CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

# DEVELOPMENT AND CHARACTERIZATION OF FUNCTIONAL INGREDIENTS FROM OLIVE POMACE: BIOACTIVITY AND POTENTIAL APPLICATION

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

***Tânia Isabel Bragança Ribeiro***

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January 2021

*To my family and friends:*

***“Science and everyday life cannot and should not be separated”***

*Rosalind Franklin*

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# Resumo

Atualmente, grande volume dos resíduos gerados ao longo da cadeia alimentar Europeia é produzido durante o processamento alimentar (19-39%). A indústria de extração de azeite não é exceção, gerando anualmente grandes quantidades de subprodutos e resíduos com elevado impacto fitotóxico, mas que são também ricos em diversos compostos bioativos e nutrientes com potencial aplicação como ingredientes alimentares. Assim sendo, é imprescindível a adoção de estratégias de redução do desperdício e de valorização dos subprodutos, no sentido de melhorar a sustentabilidade económica e ambiental da indústria alimentar. Atualmente, o bagaço de azeitona é o subproduto de maior relevo da indústria de azeite e a sua valorização adequada é também o maior desafio do sector para alcançar uma cadeia produtiva sustentável alinhada com os princípios da Bioeconomia Circular. Esta tese visou alcançar uma valorização "zero desperdício" para o bagaço de azeitona, priorizando os produtos de alto valor acrescentado sobre os de baixo valor através da produção e caracterização de ingredientes alimentares, nomeadamente ingredientes em pó ricos em vários compostos bioativos, seguida da validação *in vitro* das suas atividades biológicas ligadas a benefícios para a saúde, como a prevenção e redução de doenças cardiovasculares, diabetes, problemas intestinais e posterior aplicação no desenvolvimento de alimentos funcionais.

Uma estratégia de "desperdício zero" foi alcançada para o bagaço de azeitona. Esta estratégia consiste num procedimento simples de fracionamento, em que se obteve uma fração líquida que revelou um alto teor de hidroxitirosol (513,61-625,76 mg/100 g de peso seco) e uma fração de polpa caracterizada por ser uma boa fonte de fibra alimentar antioxidante. Adicionalmente, obteve-se ainda uma fração de caroços que exibiu valores caloríficos significativamente elevados (18,65-18,94 MJ/kg). Estes resultados validaram o potencial valor acrescentado das frações líquidas e de polpa como ingredientes alimentares e como biocombustível no caso da fração de caroços.

Após validação do potencial da fração líquida e de polpa do bagaço de azeitona como ingredientes alimentares, dois novos ingredientes em pó, estáveis e seguros, foram desenvolvidos sem qualquer etapa de extração, nomeadamente um pó enriquecido na fração líquida (LOPP) e um pó enriquecido na fração de polpa (POPP). O LOPP exibiu uma quantidade significativa de manitol (141 g/kg), potássio (54 g/kg) e hidroxitirosol/derivados (5 mg/g). O POPP exibiu uma grande quantidade de fibra alimentar (620 g/kg) associada a um teor significativo de fenólicos (7,41 mg equivalentes de ácido gálico/g fibra) com atividade antioxidante substancial. O POPP também revelou uma composição de ácidos gordos insaturados semelhante à do azeite (76% do total de ácidos gordos), sendo ainda uma fonte razoável de proteína (12%). As suas propriedades funcionais (solubilidade, capacidade de retenção de água e de óleo), capacidade antioxidante e atividade antimicrobiana foram também avaliadas, assim como a sua segurança biológica. Em síntese, a multifuncionalidade e aplicabilidade dos pós de bagaço de azeitona como fortificantes nutricionais, promotores de benefícios para a saúde e conservantes foram atestadas.

A digestão simulada *in vitro* permitiu concluir que os fenólicos e minerais do LOPP foram afetados negativamente pela digestão, no entanto, uma bioacessibilidade significativa de potássio e hidroxitirosol foi verificada ($\geq$ 57%) e as suas bioatividades foram apenas moderadamente afetadas. Em relação às suas bioatividades, pelo menos metade da atividade antioxidante (capacidade de

absorção do radical de oxigénio), de inibição da α-glucosidase e de inibição da enzima conversora de angiotensina (91,98%) foram retidas e bioacessíveis após a digestão. Por sua vez, a digestão do POPP revelou uma perda significativa de fenólicos na digestão oral (62,48%), mas o papel da fibra como veículo de fenólicos permitiu recuperar uma quantidade significativa de fenólicos no estômago (77,11%) e um índice de bioacessibilidade de pelo menos 50% (tirosol e o seu glucosídeo). A fibra do POPP também interagiu positivamente com os ácidos gordos, diminuindo e facilitando bioacessibilidade dos saturados (5-6%) e dos insaturados (4-11%), respetivamente.

Após realização da digestão *in vitro* dos pós de bagaço de azeitona, as frações disponíveis no cólon de ambos os pós exibiram uma composição abundante em compostos associados a potenciais benefícios para a saúde intestinal, nomeadamente fibra e compostos fenólicos e fibra. Assim sendo, procedeu-se a fermentação fecal *in vitro* das frações não digeridas retidas no cólon após a digestão gastrointestinal simulada *in vitro* do LOPP e POPP. Os resultados da análise do gene 16S rRNA e dos ácidos gordos de cadeia curta mostraram que estes pós não tiveram um impacto negativo na diversidade da microbiota intestinal e que promoveram uma maior produção de ácidos de cadeia curta (acetato>butirato>propionato) do que os frutooligossacarídeos. A identificação dos metabólitos fenólicos dos pós de bagaço de azeitona por LC-ESI-UHR-QqTOF-MS seguida das análises de clustering supervisionada e clustering hierárquico permitiram avaliar os padrões destes metabólitos ao longo da fermentação fecal *in vitro*, bem como explicar as potenciais atividades biológicas destes pós no intestino. Ambos os pós apresentaram um conteúdo fenólico e uma capacidade de absorção de radical de oxigénio significativas e capacidade de inibição da adesão de patógenos como o *Bacillus cereus* (22,03%) e *Listeria monocytogenes* (20,01%), sobretudo pelo POPP.

Por fim, para validar o desempenho dos pós de bagaço de azeitona como ingredientes funcionais, LOPP e POPP foram incorporados em iogurte para aumentar o seu conteúdo em fibra alimentar, hidroxitirosol e ácidos gordos insaturados. A adição de POPP (2%) ao iogurte permitiu cumprir a condição de "fonte de fibra", por sua vez LOPP (1%) forneceu 5 mg de hidroxitirosol/ derivados num iogurte (120 g). A adição de POPP e azeite juntamente com LOPP ou POPP permitiu uma melhoria do perfil de ácidos gordos insaturados dos iogurtes. Além disso, todos os iogurtes fortificados apresentaram um maior teor de fenólicos e atividade antioxidante. A digestão *in vitro* reduziu a atividade antioxidante de todas as formulações e mostrou que a incorporação de azeite juntamente com o LOPP e POPP aumentou a bioacessibilidade de hidroxitirosol e do hidroxitirosol glucosídeo, respetivamente. Contudo, todas as formulações com os pós de bagaço de azeitona podem ser consideradas boas fontes de hidroxitirosol, lípidos insaturados e fibras para os consumidores.

Em suma, os resultados deste trabalho irão contribuir para a sustentabilidade da indústria do azeite no contexto da bioeconomia circular, através do desenvolvimento de ingredientes de valor acrescentado destinados à indústria alimentar com potencial impacto positivo na saúde, proporcionando ainda um biocombustível sólido.

**Palavras-chave**: bagaço de azeitona, ingredientes em pó, bioacessibilidade, benefícios para a saúde

# Abstract

Currently, a large amount of the total European food supply chain waste (between 19 and 39%) is produced during food processing. The olive oil industry is no exception, generating large quantities of by-products and wastes annually with high phytotoxic impact, but also rich in several bioactive compounds and nutrients with potential application as food ingredients. Thus, it is imperative to implement strategies to reduce waste and valorise by-products to improve the food industry's economic and environmental sustainability. Olive pomace is the most prominent by-product of the modern olive oil industry, and its proper valorisation is one of the major obstacles to olive oil industry achieve a sustainable production chain aligned with the principles of the Circular Bioeconomy concept. This project aimed at achieving a “zero waste” valorising approach to olive pomace that prioritised the high over low added-value products, through the production and characterisation of food ingredients, namely high-value powdered ingredients rich in several bioactive compounds, followed by the validation of their biological activities related with health benefits as prevention and reduction of the prevalence of cardiovascular diseases, diabetes and gut disorders and, their application in the development of functional foods.

A “zero waste” strategy was achieved for olive pomace. This strategy is based on a feasible fraction approach, and it was obtained a liquid fraction that revealed a high amount of hydroxytyrosol (513.61-625.76 mg/100 g dry weight) and pulp fraction that was characterised to be a good source of antioxidant dietary fibre. Additionally, it was achieved a stones fraction that exhibited substantial higher heating values (18.65-18.94 MJ/kg). These results supported the potential value-added of the liquid and pulp fractions from olive pomace as functional food ingredients and as biofuel to stones.

After validating the liquid and pulp fraction potential as food ingredients, new two stable and safe, powdered ingredients without any extraction step were developed, namely a liquid-enriched powder (LOPP) and a pulp-enriched powder (POPP). LOPP exhibited a significant amount of mannitol (141 g/kg), potassium (54 g/kg) and hydroxytyrosol/derivatives (5 mg/g). POPP exhibited a high amount of dietary fibre (620 g/kg) associated with a significant amount of bound phenolics (7.41 mg gallic acid equivalents/g fibre dry weight) with substantial antioxidant activity. POPP also revealed an unsaturated fatty acid composition similar to that of olive oil (76% of total fatty acids) and a reasonable source of protein (12%). Their functional properties (solubility, water-holding and oil-holding capacity), antioxidant capacity and antimicrobial activity were also assessed, and their biological safety was validated. The multifunctionality and applicability as nutritional enhancers, health-benefits promoters and preservatives of the olive pomace powders were recognised.

The *in vitro* simulated digestion allowed concluding that phenolics and minerals from LOPP were negatively affected by digestion. However, a significant bioaccessibility of potassium and hydroxytyrosol was verified ($\geq$ 57%) and its bioactivities were only moderately affected. Regarding its bioactivities, at least half of the antioxidant activity (measured by Oxygen Radical Absorbance Capacity assay), $\alpha$-glucosidase inhibition activity and angiotensin-converting enzyme inhibitory activity (91.98%) were retained and bioaccessible after *in vitro* digestion. In its turn, POPP digestion

revealed a significant loss of phenolics in the oral step (62.48%), but the dietary fibre role as phenolics' carrier allowed recovering a significant amount of phenolics in the stomach (77.11%) and a bioaccessibility index of at least 50% (mainly for tyrosol and its glucoside). POPP dietary fibre also positively interacts with lipids decreasing the saturated fatty acids bioaccessibility (5-6%) and facilitating the unsaturated fatty acid bioaccessibility (4-11%).

After the *in vitro* digestion system of both olive pomace powders, the colon-available fractions LOPP and POPP exhibited an abundant composition in phenolics, but also dietary fibre with potential gut health benefits as antioxidants, antimicrobial and prebiotic agents. So, the gut's potential beneficial effects were analysed by *in vitro* faecal fermentation of the undigested fractions retained in the colon after *in vitro* simulated gastrointestinal digestion. The 16S rRNA gene analysis results showed that LOPP and POPP did not negatively impact gut microbiota diversity. The short-chain fatty acids analysis showed a higher production of these fatty acids (acetate > butyrate > propionate) by LOPP and POPP than by fructooligosaccharides. The identification of phenolic metabolites by LC-ESI-UHR-QqTOF-MS followed by supervised cluster and hierarchical clustering analysis allowed to evaluate the metabolite patterns of olive pomace powders phenolics throughout faecal fermentation, explaining the potential biological activities exhibited in the gut by these powders. Both powders showed significant total phenolic content and oxygen radical absorbance capacity during faecal fermentation. But also, mucin-adhesion inhibition ability against pathogens as *Bacillus cereus* (22.03%) and *Listeria monocytogenes* (20.01%), specially POPP.

Finally, to validate olive pomace powders' performance as functional ingredients, LOPP and POPP were incorporated into yoghurt to increase its content in dietary fibre, hydroxytyrosol, and unsaturated fatty acids. POPP (2%) and LOPP (1%) addition to yoghurt allowed fulfilling the condition on being a "source of fibre" and provides 5 mg of hydroxytyrosol and derivatives in a standard yoghurt (120 g), respectively. The addition of olive oil, together with olive pomace powders, was also investigated in order to understand the lipids-phenolics interaction, and not only POPP addition improved the yoghurts' unsaturated fatty acids profile, but the olive oil addition together both olive pomace powders also improved too. Besides that, all yoghurts fortified with olive pomace powders exhibited higher total phenolic content and antioxidant activity. The *in vitro* digestion of yoghurts showed that olive oil incorporation together with olive pomace powders increased the bioaccessibility of hydroxytyrosol (LOPP + olive oil) and hydroxytyrosol glucoside (POPP + olive oil). Despite the reduction of antioxidant activity after digestion, the yoghurts formulations with olive pomace powders could be considered suitable carriers to deliver hydroxytyrosol, unsaturated fatty acids and fibre to the consumers.

In short, the results from this work will contribute to the sustainability of the olive oil industry in circular bioeconomy context, through the development of value-added ingredients to the food industry with a positive impact in health, offering also a solid biofuel.

**Keywords**: olive pomace, powder ingredients, bioaccessibility, health benefits

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# Abbreviations

**AAPH** – 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride

**ACE** – angiotensin-converting enzyme

**ADF** – Antioxidant dietary fibre

**AOX** – Antioxidant activity

**ABTS** – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

**BE** – Bioeconomy

**BI** – Bioaccessibility index

**BPC** – Bound phenolic compounds

**CFU** – Colony-forming unit

**CBE** – Circular bioeconomy

**CE** – Circular economy

**DAD** – Diode Array Detector

**DF** – Dietary fibre

**DPPH** – 2,2-diphenyl-1-picrylhydrazyl

**DW** – dry weight

**EC** – European Commission

**EDOP** – Extracted dried olive pomace

**FOS** – Fructooligosaccharides

**FPC** – Free phenolic compounds

**GAE** – Gallic acid equivalent

**GUAE** – Galacturonic acid equivalent

**GI** – gastrointestinal

**HPLC** – High-Performance Liquid Chromatography

**HYD** – Hydroxytyrosol

**IDF** – Insoluble dietary fibre

**LCA** – Life Cycle Assessment

**L-OP** – Liquid fraction from olive pomace

**LOPP** – Liquid-enriched olive pomace powder

**MAE** – Microwave-assisted extraction

**MH** – Mueller-Hinton

**MIC** – Minimal Inhibitory Concentration

**MRSA** – Methicillin-resistant *Staphylococcus aureus*

**MUFA** – Monounsaturated fatty acid

**MW** – Molecular weight

**OD** – Optical density

**OM** – Olive mill

**OMWW** – Olive mil wastewater

**OO** – Olive oil

**OP** – Olive pomace

**ORAC** – Oxygen Radical Absorbance Capacity

**PBS** – Phosphate-buffered solution

**PLE** – Pressurised liquid extraction

**P-OP** – Pulp fraction from olive pomace

**POPP** – Pulp-enriched olive pomace powder

**PUFA** – Polyunsaturated fatty acid

**RI** – Recovery index

**RID** – Refractive Index Detector

**RT** – Retention time

**SCFA** – Short-chain fatty acid

**SFA** – Saturated fatty acid

**SDF** – Soluble dietary fibre

**SFE** – Supercritical fluid extraction

**SGD** – Simulated gastrointestinal digestion

**S-OP** – Stones fraction from olive pomace

**TE** – Trolox Equivalent

**TPC** – Total phenolic compounds

**UAE** – ultrasound-assisted extraction

**UFA** – Unsaturated fatty acid

**USM** – Unsaponifiable matter

## Scope and Outline

This thesis is organized into five major parts, which are subdivided into chapters. Figure 1 is the schematic layout of the thesis structure.

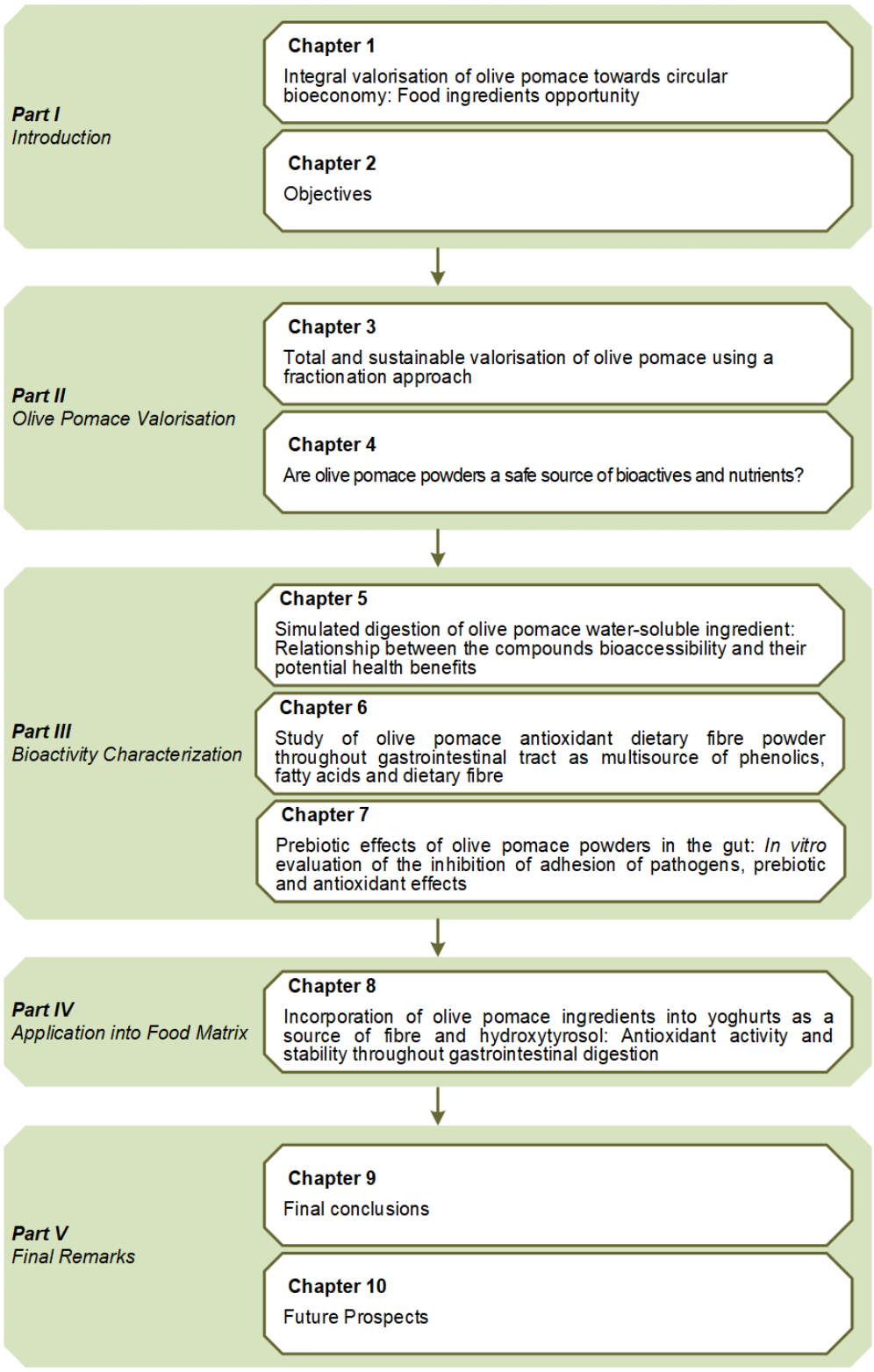



**Figure 1. Thesis outline.**

The first part is composed of two chapters, and Chapter 1 corresponds to a literature review on the valorisation of olive pomace by the development of food ingredients towards a circular bioeconomy and Chapter 2 corresponds to the objectives of this thesis. Parts II, III and IV encompass the experimental work. Part II includes Chapters 3 and 4, which focus on the integral valorisation of olive pomace by the development of powdered food ingredients (Chapter 3) and the chemical, biological safety and bioactive characterization of the powders (Chapters 3 and 4). Part III comprises Chapters 5, 6 and 7, focusing on the olive pomace powders' bioactivity characterisation through gastrointestinal digestion simulation. Explicitly, Chapters 5 and 6 encompass the simulation of gastrointestinal digestion of the liquid-enriched powder and pulp-enriched powder obtained from the olive pomace, and how it affects their chemical composition as multifunctional powders and their different biological properties, including the antioxidant capacity, antihypertensive and antidiabetic activity. Finally, Chapter 7 describes the different potential gut health benefits of the developed olive pomace powders using an *in vitro* faecal model. Part IV includes Chapter 8, which focuses on the potential application of the olive pomace powders into yoghurts to produce a functional dairy product, as a proof of concept. Finally, Part V comprises Chapters 9 and 10, corresponding to the general conclusions (Chapter 9) and future work (Chapter 10).

Figure 2 is the olive pomace powdered ingredients' scheme obtained, used, and evaluated throughout this thesis.

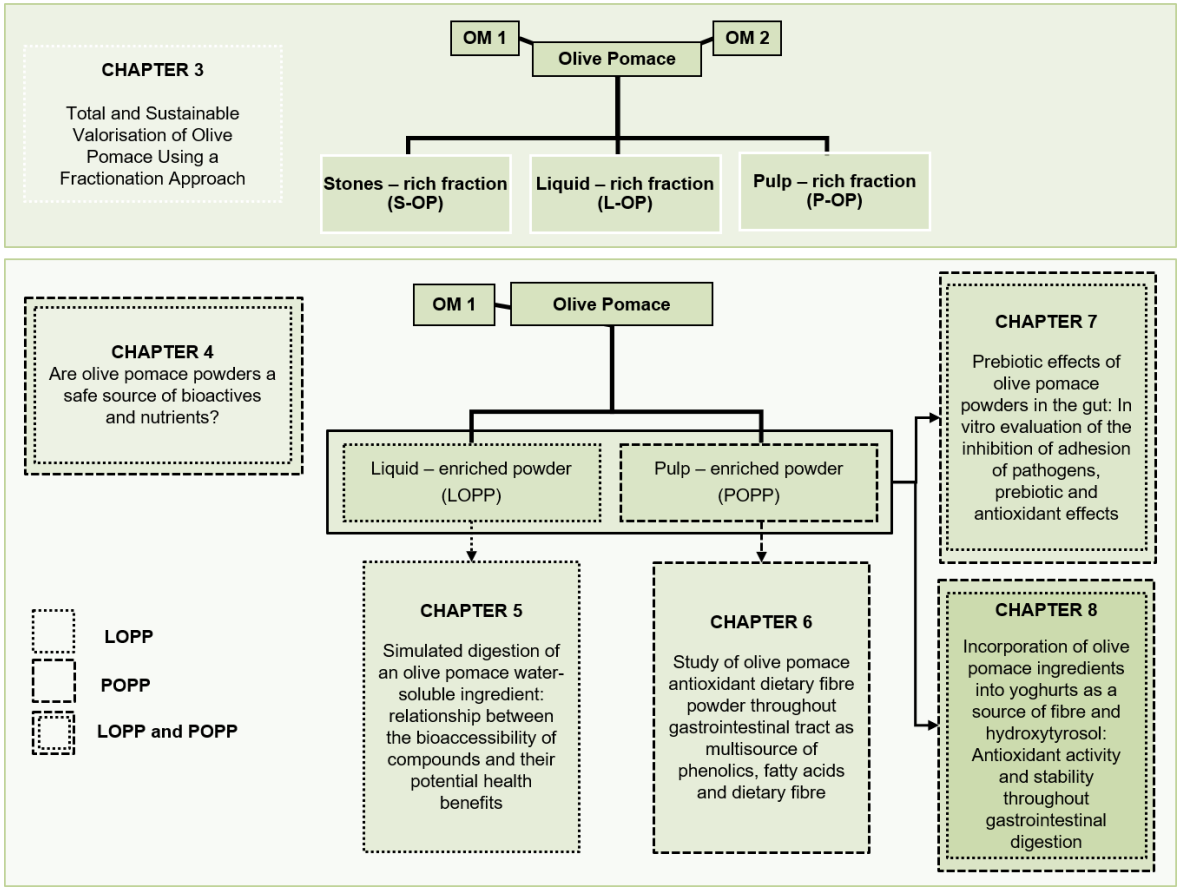



**Figure 2. Scheme of the olive pomace powdered ingredients obtained, used, and evaluated throughout this thesis.**

The core of this thesis is composed of seven articles under preparation (Chapter 1), already published (Chapter 3 to 7) or accepted (Chapter 8) in international peer-reviewed journals, according to the following list:

Chapter 1

Ribeiro, Tânia B, Nunes, J., Vicente, A. A., & Pintado, M. (2020). Integral valorisation of olive pomace towards circular bioeconomy: Food ingredients opportunity (Review under preparation)

Chapter 3

Ribeiro, Tânia B, Oliveira, A. L., Costa, C., Nunes, J., Vicente, A. A., & Pintado, M. (2020). Total and Sustainable Valorisation of Olive Pomace Using a Fractionation Approach. *Applied Sciences*, *10*(19), 6785. https://doi.org/10.3390/app10196785

Chapter 4

Ribeiro, Tânia B, Oliveira, A., Coelho, M., Veiga, M., Costa, E. M., Silva, S., Nunes, J., Vicente, A. A., & Pintado, M. (2020). Are olive pomace powders a safe source of bioactives and nutrients? *Journal of the Science of Food and Agriculture*, *n/a*(n/a). https://doi.org/10.1002/jsfa.10812 (in press)

Chapter 5

Ribeiro, Tânia Bragança, Oliveira, A., Campos, D., Nunes, J., Vicente, A. A., & Pintado, M. (2020). Simulated digestion of an olive pomace water-soluble ingredient: relationship between the bioaccessibility of compounds and their potential health benefits. *Food & Function*, *11*(3), 2238–2254. https://doi.org/10.1039/C9FO03000J

Chapter 6

Ribeiro, Tânia Bragança, Campos, D., Oliveira, A., Nunes, J., Vicente, A.A. & Pintado, M. (2020). Study of olive pomace antioxidant dietary fibre powder throughout gastrointestinal tract as multisource of phenolics, fatty acids and dietary fibre. *Food Research International*, 110032. https://doi.org/10.1016/j.foodres.2020.110032

Chapter 7

Ribeiro, Tânia Bragança, Costa, C. M., Bonifácio – Lopes, T., Silva, S., Veiga, M., Monforte, A. R., Nunes, J., Vicente, A. A., & Pintado, M. (2021). Prebiotic effects of olive pomace powders in the gut: In vitro evaluation of the inhibition of adhesion of pathogens, prebiotic and antioxidant effects. *Food Hydrocolloids*, 112, 106312. https://doi.org/10.1016/j.foodhyd.2020.106312

Chapter 8

Ribeiro, Tânia Bragança, Bonifácio – Lopes, T., Morais, P., Miranda, A., Nunes, J., Vicente, A.A., Pintado, M. (2021). Incorporation of olive pomace ingredients into yoghurts as a source of fibre and hydroxytyrosol: Antioxidant activity and stability throughout gastrointestinal dig estion. *J. Food Eng.,* 297,110476. https://doi.org/10.1016/j.jfoodeng.2021.110476

# PART I

# Introduction

# Chapter 1.
# Integral valorisation of olive pomace towards circular bioeconomy: Food ingredients opportunity

## 1.1. Current scenario

The food sector is a significant contributor to the depletion of natural resources, greenhouse gas emissions (GHG) and waste accumulation (Teigiserova *et al.*, 2019). The growing global population demands more food, which has to lead to an intensification in food production/ processing and subsequent food waste generation and accumulation (Ravindran and Jaiswal, 2016). Food waste accumulation has been rising and tends to continue growing exponentially along with the global population. According to FAO, 13.8% of global produced food is wasted, representing not only a poor use of natural resources but also an environmental and economic problem (FAO, 2019; Torres-León *et al.*, 2018). Each ton of food waste embodies high management costs and greenhouse gas (GHG) emissions (Torres-León *et al.*, 2018).

Nowadays, it is imperative to implement changes in the food sector regarding waste prevention and valorisation to improve its economic and environmental sustainability (Mirabella *et al.*, 2014). Food losses or waste occur, along with the entire food system. However, food manufacturing is responsible for a vast amount of food waste. It is estimated that industrial food waste ranges between 19 and 39% of the total food waste in the European food supply chain (Garcia-Garcia *et al.*, 2019).

Food industry generates by-products or waste streams as stems, leaves, seeds, shells, pomace, bran and so forth, besides non-conformity food that does not meet the quality standards and does not make it into the production chain (González-García *et al.*, 2019). Despite the efforts to reduce waste levels by optimizing the food manufacturing processes and implementing new measures regarding non-conformity food, food production will always be linked to unavoidable and inedible by-products (Teigiserova *et al.*, 2019). Among the most prominent food processing by-products, there is a variety of peels (e.g. potato, tomato, kiwi, orange and banana), plant biomass (e.g. tomato and barley hulls), spent coffee grounds, and several pomace wastes (e.g. apple, tomato, grape and olive) (Ravindran and Jaiswal, 2016; Teigiserova *et al.*, 2019). These by-products could be discarded, with the consequent environmental and economic costs, or they can be valorised (Teigiserova *et al.*, 2019).

The conversion of by-products into high added-value products or raw materials could generate economic gains and provide environmental benefits, reducing the pollution and the excessive use of resources (Torres-León *et al.*, 2018). This vision goes in the direction of supporting the development of a circular bioeconomy (CBE) in the food industry, an approach that is being encouraged by current European policies (Ellen MacArthur Foundation, 2015; European Commission, 2018). Currently, the development of strategies to obtain different high-value products from agri-food by-products is an essential ongoing field of research (González-García *et al.*, 2019; Ravindran and Jaiswal, 2016).

At the moment, the food industry is interested in obtaining zero waste processes (Torres-León *et al.*, 2018) in order to close the loop using by-products as resources to obtain new products and applications with high added-value (Garcia-Garcia *et al.*, 2019). This interest exists because growing the economic value of food by-products, even by a small amount, may cause a significant economic advantage due to the large volumes of food currently wasted (Garcia-Garcia *et al.*, 2019) and could contribute to the Sustainability Development Goals (SDGs) (European Commission, 2018).

The implementation of a value chain according to the CBE principles is a huge challenge, and even more for industries like olive oil mills. Olive mill wastes are produced in large quantities during a short production season and comprise high moisture content. Seasonality and high water content of olive by-products could compromise the economic feasibility of future circular bioeconomy value chains and products based on these waste streams (Donner *et al.*, 2020). Olive pomace (OP) is the most critical by-product from the modern olive oil industry that needs to be valorised. Besides being the most abundant waste stream (representing approximately 65% of the initial weight of olive) (AGAPA, 2015), OP is phytotoxic and challenging to treat (Rigane *et al.*, 2012). However, OP contains valuable bioresources that can be utilized to obtain several useful chemicals, foods (Nunes *et al.*, 2016), materials (Moreno-Maroto *et al.*, 2019), biocomposites (Hammoui *et al.*, 2015; Lammi *et al.*, 2018) and fuels (Christoforou and Fokaides, 2016). Therefore, the olive oil industry's residues' recovery is being stimulated to move towards a circular bioeconomic model, reducing waste production or adding value to unavoidable by-products as OP, developing added-value secondary product lines.

Research has been focused on supporting OP use for energy (low-value use) and looking for isolated high-value components, neglecting the importance of the cascading approach (energy use should be the last option after valuable compounds have been extracted). A large extent of these investigations was focused on single-step procedures, or main efforts were put on a specific fraction of OP (e.g., phenolics), blocking a cradle-to-grave analysis aimed at obtaining multiple products out of the whole by-product. Further, complex and non-sustainable processes are often applied, compromising the economic and environmental sustainability of added-value products obtained from OP.

In order to bridge this gap, and supply insights for future OP value chain investments, the overall aim of this review is to deliver an overview of the emerging bioconversion processes using OP, and the ability of these processes to supply high-value products to the market. Specifically, we have focused on using OP as food ingredients, and the justification of this use as a practical strategy within a framework encompassing CBE and sustainable resource policy. The review is structured in four sections: the first section explains the CBE concept, cascading biomass use and bio-based value pyramid. The next section is a brief description of waste in the olive value chain. The third section analyses the state-of-the-art olive oil by-product valorisation and the relevance of food ingredients as the high-value use of OP. Finally, we reviewed whole valorisation strategies for OP as integrated value chains of OP, including food ingredients and other high value uses based on the most promising bioeconomy sectors, but also the opportunity of powdered food ingredients.

## 1.2. Implementation of circular bioeconomy in the food industry

### 1.2.1. Circular bioeconomy concept

Circular economy (CE) and bioeconomy (BE) are always considered when a sustainable economy is discussed. These two concepts have so far been developed in parallel. However, the need of connection and support of each other has been identified in order to face the actual consuming panorama, according to the updated BE strategy of the European Commission (EC): *"European Bioeconomy needs to have sustainability and circularity at its heart"* (European Commission, 2018). As a response, BE and CE models were merged, leading to the new CBE concept (Stegmann *et al.*, 2020).

Other publications suggested a more comprehensive view of CBE *as "more than bioeconomy or circular economy alone"* and Ellen MacArthur Foundation (2013), even after the term CBE appeared, implied that BE is an integral CE element. Despite the different interpretations of the CBE concept, it is clear that synergy between BE and CBE is much needed in the present scenario, and neither of them is complete without each other (Venkata Mohan *et al.*, 2019). Regarding the current targets for sustainable growth, CBE can directly contribute to 11 out of the 17 United Nations SDGs (Figure 1.1) (Lokesh *et al.*, 2018).


**Figure 1.1. The potential contribution of the circular bioeconomy-based value chains to achieve the United Nations Sustainability Development Goals and the potential of value chain mapping and analysis in quantifying these goals** (Lokesh *et al.*, 2018)**.**

CBE is defined by Carus & Dammer (2018) as "*the sustainable, cascading processing of biological residues into biobased products, which can be shared/reused/remanufactured and recycled, or released safely to the biosphere via organic and nutrient cycles*" (Figure 1.1). More recently, Stegmann *et al.*, (2020), after a literature review and analysis of keywords used in scientific publications about CBE, suggested the following CBE definition (Figure 1.1): *"The circular bioeconomy focuses on the sustainable, resource-efficient valorization of biomass in integrated, multi-output production chains (e.g. biorefineries), while also making use of residues and wastes and optimizing the value of biomass overtime via cascading* (Figure 1.3). *Such optimization can focus on economic, environmental or social aspects and ideally considers all three pillars of sustainability. The cascading steps aim at retaining the resource quality by adhering to the bio-based value pyramid* (Figure 1.3) *and the waste hierarchy* (Figure 1.3) *where possible and adequate"*.

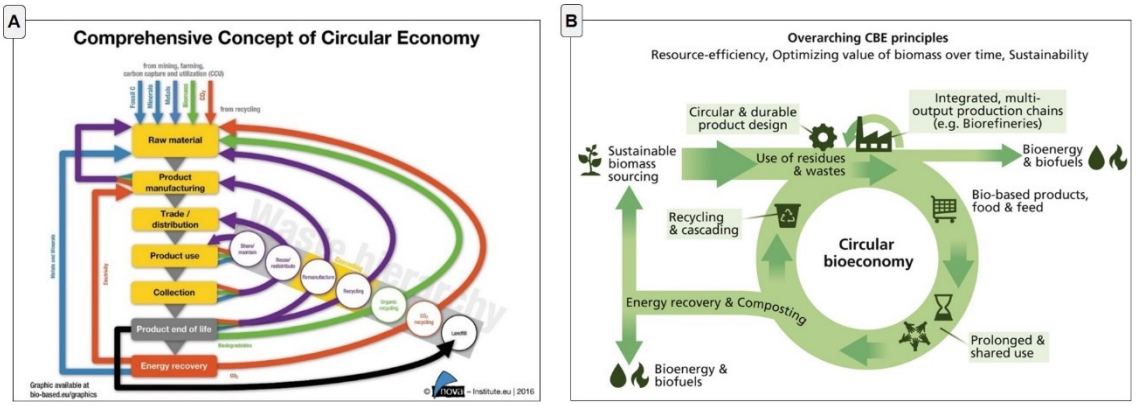



**Figure 1.2. Concept of Circular Bioeconomy. (A) A comprehensive concept of the circular economy, according to Carus & Dammer (2018) (Nova Institute. http://bio-based.eu/graphics/). (B) The circular bioeconomy and its elements according to the literature review carried out by Stegmann *et al.* (2020).**

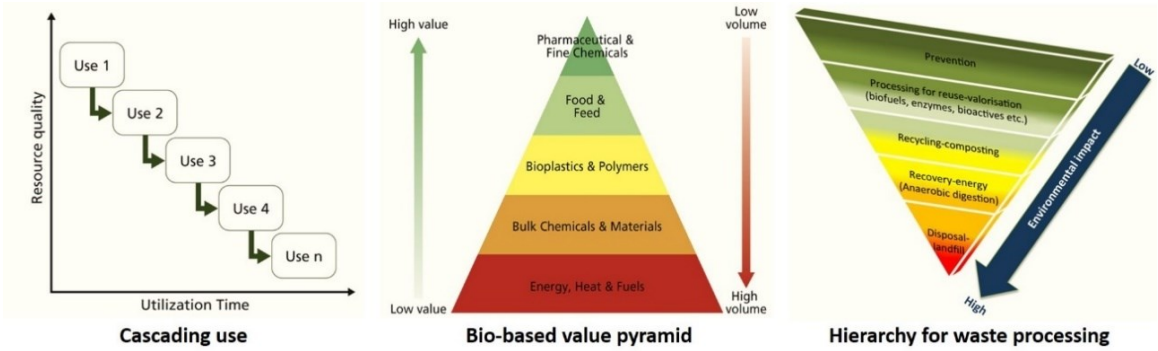



**Figure 1.3. Cascading use of biological resources [Adapted from Stegmann *et al.*, (2020) and Ravindran & Jaiswal, (2016)].**
In line with the EC, cascading use of biomass could be defined as biomass processing into a bio-based final product, which is used at least once more either for material or energy purposes. Cascading is also sometimes interpreted as an order of priority, aiming for the highest added value. The bio-based value pyramid is a commonly used way to classify biomass use according to their value and volume. The waste hierarchy introduced by the EU Directive 2008/98/EC on waste (Waste Framework Directive) provides a priority order for waste management with waste prevention as the first priority, followed by reuse, recycling, recovery and disposal.

After the full understanding of the CBE concept, elements and its potential to attain the global sustainability goals (Lokesh *et al.*, 2018; Venkata Mohan *et al.*, 2019), there is now the need to understand how the food industry could become more sustainable and resource-efficient using its by-product streams to supply higher-value products to the market towards a CBE.

Food by-products are residues in liquid or solid form, usually of high organic load, derived from raw materials processing to obtain food. These materials are removed from the production process as unwanted substances and denominated as “wastes” in past European legislation (European Commission, 2006a). Most recently, the term “food by-products” was introduced for EC to advise that “food wastes” are key substrates for the recovery of functional compounds and to develop new products with market value (European Commission, 2008).

By-products valorisation has a well-defined role to play in order to implement a CBE in the food industry sector and increase sector sustainability (Garcia-Garcia *et al.*, 2019; Venkata Mohan *et al.*, 2019). However, the CBE is still an emerging concept, and its application into food by-products is still a challenge (Donner *et al.*, 2020).

The cascading use of biomass is a vital characteristic of the CBE to optimize biomass value over time. However, food by-products were usually used in low added-value applications such as energy generation (mainly), composting and animal feed, leading to the loss of the remaining economic and biological value (Campos *et al.*, 2020a). Ideally, a CBE cascading use of biomass would follow a movement down the bio-based value pyramid (Figure 1.3) and the waste hierarchy (Figure 1.3), going from high value to lower value biomass applications (Stegmann *et al.*, 2020). Staying on the upper part of both hierarchies is vital for CBE and a key focus for future research and technology development (Teigiserova *et al.*, 2019).

Several studies of valorisation of food by-products have highlighted and untapped potential for the extraction of valuable biomolecules from food by-products, leading to high-value compounds from bioplastics, enzymes, antioxidants, proteins, nutraceuticals and colourants (Galanakis, 2012; Mirabella *et al.*, 2014; Ravindran and Jaiswal, 2016; Torres-León *et al.*, 2018). However, in practice, applications on the lower use of biomass might still be chosen from an environmental and economic perspective (Stegmann *et al.*, 2020). One of the main reasons for this unsuccess in obtaining high added-value products arises from the existence of a well-established market for these products from other sources (Teigiserova *et al.*, 2019). Recent studies of Teigiserova *et al.*, (2019) and Stegmann *et al.* (2020) identified that the most promising sectors are high-value biomass uses such as bioplastics, pharmaceuticals, food and feed additives. Other lower to medium value applications on the bio-based value pyramid (Figure 1.3) were also recognized as favourable sectors, such as biocomposites and sustainable building materials. Low-value applications, like bioenergy and biofuels, despite the broader size of their markets, were identified as disappointing sectors, due to their low price and lack of policy support (Stegmann *et al.*, 2020).

Another vital part of CBE is the integrated and multi-output value chains (e.g. biorefineries) (González-García *et al.*, 2019; Ravindran and Jaiswal, 2016; Teigiserova *et al.*, 2019). Integrated value chains have been identified as one of the most promising pathways to improve the biomass's

total value and attain a resource-efficient CE (Lokesh *et al.*, 2018). In this case, aligned with CBE principles, the value chain is designed to valorise food by-products into different valuable products in a closed-loop system (Teigiserova *et al.*, 2019). Further, the goal is to move towards integrated value chains, i.e. a combination of several conversion technologies that allow for more flexibility, cost reduction and production of fine chemicals, materials, bulk chemicals, fuel and energy (Stegmann *et al.*, 2020). However, it is crucial to ensure that integrated value-chains conversion technologies are environmentally efficient.

To ensure that all extraction technologies and even more that the overall system performance is environmentally efficient, it is essential to use holistic environmental assessments methods, such as Life Cycle Assessment (LCA). EC highlighted LCA as a preferred tool to assess the environmental performance of products and systems. Briefly, LCA is a methodology used for quantifying environmental impacts of the products considering their entire lifetime from production to final disposal (Teigiserova *et al.*, 2019).

## 1.3. Olive oil extraction: Waste and by-product production

Olive oil extraction represents an important agro-industrial activity in Mediterranean countries. Mediterranean countries alone produce 98% of the total worldwide olive oil production (Esteve *et al.*, 2015; Rodrigues *et al.*, 2015). The leading olive oil producers are Spain, Italy and Greece (Dermeche *et al.*, 2013). Despite its relatively small size, Portugal ranks as the world's 8$^{th}$ largest olive oil producer (FAOSTAT, 2018).

Olive oil extraction is one of the most heavily polluting industries in the food sector (Filotheou *et al.*, 2015). The mechanical extraction of olive oil generates large quantities of by-products with high phytotoxic impact on land and water environments. Many LCA analyses have pointed out the olive by-products as the major obstacle preventing this sector from achieving a sustainable production chain (Muíño *et al.*, 2017).

Olive by-products production (Figure 1.4) begins in olive orchards with the pruning of olive trees (usually every two years) to improve olive oil production by the elimination of unproductive and old branches, resulting in a large amount of biomass (Negro *et al.*, 2017). Once olives are picked and brought to the olive mill, the leaves collected with olives are separated. The next step is to extract olive oil itself, including processes such as milling, mixing or malaxation, and separation in a horizontal centrifuge or decanter (Manzanares *et al.*, 2017; Negro *et al.*, 2017).

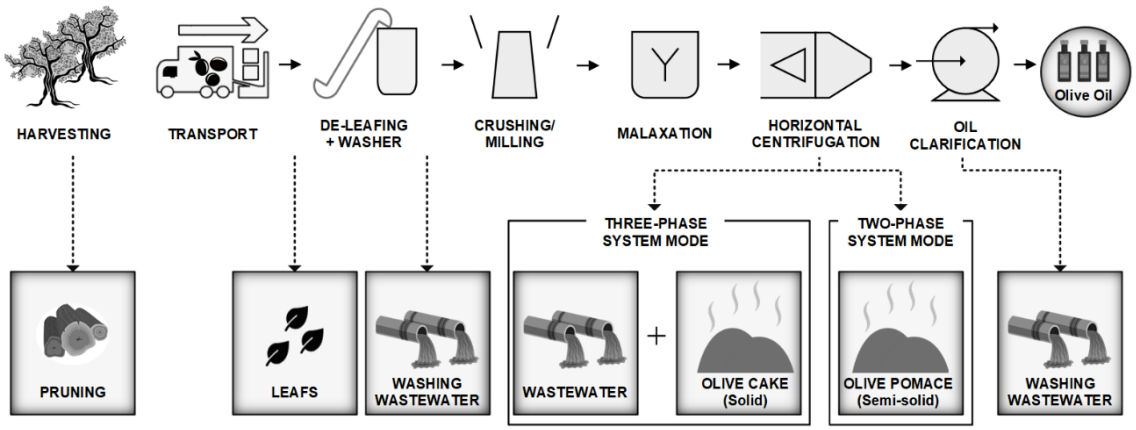



**Figure 1.4- Simplified process for olive oil production and main waste and by-products using the three-phase and two-phase olive oil extraction system.** Based on Negro *et al.* (2017) and Roselló-Soto, Koubaa, *et al.* (2015).

The by-products composition depends on the process applied to separate olive oil from the remaining crushed olive paste (Negro *et al.*, 2017). The traditional discontinuous pressing process (employed mainly by small producers) was at first replaced by a continuous process using a three-phase system, and later increasingly replaced by a two-phase system (Rincón *et al.*, 2012), where the main differences between these last two systems are the addition of water. As a consequence, the reduction of the amount of olive-mill wastewater (OMWW) produced. The three-phase system produces two primary wastes: a solid cake (olive cake or olive kernel), and large amounts of liquid phase (OMWW) that contains rinsing water, olive mill water coming from the decantation stage and water coming from the separator (Rodrigues *et al.*, 2015). On the other hand, the two-phase system can be considered a modified version of the three-phase method and allows separating the oil from olive paste without the addition of water, and it eliminates the problem of the vegetable wastewater. The two-phase system only produces a small amount of residual water that naturally evaporates in tanks and a semisolid residue, the olive pomace (OP) (Nunes *et al.*, 2016). A substantial reduction of liquid-residues and water consumption is reached using the two-phase process; nevertheless, this process leads to a slight increase in solid wastes (Rincón *et al.*, 2012). According to different authors, one hectare of olive tree originates about 2500 kg of olives (Rodrigues *et al.*, 2015) and approximately 40-70 kg of OP are produced per 100 kg of olives (AGAPA, 2015; Nunes *et al.*, 2016; Romero-García *et al.*, 2014; Ruiz *et al.*, 2017). Table 1.1 summarizes the amount of the main by-products from olive oil extraction industry.

Currently, the two-phase system is the most widely implemented process. In Spain (the largest olive oil producer) and Portugal, more than 90% of olive mills use the two-phase system (Duarte *et al.*, 2011; Ruiz *et al.*, 2017) So, OP is nowadays the most abundant and relevant by-product in the olive oil industry (Dermeche *et al.*, 2013). OP is phytotoxic, non-biodegradable and challenging to treat because of its rich composition in organic compounds and high moisture content (Esteve *et al.*, 2015; Lama-Muñoz *et al.*, 2012). OP is a significant pollutant, and the proper treatment/valorisation of this by-product is mandatory for olive producers and detrimental for producers' economic profit. The OP is a semisolid residue that comprises the olive pulp (30-50%), skin (15-30%), stone (30-45%), water

(50-70%) and oil residues (4-18%) (Aliakbarian *et al.*, 2011; Dermeche *et al.*, 2013; Galanakis, 2011; Lama-Muñoz *et al.*, 2012; Rigane *et al.*, 2012; Rincón *et al.*, 2012; Rodríguez-Gutiérrez *et al.*, 2014), which corresponds to ca. 65-80% of the initial fruit weight (AGAPA, 2015; Lama-Muñoz *et al.*, 2012; Rodrigues *et al.*, 2015). Regarding bioactive compounds, OP contains a high quantity of phenolic compounds. Almost all the olive fruit's phenolic content (~98 %) remains in the OP after olive oil extraction (Araújo *et al.*, 2015; Nunes *et al.*, 2018). Besides that, OP is a significant source of polysaccharides, lipids, minerals and proteins of putative technological/ bioactive importance for different industries such as food, nutraceuticals and cosmetics (Esteve *et al.*, 2015; Filotheou *et al.*, 2015; Nunes *et al.*, 2016).

**Table 1.1. Main characteristics of the different types of olive by-products** (AGAPA, 2015; Nunes *et al.*, 2016; Romero-García *et al.*, 2014; Ruiz *et al.*, 2017)**.**

| By-product | Location | Olive oil extraction system | Estimated production | Current application | Ref. |
|---|---|---|---|---|---|
| ***Olive tree pruning*** | Olive orchards | - | 1.5–3 ton/ha/year | Firewood | (Ruiz *et al.*, 2017) |
| ***Olive leaves*** | Olive mills | Both | 4–7% of the olive weight | Animal feed | (Ruiz *et al.*, 2017) |
| ***Olive pomace*** | Olive mills | Two-phase system | 40-70% of the olive weight | Production of pomace olive oil and extracted dry olive pomace<br>Extracted dry olive pomace used as fuel | (AGAPA, 2015; Nunes *et al.*, 2016; Romero-García *et al.*, 2014; Ruiz *et al.*, 2017) |
| ***Olive stones*** | Olive mills | Recovered after oil separation in both systems. | 8–15% of the olive weight | Biofuel | (Romero-García *et al.*, 2014; Ruiz *et al.*, 2017) |
| ***Olive cake*** | Olive mills | Three-phase system | 40-45% of the olive weight | Production of pomace olive oil and extracted dry olive cake<br>Extracted dry olive cake used as fuel | (Romero-García *et al.*, 2014; Ruiz *et al.*, 2017) |
| ***Olive mill wastewater*** [*1] | Olive mills | Three-phase system | 40–50% of the olive weight | None | (Romero-García *et al.*, 2014; Ruiz *et al.*, 2017) |

[*1] Wastewater is also generated in the two-phase system during olive fruits cleaning and the washing process from the secondary centrifuge of virgin olive oil but in much lower amounts. The standard practice is the disposal of the junction of these two types of wastewater by pouring them into an open-air pond for evaporation (Romero-García *et al.*, 2014).

## 1.4. Uses of olive pomace

### 1.4.1. Conventional uses of olive pomace

The main post-treatment for OP is a drying - solvent extraction (using hexane) process of pomace olive oil, in which the remaining solid (EDOP) is used mainly as an energy source (AGAPA, 2015).

Only after these prior drying and oil extraction processes, OP is suitable to be used as fuel (Kinab and Khoury, 2015; Manzanares *et al.*, 2017). The conversion of olive by-products into thermal and electrical power is particularly attractive for low moisture solid olive by-products as EDOP or olive stones (Negro *et al.*, 2017). EDOP or olive stones have been indicated as containing a considerable amount of energy (net calorific value (LHV) = 15.58-19.81 MJ/kg) (Christoforou and Fokaides, 2016).

This production of olive oil pomace and EDOP was also negatively affected by adopting the two-phase system in olive mills. Traditionally, pomace olive oil was extracted from the dried pomace (ca. 8% moisture) with *n*-hexane (Gómez-de la Cruz *et al.*, 2015). The high amount of water present in the OP (ca. 65% moisture) was making the basic operations of transportation, storage and drying much more expensive and complex, while at the same time reducing the profits rendered from pomace olive oil (Moral and Méndez, 2006). First, OP needs to be stored in ponds outside factories due to its high-water content, sometimes up to 6 months. Then, before drying, an additional step of physical extraction using horizontal centrifugal machines or decanters is performed to extract 40-60% of pomace olive oil (Moral and Méndez, 2006). The final products obtained in these extracting units are the pomace olive oil, EDOP and olive stones (Manzanares *et al.*, 2017; Negro *et al.*, 2017).

Pomace olive oil is typically used in the soap industry (Kinab and Khoury, 2015), but it is possible to be used for biodiesel production (Lama-Muñoz *et al.*, 2014). Olive stones and extracted OP are both explored for energy production. However, stones are considered a more suitable solid biofuel than extracted OP (Kinab and Khoury, 2015; Manzanares *et al.*, 2017). The EDOP was widely used as solid fuel, but the presence of some impurities in its composition, such as oil residues and low weight particles (pulp and olive stones <1 mm) decrease its value as fuel (Manzanares *et al.*, 2017; Mata-Sánchez *et al.*, 2015). These impurities are considered harmful compounds related to uncontrolled pollutant emissions, corrosion and slagging, compromising EDOP performance as biofuel (Mata-Sánchez *et al.*, 2015).

Consequently, the use of olive stones instead of EDOP has been progressively increased, and the practice of removing most of the olive stone contained in OP has become even more usual (Manzanares *et al.*, 2017; Negro *et al.*, 2017). Olive stones recovery from OP or EDOP has been considered a “best practice” with economic and environmental viability in the olive oil sector (Pattara *et al.*, 2010). Recently, Restuccia *et al.* (2018) also highlighted all the advantages of de-stoning olives before olive oil extraction regarding olive oil quality and process sustainability. Therefore, currently, EDOP contains even fewer stones, reducing its value since worse combustion properties (Negro *et al.*, 2017).

The generation of energy from olive by-products is well established in olive oil-producing countries (Negro *et al.*, 2017). In Andalusia, Spain (50% of EU-28 olive oil), 80% of olive by-products were used for energy generation (47% for electricity generation and 33% for thermal energy) (AGAPA, 2015; Berbel and Posadillo, 2018). The olive oil industry is the principal user of thermal energy obtained from olive by-products (Berbel and Posadillo, 2018; Moral and Méndez, 2006). EDOP is usually used to dry OP in pomace olive oil extraction units (Christoforou and Fokaides, 2016), and olive stones are often employed in industrial boilers to obtain process steam because they cause

fewer corrosion problems (Negro *et al.*, 2017). The domestic use of olive stones as a substitute for wood pellets is usual but smaller (Berbel and Posadillo, 2018). On the other hand, olive stones and EDOP are also applied to generate electrical energy or cogeneration (simultaneous steam and electricity production) through combustion. Currently, this is the dominant energy use of olive by-products in Andalusia (AGAPA, 2015).

Besides the well-established direct combustion of olive stones and EDOP for electricity generation or thermal use, other thermochemical technologies have been investigated in recent years for olive stones and EDOP valorisation, such as pyrolysis or gasification (Christoforou and Fokaides, 2016). Pelletizing has been applied as pre-treatment of OP to add more value to olive by-products than direct energy use and composting (Berbel and Posadillo, 2018). Recent studies have examined the thermal characteristics of pellets made from OP or mixtures of OP with other biomass, reporting better results using blends with OP < 50% (Miranda *et al.*, 2012).

Composting and the biofertilizers development from OP have received considerable attention (Muktadirul Bari Chowdhury *et al.*, 2013), representing 14.3% of olive by-products utilisation in Andalusia (AGAPA, 2015). OP is rich in minerals and organic compounds except for sulphur, representing a possible source of nutrients for plant growth and development. Muscolo *et al.* (2019) compared three different biological methods: anaerobic digestion, aerobic digestion, and crude agricultural waste management system (sulphur-bentonite processing procedure) to transform OP in fertilisers. Aerobic digestion showed economic advantages over other alternatives, exhibiting the most significant fertiliser effects even if the production time is longer than the other two. The other two options represent processes with reduced greenhouse gas emissions. From an environmental point of view, composting of OP revealed a higher environmental impact than OP pellets' production or feed additives upon removing olive stones in a recent LCA study in Turkey (Duman *et al.*, 2020).

OP use as feed is considered a medium-value use; however, only 5% of olive by-products were consumed as animal feed in Andalusia. Hence, as described, 95% of all by-products are devoted to low-value uses, particularly energy use (AGAPA, 2015). After the removal of olive stones, OP can be easily used as animal feeding additive. However, OP as feed should be limited to a certain percentage (10% of the total diet but 5% is usually recommended) due to its fat-rich composition (Berbel and Posadillo, 2018). The use of OP in low doses resulted in beneficial effects for the meat composition, mainly to its composition in oleic acid and polyunsaturated fatty acids (Restuccia *et al.*, 2018). Recently, an LCA study of dairy goat production in Spain revealed that OP as a feed additive reduced GHG emissions attributed to $N_2O$ emissions from feed production stages (Pardo *et al.*, 2016). Feed application of OP can not only improve the quality of meat but also render livestock more sustainable.

As described, 95% of all by-products are devoted to low-value uses, particularly to energy use by direct combustion (AGAPA, 2015). The use of OP as solid fuel faces several disadvantages due to its impurities in case of EDOP, and it is a low-value application diminishing the economic income from by-products valorisation (Stegmann *et al.*, 2020).

From the viewpoint of a value chain, OP and EDOP are the by-products with better perspectives to be used as raw material to obtain medium to high-value products, once olive stones are already considered a suitable and high-value solid biofuel.

### 1.4.2. High-value use of olive pomace as a source of functional food ingredients

Nowadays, the food industry is facing the challenge of achieving more sustainable production chains (Teigiserova *et al.*, 2019) while attending to consumers' increasing demand for healthier and functional foods (Torres-León *et al.*, 2018). The reuse of some of its by-products as food ingredients has been emerging as a solution to increase the nutritional composition of food (Coderoni and Perito, 2020; O'Donnell *et al.*, 2015; Torres-León *et al.*, 2018). Food by-products have been proposed as natural sources of numerous macro and micronutrients and bioactive compounds such as proteins, dietary fibre, fatty acids, vitamins, minerals, phytochemicals such as phenolics and carotenoids, bioactive peptides and prebiotics with several potential health benefits (Galanakis, 2012; Torres-León *et al.*, 2018). These novel food ingredients or products have been defined as waste-to-value products (Aschemann-Witzel and Peschel, 2019) or value-added surplus products (Bhatt *et al.*, 2018), to highlight the circular approach that transforms surplus or even by-products, produced during the manufacturing of other foods, into new value-added ingredients or foods with higher nutritional properties and/or health benefits (Coderoni and Perito, 2020).

Consumers' perception and acceptance of these novel foods based on ingredients obtained from by-products have also been recently examined (Aschemann-Witzel and Peschel, 2019; Bhatt *et al.*, 2018; Coderoni and Perito, 2020). These researches suggest a strong potential for such foods to lead position as a new category of foods, as organic foods (Bhatt *et al.*, 2018). Indeed, a core of consumers concerned with sustainability and health seems to emerge, and those consumers value reading food labels and think that food could have environmental or health benefits and are more likely to be willing to buy this new category of foods (Coderoni and Perito, 2020).

The development of novel food products using food by-products offers the opportunity of obtaining new natural-based ingredients that could generate economic gains, contribute to reducing nutritional problems, produce beneficial health effects and reduce the environmental load of these by-products (Torres-León *et al.*, 2018). So, presently, food industries are becoming more and more interested in "zero waste" food systems (by-products are re-inserted into the food chain) in line with the CBE approach to answer the sustainability challenges of the sector and achieve the UN's SDGs (Coderoni and Perito, 2020; Torres-León *et al.*, 2018).

Olive oil is an industry where the conversion of food by-products into novel functional food ingredients is already a reality. The conversion of food by-products into novel functional food ingredients involves several steps as demonstrated in Figure 1.5, where the approach for functional foods development, particularly those expecting health claims, is not yet approved by the European Food Safety Authority (EFSA). The bioactive compounds identified are related to those present in olive oil by-products.

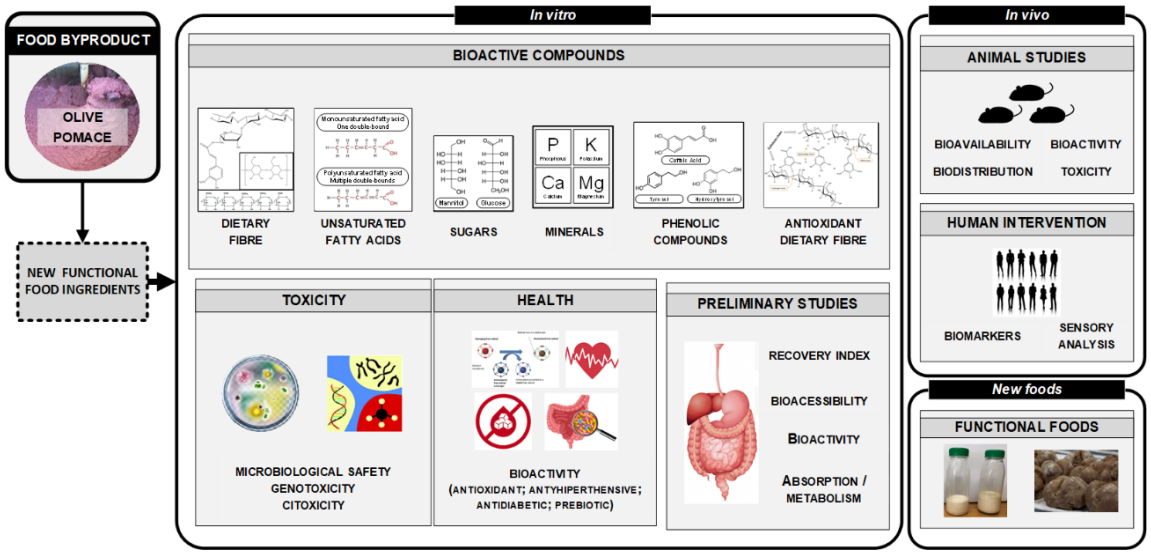



**Figure 1.5. Steps for the conversion of olive by-products into new functional food ingredients.**

### 1.4.2.1. Phenolic composition

OP is considered a rich source of phenolic compounds since 98% of olive phenolics remain in this solid residue (Araújo *et al.*, 2015; Nunes *et al.*, 2018). The main phenolic compound and related derivatives identified in OP is hydroxytyrosol (HYD) (Araújo *et al.*, 2015; Nunes *et al.*, 2018; Rubio-Senent *et al.*, 2012). Other OP phenolics also found in high amounts are oleuropein (Cioffi *et al.*, 2010; Klen and Vodopivec, 2012), tyrosol (Cioffi *et al.*, 2010; Nunes *et al.*, 2018), elenolic acid derivatives such as dialdehydic form of elenolic acid linked to HYD (3,4-DHPEA-EDA) (Suárez *et al.*, 2009), oleuropein derivatives such as oleuropein aglycone (3,4-DHPEA-EA) or demethyloleuropein (Klen and Vodopivec, 2012; Suárez *et al.*, 2009) and protocatechuic acid (Alu'datt *et al.*, 2010). Phenolic compounds such as comselogoside, verbascoside, caffeic acid, vanillin, vanillic acid, apigenin, luteolin, luteolin- 7-*O*-glucoside and rutin have also been identified in the OP, but at lower concentrations (Nunes *et al.*, 2018; Suárez *et al.*, 2009).

HYD is an oleuropein precursor (derivative from hydrolysis of oleuropein) and, compared to tyrosol, it has an additional OH group in its benzene ring. It is one of the most potent antioxidant compounds after gallic acid and one of the most potent antioxidants from OP, followed by oleuropein, caffeic acid and tyrosol (Martínez *et al.*, 2018).

HYD consumption has been associated with several health benefits reported in a significant number of publications (Cicerale *et al.*, 2012; Martínez *et al.*, 2018; Parkinson and Cicerale, 2016). *In vitro* and *in vivo* studies and clinical trials have confirmed several potential activities with significant impact in human health as an antioxidant, anti-inflammatory, anti-atherosclerotic and antimicrobial agent (Cicerale *et al.*, 2012; Martínez *et al.*, 2018; Parkinson and Cicerale, 2016). The HYD anti-inflammatory actions are exerted by attenuation of the expression of pro-inflammatory cytokines as TNF$\alpha$ and interleukin 1 beta (IL1$\beta$) (Gong *et al.*, 2009). On the other hand, HYD can reduce the size of atherosclerotic lesions increasing the low-density lipoprotein (LDL) resistance against oxidation

reducing the size of atherosclerotic lesions in clinical trials (Hernáez *et al.*, 2015), but also to possess significant anti-inflammatory actions in an animal model of inflammation and attenuate the expression of pro-inflammatory cytokines often observed in inflammatory disease as TNF$\alpha$ and interleukin 1 beta (IL1$\beta$) (Gong *et al.*, 2009). The potent antioxidant activity (AOX) of HYD plays a vital role in the prevention of diseases linked to the damaged tissues resulting from the production of reactive oxygen species (ROS) such as cancer, diabetes, cardiovascular and neurodegenerative diseases. HYD and tyrosol's antimicrobial activity has been linked to the bacterial ATP synthase's binding and inhibition capacity (Amini *et al.*, 2017). Other benefits associated with HYD include dermo protective effects, ocular health benefits, and osteoporosis (Eliche-Quesada *et al.*, 2018). More recently, the potential health benefits of HYD to gut health have also been reported (Conterno *et al.*, 2019; Mosele *et al.*, 2014; Žugčić *et al.*, 2019). All the health-benefits activities reported to HYD have also been associated with tyrosol but in less extent (Cicerale *et al.*, 2012; Conterno *et al.*, 2019; Hernáez *et al.*, 2015; Mosele *et al.*, 2014; Zbakh and El Abbassi, 2012; Žugčić *et al.*, 2019).

HYD actions as protector of the cardiovascular system are well established in the literature and accepted by the EFSA (EFSA, 2011). HYD and its derivatives (tyrosol, oleuropein, HYD glucoside, etc.) have been approved with the claim “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” by EFSA for olive oil (20 g) that contains at least 5 mg of HYD and its derivatives (EFSA, 2011). Besides health benefits, HYD has also been applied as a natural antioxidant in foods (Soni *et al.*, 2006; Suárez *et al.*, 2009). HYD showed to avoid the oxidative deterioration of fats and oils (Pazos *et al.*, 2008), but also to reduce the lipid oxidation, microbial contamination and amine formation in meat products (Martínez *et al.*, 2018) and other products (Quaglia *et al.*, 2016).

Nowadays, several HYD-based products are being developed and sold (Khymenets *et al.*, 2016), since EFSA has confirmed that its use does not provide adverse effects to consumers' health (EFSA, 2017). Extracts of olive phenolics including HYD have also been employed in functional beverages (Kranz *et al.*, 2010; Zbakh and El Abbassi, 2012), plant-based foods as bean purée, potato purée and tomato juice (De Toffoli *et al.*, 2019) and cereal foods such as bread and pasta (Cedola *et al.*, 2020). The impregnation of food packaging films with olive phenolic extracts has also been applied to prevent food products' oxidation (Cejudo Bastante *et al.*, 2019; Moudache *et al.*, 2017). A summary of the application of HYD and other olive by-products phenolics extracts in different categories of food products is reported in Table 1.2.

**Table 1.2. Applicability of hydroxytyrosol and other olive phenolics on food products and related beneficial effects.**

| Food | Source of hydroxytyrosol (HYD) and other olive phenolics | Concentration of hydroxytyrosol and other olive phenolics | Beneficial effects | Ref. |
|---|---|---|---|---|
| ***Meat products*** | | | | |
| Fermented sausages | Olive-mill wastewater purified extract | Dipping solution with 2.5–5% of the extract | Inhibition of fungal growth and spore germination *in vivo* and *in situ.*<br>Reduction of oxidative changes. | (Chaves-López *et al.*, 2015) |
| Minced lamb meat enriched in omega-3 fatty acids (with fish oil) | Commercial olive pomace extract Hytolive® - 10.5% HYD and 1.6% tyrosol | 100, 200 and 400 mg of gallic acid equivalents (GAEI/kg meat | Reduced lipid and protein oxidation<br>Increased the shelf-life - Maintained an acceptable colour for a longer time period<br>Olive odour/ flavour no affect acceptability. | (Muíño *et al.*, 2017) |
| Chicken sausages (minced pork fat + chicken meat with walnuts) | Olive-mill wastewater extracts: HYD 23% and HYD 7%<br>Olive leaves extract: HYD 7% | 50 ppm | Reduced lipid and protein oxidation.<br>Higher stability and acceptability of sausages with HYD 23% and olive oil 20% | (Nieto *et al.*, 2017) |
| ***Fat-rich products*** | | | | |
| Food systems rich in fish lipids:<br>- Cod liver oil<br>- Emulsion cod liver oil-in-water<br>- Minced horse mackerel muscle | HYD extract from olive pomace (90-95% purity) - hydrothermal treatment. | 10, 50 and 100 ppm | Increased oxidative stability in similarity with the propyl gallate | (Pazos *et al.*, 2008) |
| French potatoes frying with high oleic sunflower or canola oil | Hydroalcoholic olive leaf extract | 630 mg caffeic acid equivalent/kg oil | Increased tocopherols retention in the frying<br>Decreased the formation of the polar compounds<br>Improved oil thermal stability | (Jiménez *et al.*, 2017) |
| Echium oil (rich in stearidonic acid with high conversion efficiency to eicosapentaenoic acid) | Synthetic commercial HYD extract | 200 ppm | Increased oxidative stability at higher temperatures (> 70º C) | (Bañares *et al.*, 2019) |
| ***Plant products*** | | | | |
| Plant-base foods:<br>- bean purée – proteins/neutral pH<br>- potato purée – starch/ neutral pH<br>- tomato juice – fibre/low pH | Olive-mill wastewater concentrate | 0.44, 1.00, 2.25, 5.06 g/kg | Highest phenolic enrichment in tomato juice and lowest in bean purée.<br>Only bean purée compensated the negative sensorial impact of added concentrate. | (De Toffoli *et al.*, 2019) |
| Decayed pomegranate fruit by *Penicillium adametzioides* | Olive-mill wastewater concentrate (ultrafiltration)<br>Olive-mill wastewater purified extract (Reverse osmosis) | *In vitro* experiment: 1, 2, 4 and 8 mg/mL of total phenols<br>*In vivo* experiment: spray for 2s at 4 and 8 mg/mL of total phenols | Both extracts (4 and 8 mg/ mL): *In vitro* inhibition of mycelial growth and germination of fungi isolate<br>Purified extract: *In vivo r*eduction of the *P. adametzioides p*ercentage disease index. | (Quaglia *et al.*, 2016) |
| Fruit smoothie | Commercial olive leaf extract | 0, 5, 10, 15,20 and 25 mg/100 g | Nutritional enrichment.<br>Bitterness masking was achieved using sucrose and sodium cyclamate. | (Kranz *et al.*, 2010) |

| | | | | |
|---|---|---|---|---|
| Wine | Olive pomace HYD-enriched extract (HT80®, patent WO2007093659A1) | 50 and 80 mg/L of HYD | Improve wine antioxidant /antimicrobial activities.<br>Potential to partially replace $SO_2$ addition. | (Ruiz-Moreno *et al.*, 2015) |
| ***Cereal products*** | | | | |
| Baked snacks with extra virgin olive oil | Olive leaf extract | 400 mg/kg | Improve oxidative stability and shelf life.<br>Higher phenolic content and AOX.<br>Extract addition did not influence taste. | (Difonzo *et al.*, 2018) |
| Biscuits | Olive-mill wastewater purified extract (95% purity) | 6.25 mg of HYD/30 g (average biscuit portion size) | High bioavailability of HYD bioavailability.<br>Significant lowered oxidised-LDL plasma levels. | (Mateos *et al.*, 2016) |
| Cereal foods:<br>- Bread (B)<br>- Spaghetti (S) | Olive-mill wastewater purified extract - OMWW<br>Olive pomace powder (< 500 μm) - OP | OMWW instead water<br>10% (w/w) OP<br>OMWW instead water+10% (w/w) OP | OP: Higher phenolic content and AOX.<br>OMWW+OP: Higher negative effect on odour/taste<br>OP: Higher quality index to bread. | (Cedola *et al.*, 2020) |
| ***Dairy products*** | | | | |
| Cheese 'Fior di latte' | Olive-mill wastewater concentrate extract | 250-500 μg total polyphenols/mL governing liquid (brine + whey) | Retarded *Pseudomonas fluorescens* and *Enterobacteriaceae* spp. growth.<br>Extended shelf-life: 2-4 days. | (Roila *et al.*, 2019) |
| Milk beverage with functional lactic acid bacteria | Olive-mill wastewater concentrate extract | 100 and 200 mg/L | Health benefits: number of viable functional microorganisms > Log 6.0 CFU/mL.<br>No negative effects on sensorial attributes.<br>High total phenolics concentration: 500 mg/kg (100 mL = 20 g of olive oil). | (Servili *et al.*, 2011) |
| Yoghurt | Commercial oleuropein | 0.1 and 0.2 mg/ml of milk | No interference with yoghurt production.<br>Oleuropein stable up to 35 days of storage.<br>No negative effects on sensorial attributes. | (Zoidou *et al.*, 2017) |
| ***Packaging*** | | | | |
| Packaging of multilayer polyethene film (2 LDPE) applied in minced pork meat | Olive leaf extract | 2, 5, 10 and 15% in the adhesive formula | Increase of oxidative stability.<br>Extension of the shelf life (about two days). | (Moudache *et al.*, 2017) |
| Strawberry edible coating of pectin – fish gelatine | Purified HYD + 3,4-dihydroxyphenylglycol (DHPG) from olive tree by-products (ion exchange chromatographic system) | Hydroxytyrosol:0.03-1.8 mg/mL<br>DHPG: 0.03-0.18 mg/mL | Improved amount of natural antioxidant - Vehicle for the delivery of HYD and DHPG.<br>Delay the mould growth.<br>Extension of shelf-life. | (Bermúdez-Oria *et al.*, 2017) |
| Chitosan – olive pomace flour as the packaging of walnuts | OPF: Olive pomace flour (Lyophilization, < 500 nm)<br>OPFM: Olive pomace flour microparticles (spray drying) | OPF: 10, 20 and 30%<br>OPFM: 10, 20 and 30% | OPFM (10%): Films with better structure and performance<br>Preservation of unsaturated fatty profile: Chitosan action until the 26th day, but OPF and OPFM were responsible by the packaging protective effect after (until the 31st day). | (de Moraes Crizel *et al.*, 2018) |

Numerous patents have been developed to recover phenolic compounds and principally HYD from olive oil by-products (Sabatini, 2010). Even though OP is currently the olive by-product produced at the highest amount, the olive phenolics extracts applied in food products reported have been mainly obtained from olive wastewater, or olive leaves (Martínez *et al.*, 2018).

Despite the OP potential as phenolics source has been neglected in comparison to other olive mill by-products, the recovery of phenolic compounds from OP has also been widely investigated (Alu'datt *et al.*, 2013; Cioffi *et al.*, 2010; Japón-Luján and De Castro, 2007; Lafka *et al.*, 2011; Leouifoudi *et al.*, 2015; Obied *et al.*, 2007; Obied *et al.*, 2005; Pérez-Serradilla *et al.*, 2008; Roselló-Soto *et al.*, 2015a; Rubio-Senent *et al.*, 2012, 2015b; Suárez *et al.*, 2010; Uribe *et al.*, 2015; Xie *et al.*, 2019). New phenolic-rich extracts from OP have been tested concerning their health benefits and technological properties as food ingredients (Table 1.2).

Convective hot-air drying has been applied in OP to stabilize it, avoiding modification by microorganisms without affecting the phenolic compounds' bioactive properties (Uribe *et al.*, 2014, 2013). Rotary drying and hot-air drying are the most common drying techniques of OP (Sinrod *et al.*, 2019), but other more sophisticated drying methodologies as freeze-drying (Nunes *et al.*, 2018), vacuum drying (Pérez-Jiménez *et al.*, 2015) and drum-drying (Sinrod *et al.*, 2019) have also been explored. Other pre-treatments have been employed to improve the extraction efficiency of OP phenolics such as acidification (pH 2-3) to promote protein precipitation and increase free phenolic compounds; separation of solid residue from the liquid phase by centrifugation to increase the efficiency of phenolics from the two fractions (Suárez *et al.*, 2009) and defatting by soxhlet/solid-liquid extraction with petroleum ether or *n*-hexane to eliminate the possible interference of lipids with phenolic compounds (a decrease in phenolic content could occur) (Alu'datt *et al.*, 2010; Lafka *et al.*, 2011; Leouifoudi *et al.*, 2015) were applied.

Regarding the methodologies applied to obtain the food antioxidants from OP, some conventional methodologies such as solid-liquid extraction using different solvents like water (Nunes *et al.*, 2018), ethyl acetate (Mulinacci *et al.*, 2005; Suárez *et al.*, 2010) ethanol (Mulinacci *et al.*, 2005) and ethanol/water mixtures (Japón-Luján and De Castro, 2007) combined or not with technologies such as Soxhlet (Mulinacci *et al.*, 2005) and thermal treatment (Rubio-Senent *et al.*, 2012, 2015b) have been explored. Several novel extraction methods including supercritical fluid extraction (SFE) with $CO_2$ (Lafka *et al.*, 2011), pressurised liquid extraction (PLE) (Pérez-Serradilla *et al.*, 2008), ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) (Xie *et al.*, 2019) were also used for enhancing the extraction of HYD and other phenolics from OP. More recently, UAE extraction has also been applied using natural deep eutectic solvents (Chanioti and Tzia, 2018) and cyclodextrins (Albahari *et al.*, 2018), improving the extraction yield and extracts' stability, but also was achieved a patented hydrothermal pre-treatment that allowed to obtain an antioxidant-enriched liquid rich in HYD (Rubio-Senent *et al.*, 2012, 2015b). Some of the principal extraction methodologies applied to OP were summarised in Table 1.3.

**Table 1.3. Literature review of methodologies applied to recovery of phenolic compounds from olive pomace.**

| Methodology: Solvent, extraction conditions | Disadvantages | Ref. |
|---|---|---|
| **Solid-liquid extraction**<br>(1) Solvent: 100% Water<br>Time: 1 h<br>Temperature: 40 ºC<br>Agitation: 600 RPM/ min<br>(2) Separation of liquid from solid fraction<br>Liquid fraction solvent: Ethyl acetate but previously defatted using *n*-hexane<br>Solid fraction solvent: Ethanol/water (80:20, v/v) followed by acidification (pH 2)<br>(3) Solvent: ethanol<br>Time of extraction: 2 h<br>Defatting of extract with hexane | Continuous agitation,<br>Long surface contact<br>Stable temperatures<br>Time and energy-consuming<br>Requires high amounts of solvents | (Nunes *et al.*, 2018; Suárez *et al.*, 2010; Suárez *et al.*, 2009) |
| **Soxhlet extraction**<br>Solvent: Ethanol<br>Time of extraction: 15 h<br>Acidification to pH 2 | Stable temperatures<br>Time and energy-consuming<br>Requires high amounts of solvents | (Leouifoudi *et al.*, 2015) |
| **Hydrothermal Treatment**<br>Patent PCT/ES2011/070583<br>Temperature: 160 ºC<br>Time: 15-90 min<br>Separation of liquid from solid | The high cost of implementation | (Rubio-Senent *et al.*, 2012) |
| **Supercritical fluid extraction with $CO_2$**<br>SC-$CO_2$ flow: 2 g/min<br>Temperature: 40 ºC<br>Pressure: 350 bar<br>Time: 60 min<br>Solvent: Methanol | The high cost of implementation | (Lafka *et al.*, 2011) |
| **Pressurized liquid extraction**<br>Pressure: 10 bar<br>Temperature: 200 ºC<br>Time: 27 min<br>Solvent: Ethanol/water (80:20, v/v) | The high cost of implementation | (Pérez-Serradilla *et al.*, 2008) |
| **Microwave-assisted extraction**<br>Solvents: acetonitrile, ethanol, chloroform, ethyl acetate, n-butyl alcohol, acetone, and tetrahydrofuran.<br>Time: 3, 5, 10, 20, 30 and 40 min<br>Microwave power: 100, 200, 400, 600, 800 and 1000 W<br>Temperature: 30, 40, 50, 60, 70 and 80<br>Agitation: 200 RPM/ min | The high cost of implementation | (Xie *et al.*, 2019) |
| **Ultrasound-assisted extraction**<br>(1) Solvents: acetonitrile, ethanol, chloroform, ethyl acetate, n-butyl alcohol, acetone, and tetrahydrofuran.<br>Probe diameter: 13.0 mm<br>Time: 40 min<br>(2) Solvents: Natural deep eutectic solvents (Component 1: Choline chloride; Component 2: Citric acid or Lactic acid or Maltose or Glycerol<br>ultrasound sonication bath (60 kHz, 280 W)<br>Temperature: 40 or 60 ºC<br>Time: 30 min<br>(3) Solvent: cyclodextrins (0 g/100 mL; 0.8 g/100 mL and 1.6 g/100 mL)<br>Time:3, 6, 9, 20, and 25 minutes<br>Probe diameters: 12.5 and 130 25 mm<br>Power amplitude of ultrasound waves: 50, 75 and 100% | High cost of implementation | (Albahari *et al.*, 2018; Chanioti and Tzia, 2018; Xie *et al.*, 2019) |

#### 1.4.2.2. Antioxidant dietary fibre and carbohydrates

Despite the health and technological benefits of OP phenolics, these compounds represent a minor part of OP. OP is mainly composed of dietary fibre (DF) (Galanakis, 2011; Uribe *et al.*, 2015) and carbohydrates (Lama-Muñoz *et al.*, 2012; Rodríguez-Gutiérrez *et al.*, 2014; Uribe *et al.*, 2015).

DF are carbohydrates polymers with ten (oligosaccharides) or more (polysaccharides) monomeric units resistant to digestion by humans' enzymes. DF includes cell wall components such as cellulose, hemicelluloses, lignin and pectic substances, but also non-structural components such as gums, resistant starch, inulin, that may be associated with other non-carbohydrate components (e.g., phenolics, waxes, saponins, cutin, phytates, resistant protein) (Elleuch *et al.*, 2011; Galanakis *et al.*, 2010a).

Olive pomace contains crushed olive stones (around 10% by wet weight, wet basis) and pulp fraction with different structural and chemical composition (Figure 1.6.). Olive stones have been emphasized by their lignocellulosic composition [cellulose (28 – 40%), hemicelluloses (18 – 32%) and lignin (25 – 27%)] (Ghanbari *et al.*, 2012; Matos *et al.*, 2010; Rodríguez *et al.*, 2008). The whole olive stone comprises the wood shell (stone) and the seed (Rodríguez *et al.*, 2008). Olive pulp/skin contains considerable amounts of cellulose (11 – 23%), hemicellulosic polymers (14 – 17%) mainly rich in xylans and glucuronoxylans (14%) or xyloglucans (15%) and mannans (2 %) and a considerable amount of pectic polysaccharides (30 – 39%) (Lama-Muñoz *et al.*, 2012; Miranda *et al.*, 2019).

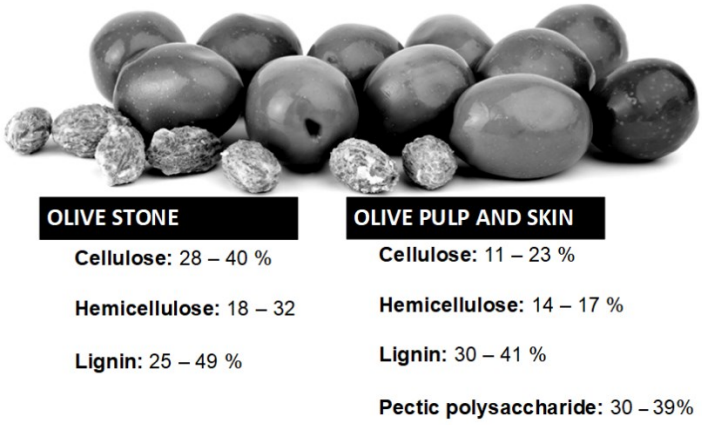



**Figure 1.6. Review of structural carbohydrate composition of olive pomace as mixture of olive stones and olive pulp/ peel** (Ghanbari *et al.*, 2012; Lama-Muñoz *et al.*, 2012; Matos *et al.*, 2010; Miranda *et al.*, 2019; Rodríguez *et al.*, 2008)**.**

The physiological effects or health benefits of DF are well-known. However, DF's physiological effects depend on the relative amount of soluble and insoluble fractions. Soluble dietary fibres (SDF) include pectic substances, gums and some hemicelluloses, whereas cellulose, other types of hemicelluloses and lignin are included in the insoluble dietary fibre (IDF) fraction. IDF represents the main DF fraction of OP (approximately 92%). IDF is known by their porosity, low-density, and ability to increase faecal bulk and decrease intestinal transit (Elleuch *et al.*, 2011).

The vital role of fibre in preventing several diseases has led to the development of a large and potential market for fibre-enriched food products, ingredients, and gelling agents. Nowadays, food by-products have been used as raw material to obtain new DF sources (Galanakis *et al.*, 2010a).

Regarding the extraction of polysaccharides from olive wastes, the OMWW has also been further studied than OP (Galanakis *et al.*, 2010a, 2010b; Nadour *et al.*, 2015). However, one-third of the olive-pulp cell wall comprises arabinose-rich pectin polysaccharides (Cardoso *et al.*, 2003), which turns OP into an appealing pectins source. Therefore, some studies have been reported about pectins' extraction by applying heat treatment in OP (Lama-Muñoz *et al.*, 2012; Rubio-Senent *et al.*, 2015a, 2015b). Moreover, Filotheou *et al.* (2015) described OP olive pulp as a source of efficient emulsifiers (AIS, alcohol-insoluble solids). More recently, xylooligosaccharides (XOS) were extracted from EDOP using a mild hydrothermal treatment. XOS are attractive food ingredients with recognized prebiotic properties (Miranda *et al.*, 2019).

Contrary to that observed for phenolics, only a few studies have analysed biological activities (AOX, antimicrobial activity, prebiotic effect, etc.) of the olive mill's polysaccharides by-products (Nadour *et al.*, 2015). Only the pectin extracted from OP by hydrothermal treatment (160 °C/30 min) (Rubio-Senent *et al.*, 2015a) or by gentle heat treatment (50–80 °C) (Rubio-Senent *et al.*, 2015b) reported above demonstrated both excellent technological properties (high oil holding capacity and emulsifying activity than commercial citrus pectin) and suitable biological properties (higher capacity for binding bile acids and glucose than commercial citrus pectin). Recently, these pectin extracts obtained by hydrothermal treatment (160 °C/30 min) (Bermúdez-Oria *et al.*, 2019a) and gentle heat treatment (80 °C/30 min) (Bermúdez-Oria *et al.*, 2019b) have been studied in order to determine potential anti-tumoral properties. Both revealed higher antiproliferative activity on Caco-2 and THP-1 cell lines than commercial citrus pectin. However, these studies justified the higher inhibition of proliferation using pectins OP extracts than commercial pectins associated with phenolic compounds' content (Bermúdez-Oria *et al.*, 2019b).

The association between phenolics and DF has been reported in several vegetable sources, but the bound phenolics linked to fibre have been ignored in most chemical and biological studies, i.e. discarded in the extraction residue (Macagnan *et al.*, 2016). A new concept of “antioxidant dietary fibre” (ADF) is defined as the complex between phenolic compounds and polysaccharides of the cell wall (Saura-Calixto, 1998). ADF contains over 50% of fibre DW and high AOX with the benefits of bringing polysaccharides and phenolics together (Acosta-Estrada *et al.*, 2014; Beres *et al.*, 2016).

Lignin is a clear example of ADF. Phenolics were commonly found attached to lignin (bound phenolics), which could be released and absorbed in the small intestine (Sato *et al.*, 2011). Generally, IDF has been claimed as a carrier of phenolics throughout the gastrointestinal tract (Saura-Calixto, 2011). Hydroxycinnamic acid derivatives like *p*-coumaric, caffeic and ferulic acids are mainly present in the bound form, linked to cell-walls of structural components, such as cellulose, lignin and proteins through ester bonds (Jakobek and Matić, 2019).

Overall, ADF works as a natural vehicle for phenolic compounds throughout the gastrointestinal tract until the colon, where phenolics could be metabolized during faecal fermentation, and its metabolites

could play an essential role in gut health creating an antioxidant environment (Sato *et al.*, 2011) and stimulating the growth of healthy bacteria (Liu *et al.*, 2019). Besides health benefits, ADF represents an attractive food ingredient that combines a favourable perception by consumers of both DF and antioxidants as health-promoting ingredients, but also technological advantages (e.g. increase of storage stability, improved water- and fat-holding capabilities or functioning as a non-caloric bulking agent), leading to new functional food products (Silva *et al.*, 2018). Hence, the search for natural ADF to be used by the food industry is of great interest.

Numerous fruit and vegetable by-products have been explored to develop new ADF ingredients (Quirós-Sauceda *et al.*, 2014). The occurrence of bound phenolics in OP has been reported in a few studies (Alu'datt *et al.*, 2013; Pérez-Jiménez *et al.*, 2015; Uribe *et al.*, 2015), including the chemical linkages between phenolics and pectins reported above for OP pectin-rich extracts (Bermúdez-Oria *et al.*, 2019a, 2019b). HYD revealed to be present in significant amounts in the OP bound phenolic fraction (Uribe *et al.*, 2015).

Until now, few studies explored OP as a source of ADF and even less investigated the technological and health-benefits of the association between DF and phenolics obtained from OP. Nevertheless, OP was used to replace semolina in OP, increasing the total phenolic compounds and DF amount of pasta simultaneously, which leads to higher AOX OP-fortified pasta (Simonato *et al.*, 2019). In this study, OP was considered a useful ingredient to improving traditional pasta's nutritional quality concerning the extent of starch digestion (reducing the rapidly digestible starch and increasing the slowly digestible starch and the resistant starch). OP gut health benefits were only assessed in a clinical trial after incorporation of OP into biscuits. OP-biscuits led to a significant increase in the gut microbiota's metabolic output and the levels of homovanillic acid and 3,4-dihydroxy-phenylacetic acid (DOPAC), thought to be involved in reducing oxidative LDL cholesterol (Conterno *et al.*, 2019).

Regarding carbohydrates, the major sugars in olive fruit are glucose, fructose, sucrose and mannitol. A higher amount of mannitol was found during olive maturation, i.e., as the mesocarp accumulates storage lipids. Indeed, the relative amount of mannitol in the fruit is often used to indicate the cultivar potential for oil biosynthesis (Conde *et al.*, 2008). Considerable amounts of mannitol were also expected in OP since this by-product is essentially a mixture of pulp and stones from ripe olives. Between olive by-products, olive leaves have been associated with higher amounts of mannitol (Ghoreishi and Shahrestani, 2009).

Mannitol is currently used as a natural and functional sweetener, due to its uptake insulin-independent (non-metabolizable), low–energy (1.6 kcal/g), antioxidant and cryoprotectant properties (reducer of the sugar crystallisation) (Ghoreishi and Shahrestani, 2009). Mannitol has applications in chemical, medical, and pharmaceutical industries too. Mannitol has also been associated with health benefits such as anti-hyperglycemic (Endringer *et al.*, 2014), antihypertensive (Chukwuma *et al.*, 2019) and prebiotic potential (Maekawa *et al.*, 2009).

Few studies have explored olive by-products' potential to extract mannitol and the raw material chosen has been the olive leaves. Extraction of mannitol from olive leaves showed to be viable using supercritical carbon dioxide, subcritical water extraction (Ghoreishi and Shahrestani, 2009) and using

PLE (Lama-Muñoz *et al.*, 2020). Regarding OP, only EDOP obtained after olive oil pomace extraction of OP was recently studied to recover mannitol by aqueous extraction (100 °C/ 30 min) (Manzanares *et al.*, 2020).

Hence, obtaining food ingredients rich in ADF and mannitol is of particular interest in a value chain approach to ensure CBE principles towards a more sustainable olive oil sector.

#### 1.4.2.3. Lipid fraction

OP oil fraction contains a significant amount of liposoluble bioactive compounds that can be converted into high-value products with novel applications in either food or cosmetic industries (Nunes *et al.*, 2018). The remaining oil of OP is characterized by a fatty acid profile rich in unsaturated fatty acids. OP could be considered a good source of nutritionally essential oleic and linoleic acids (Nunes *et al.*, 2018; Uribe *et al.*, 2013).

A mixture of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) composes the lipid fraction of OP (Nunes *et al.*, 2018). The proper amounts of unsaturated fatty acids (UFAs), and especially the high amount of oleic acid (representing 75% of the total fatty acids), which is a MUFA, and the lower content in PUFA (9% of the total fatty acids) make OP more stable to oxidation (Uribe *et al.*, 2013). Besides that, the ratio of oleic acid to linoleic acid is comparable to the one found in virgin olive oil (Nunes *et al.*, 2018).

Oleic acid is attractive to human nutrition for its beneficial effects on blood cholesterol and triglycerides (Lopez-Huertas, 2010). Oleic acid supplementation also showed increased gut microbial diversity, restoring the beneficial microbial population, and positively improved the bodyweight (Mujico *et al.*, 2013). The nutritional value of linoleic acid is also recognized. Linoleic is an essential fatty acid required for growth, physiological functions and maintenance, and hormone-like prostaglandins production with activities that include lowering blood pressure and constriction of smooth muscle (Uribe *et al.*, 2013).

The fatty acid composition of OP makes it suitable for animal nutrition applications, which will indirectly benefit human nutrition, too. Several studies have explored OP as a feed additive, as reported above.

Besides fatty acids, the growing interest in OP oil is due to its bioactive minor constituents, usually included in the unsaponifiable matter (USM). USM contains significant amounts of sterols, fatty alcohols, tocopherols, triterpene alcohols and squalene (Chanioti and Tzia, 2017). Among these compounds, tocopherol and triterpene compounds should be highlighted.

Regarding tocopherols, vitamin E was found in significant amount in OP. Vitamin E is a powerful liposoluble antioxidant that comprises a group of eight vitamers (α, β, δ, γ-tocopherols and α, β, δ, γ-tocotrienols) (Roselló-Soto *et al.*, 2015b). The α-tocopherol is the primary vitamin E found in OP and the most biologically active vitamin E. The α-tocopherol has a significant role in preventing lipid peroxidation and scavenging of lipid peroxyl radicals, contributing to the better oxidative stability of

OP oil (Nunes *et al.*, 2018). Among the health benefits of α-tocopherol is the ability to inhibit platelet aggregation in humans and reduce lipoprotein oxidation, which causes atherosclerosis (Roselló-Soto *et al.*, 2015b).

Triterpenic compounds as oleanolic acid, ursolic acid, maslinic acid, uvaol and erythrodiol can also be found in olive by-products. Triterpenes may have many applications such as flavourings, fragrances, pharmaceuticals, or biocontrol agents. Concerning its health benefits, studies have demonstrated that triterpene compounds display a wide variety of biological effects such as anti-inflammatory, analgesic, antimicrobial, hepatoprotective and virostatic properties (Fernández-Hernández *et al.*, 2015).

These triterpenes have been mainly extracted from olives leaves and olive pruning using conventional and emerging extraction techniques (Roselló-Soto *et al.*, 2015b). OP is a promising source of triterpenic compounds, which have been explored using conventional solid-liquid extraction techniques (Fernández-Hernández *et al.*, 2015). More recently, maslinic and oleanolic acid were extracted from OP using green technologies of MAE and UAE, in comparison with the traditional solvent extraction using ethanol: water mixtures (Xie *et al.*, 2019). UAE was considered the fastest, most effective, greener and most environmentally friendly technique for extracting maslinic and oleanolic acid from OP. Other recent study compared UAE with the conventional soxhlet extraction in terms of the extraction yield of olive pomace oil and its USM quality (Chanioti and Tzia, 2017). UAE and SE exhibited similar yields, but the UAE allowed to obtain an OP oil with higher quality.

Nowadays, there is considerable interest in recovering these valuable liposoluble compounds and further in their utilization as additives in new food products, nutraceuticals and pharmaceutical products. Therefore, the conventional extraction of OP oil using hexane should be rethought in detriment of economic and more sustainable alternatives extraction techniques, which would allow obtaining OP oil with higher bioactives contents and consequently high added-value (Chanioti and Tzia, 2019).

#### 1.4.2.4. Proteins and minerals

OP could also be considered a proper source of proteins (Esteve *et al.*, 2015) and minerals (Uribe *et al.*, 2013). However, the valorisation of proteins and minerals from OP is relatively limited until now.

OP has not been considered a reputable source of protein (not higher than 8%), but essential amino acids were about a notable 40% of the total aminoacidic pool and racemization was quite limited (Prandi *et al.*, 2019). Comparatively to other OP bioactive compounds, the studies regarding the recovery of proteins from OP are scarce. There are some studies on the recovery of protein in OP by using an alcalase-assisted method (Vioque *et al.*, 2000) and also non-thermal technologies like high voltage electrical discharges, pulsed electric fields, and ultrasound, but in the olive cake (three-phase system) (Roselló-Soto *et al.*, 2015a). On the other hand, the olive stone has been described

as a rich source of bioactive compounds, i.e. antioxidants and anti-hypertensive peptides (Esteve *et al.*, 2015).

Regarding minerals, potassium was the predominant in olive by-products, including the OP (Uribe *et al.*, 2013). Other four significant OP minerals are magnesium, calcium, iron and sodium (Rodrigues *et al.*, 2017), all recognised as critical for human nutrition. Higher potassium consumption is associated with cardiovascular disease prevention (D'Elia *et al.*, 2011) and normal blood pressure maintenance (EFSA, 2010). OP has high potassium and minimal sodium content and possesses a mixture of other minerals (magnesium and calcium) that could mask potassium's bitter taste (Inguglia *et al.*, 2017). OP characteristics could be explored by the food industry, not only in the mineral fortification to cardiovascular disease prevention/reduction, but also as taste agent/ replacer of sodium chloride.

### 1.4.3. Other high-value uses

Between the higher-value applications of biomass identified in several studies, the sectors of bioplastics and pharmaceuticals have been highlighted (Stegmann *et al.*, 2020; Teigiserova *et al.*, 2019). In the pharmaceutical sector, it could also be integrated into the segment of cosmetics and nutraceuticals.

Regarding cosmetic applications, the OMWW has been more explored (Di Mauro *et al.*, 2017; Galanakis *et al.*, 2018). Few studies explored OP as a source of compounds with applicability in cosmetics. For example, a fraction obtained from OMWW enriched in sugars and minerals obtained by a green adsorption/desorption process showed that this fraction is stable, safe for topical application and improves skin hydration without skin adverse effects after seven days of use (Di Mauro *et al.*, 2017). Another study investigated the application of different concentrations of phenols recovered from OMWW as UV booster in cosmetics. In this study, olive phenols' entrapment in silica particles and liposomes before their emulsification in cosmetics increased absorption of synthetic UV filters and phenols' water resistance (Galanakis *et al.*, 2018).

Concerning nutraceuticals first is essential to clarify that nutraceuticals are commodities derived from foods with demonstrated physiological benefits, but are used in the medicinal form (pills, capsules, and liquids). On the other hand, functional foods are defined as products that resemble traditional foods but demonstrate physiological benefits (Shahidi, 2009).

According to epidemiological, animal and *in vitro* studies, the regular intake of olive fruits and olive oil has been associated with reducing the risk of beginning and developing cardiovascular diseases, atherosclerosis, diabetes and some types of cancer (Alu'datt *et al.*, 2017). These health benefits have been extended to olive by-products such as OP, which contains the same bioactive compounds and often at higher amounts which have been exploited to develop new nutraceuticals (Araújo *et al.*, 2015; Chanioti and Tzia, 2017; Ghanbari *et al.*, 2012; Icyer *et al.*, 2016; Nunes *et al.*, 2018).

Concerning the antihypertensive action of OP oil, a study identified that the long-term intake of OP oil and OP oil supplemented in oleanolic acid (up to 800 ppm) by spontaneously hypertensive rats

reduced the endothelial dysfunction associated with hypertension by an enhancement in endothelial nitric oxide availability mainly associated to minor components as oleanolic acid (Rodriguez-Rodriguez *et al.*, 2007). Additionally, OP tablets ingestion (4 tablets/day to achieve an intake of 30 mg/day of HYD) for two months reduced participants' cardiovascular risk. The levels of total cholesterol, LDL cholesterol and urea decreased, and calcium levels increased. Besides that, the analysis of the inflammatory profile, finally, revealed a statistically significant reduction in human monocyte chemoattractant protein-1levels (Dinu *et al.*, 2020).

Another study, which extracted short-chain peptides from the olive seed using the protease thermolysin and isolated using ultrafiltration (5 kDa and 3 kDa cut-off filters), exhibited antioxidant and antihypertensive activity maintained even after *in vitro* gastrointestinal digestion (Esteve *et al.*, 2015). Besides the high *in vitro* hypotensive effect, the short-chain peptides extract <3 kDa showed antitumor activity increasing tumour cells' adhesion capacity, decreasing cancer cells' migration capacity and arresting cell cycle on S phase (Vásquez-Villanueva *et al.*, 2018). Previous works also reported breast cancer antiproliferative activity (breast cancer cell line MDA-MB-231) of HYD-rich extracts from EDOP and OP (Ramos *et al.*, 2013). An OP-water extract's therapeutic potential in intestinal bowel diseases was confirmed by data attained after its supplementation to a human intestinal cell in culture (Caco-2) (Di Nunzio *et al.*, 2018).

OP has been pointed out as promising filler for bioplastics and biocomposites to be further incorporated in polymer matrices to produce a range of biocomposites (Hammoui *et al.*, 2015; Lammi *et al.*, 2018). Lamni *et al.* (2018) indicated that dry fractionation is a promising technology for substituting wet-pretreatments and producing raw materials to produce fillers from OP. Other study indicated that the use of clean OP (particle < 125 µm and washed with acetone to remove remaining organic impurities) to reinforce plasticized wheat gluten using glycerol could be an excellent way to produce bio-based materials (Hammoui *et al.*, 2015).

## 1.5. Whole valorisation approaches

### 1.5.1. Integrated value chain options for olive pomace

Besides being a serious environmental problem, OP represents today a precious resource of useful compounds for recovery and valorisation purposes (Souilem *et al.*, 2017). The potential uses of OP are varied, being related to health (cosmetics, pharmaceuticals, food additives, etc.), biofertilizers/compost, animal feed and with the production of alternative energy, perfectly aligned with a vision of implementation of an integrated value chain in the olive oil sector into the direction to a CBE.

Some studies explored the integrated biorefinery concept regarding olive mill waste to produce different value-added products (Goldfarb *et al.*, 2017; Schievano *et al.*, 2015; Serrano *et al.* Other studies identified that olive mill wastes are excellent sources of high-value products such as cosmetics (Rodrigues *et al.*, 2015), food ingredients (Nunes *et al.*, 2016) and even so medium value products as construction materials (Moreno-Maroto *et al.*, 2019) and bioplastics. Extraction

methodologies of high value-added nutrients have been improved; however, until now research has demonstrated little success in finding a more environmentally and economically viable solution to integrate these technologies in a value chain scheme for general adoption.

The most common integrated biorefinery schemes developed have been more focused on the production of energy and antioxidants. Numerous extractions techniques, including conventional and greener, have been adopted to extract phenolics. Schievano *et al.* (2015) explored supercritical $CO_2$ ($SCO_2$) extraction for extracting value-added phenolics and mono/ polyunsaturated fatty acids followed by thermochemical (oxidation or pyrolysis) recovery of bio-oil/syngas, heat/steam and biochar. Furthermore, the same group proposed the biochar activation using $CO_2$ and KOH to obtain a sorbent material for water treatment (Goldfarb *et al.*, 2017).

The pathway of production of methane (renewable energy source) by anaerobic digestion was also explored in some value chain schemes, but prior recovery of phenolics it is imperative to improve the further bio mechanization process. Besides that, this previous step allowed to obtain high-added value compounds. Serrano *et al.* (2017) applied a thermal pretreatment in a reactor (60 min at 170 °C by vapour injection under 0.85 MPa pressure) before anaerobic digestion to recover phenolic compounds in the liquid fraction after centrifugation and purification by an industrial chromatographic system. Recently, the use of deep eutectic solvents in a study showed to have the ability to reduce the temperature of thermal pretreatments. The use of deep eutectic solvents of choline, glycolic acid and oxalic acid (1:1.7:0.3) improved the extraction of soluble sugars (6-fold) and HYD (30-fold) at 120 °C when compared with the treatment at 180 °C (Fernández-Prior *et al.*, 2020).

OP contain other add-value compounds besides phenolics, fatty acids and fermentable sugars that could be valorised before energy application. In this sense, a different cascade approach was developed by Kazan *et al.* (2015) to recover proteins and lignin, besides the oil and fermentable sugars from OP. For that, a sequence of $SCO_2$ to extract residual oil followed by high pressure to obtain proteins (at 220 °C, under a water flow of 2.26 mL $min^{-1}$ at 88 °C, during 30 min) and lignin/sugar recovery using liquid hot water hydrolysis and high-pressure enzymatic hydrolysis were applied. The total recovery of protein, sugar and lignin were 10.8%, 93.7% and 94.4%, respectively (Kazan *et al.*, 2015). This approach allowed to use the sugar-rich hydrolysate to bioethanol production but also obtain oil, protein and lignin fractions that can be further utilized in the formulation of high value-added products (Kazan *et al.*, 2015). Mannitol is another added-value compound which was recovered from OP in a biorefinery context. A pretreatment using water extraction (100 ºC, 30 min) allowed the recovery of a high amount of glucose, phenols and mannitol, which could be further applied in the development of antioxidants and sweeteners (Manzanares *et al.*, 2020).

The olive oil industry's current challenge is the implementation of a food processing chain based on the integrated value chain concept using environmental technologies, aiming full use of olive and transforming its by-products such as OP into high added-value products. However, the valorisation schemes developed to OP until now have been neglected some high-value OP fractions. There is an evident lack of processes that add high value uses to the whole OP. An example of high total use of OP was proposed by Guermazi *et al.* (2017). In this study, a new facility composed of a de-stoner

and a two-phase extraction system is proposed and allowed to obtain not only an extra virgin olive oil rich in natural antioxidants, but also an olive paste with a smoky taste (fermentation 28 days + 10% of brine) for food consumption, and oil from stones (solvent extraction) for cosmetic application and fragmented stone to produce energy.

### 1.5.2. New opportunity for powdered food ingredients

As previously described, many extraction techniques have been widely investigated to recover predominantly phenolic compounds from OP. Most of the techniques employed are solid-liquid extraction methods, not considered “green” practices due to the use of organic solvents. The novel eco-friendly extraction techniques with shorter extraction times and lower or even no-consumption of organic solvents, as reported above, have also been applied with suitable yields. However, these novel techniques possess higher total operational costs, which might force producers to make a substantial investment and/to raise final sale prizes of functional ingredients. Moreover, these bioactive compounds extraction approaches without being integrated into a value chain, only allowed to valorise a small fraction of the OP or the other by-products (García-Lomillo *et al.*, 2014).

Besides these extraction techniques, it is possible to obtain value-added ingredients without any extraction step. Saura-Calixto (1998) and García–Lomillo *et al.* (2014) have proposed the development of powdered products from wine pomace. The powdered products meet the requirements of the food industry to be cheap, environmentally friendly and a natural source of bioactive compounds, with the advantage of preserving proteins, lipids, polysaccharides and phenolics together and in the association, ascribing multifunctional properties to these new food ingredients (Rubio-Senent *et al.*, 2015b).

In recent years, a substantial number of works have shown the importance of phenolics' interactions with lipids, proteins, and carbohydrates in bioaccessibility and bioavailability of phenolics. Moreover, phenolics' interactions with nutrients could protect them from oxidation during their passage throughout the gastrointestinal tract. On the other hand phenolics interaction with lipids could protect lipids from oxidation and reduce fat absorption, but proteins-phenolics interactions can reduce protein absorption and enzymes activities (Jakobek, 2015; Jakobek and Matić, 2019).

It has been demonstrated that there are positive effects of phenolics interaction with the other food components regarding their bioactivity and bioaccessibility (Jakobek, 2015). Consequently, the development of food ingredients that contains a mixture of nutrients and bioactives could increase the bioactivity and health benefits of compounds associated with the synergies observed among them. In this sense, powderization by the reduction of particles size and posterior size standardization (after the previous drying) have been applied to develop new functional ingredients from food by-products as an economical and sustainable added-value strategy that allowed a whole valorisation of by-products (Crizel *et al.*, 2016; Gouw *et al.*, 2017; Gullon *et al.*, 2015).

Fruit pomace powders were investigated regarding their potential to supply DF and phenolics benefits (Crizel *et al.*, 2016; Gouw *et al.*, 2017). Other studies evaluated the effect of *in vitro*

gastrointestinal digestion in recovery and bioaccessibility index of phenolic and flavonoid compounds of pomegranate peel powder and the capacity to produce short-chain fatty acids (SCFAs) during *in vitro* faecal fermentation (Gullon *et al.*, 2015). All these studies reported that using by-products powders might be exciting and viable in the food industry due to their low cost and nutritional properties related to human nutrition. The development of powders from OP has been reported (Pérez-Jiménez *et al.*, 2015; Speroni *et al.*, 2019; Ying *et al.*, 2017). Nevertheless, few works have analysed the effect of digestion in potential health benefits of OP powders as food ingredients (Conterno *et al.*, 2019) or tested their incorporation in foods (Simonato *et al.*, 2019; Ying *et al.*, 2017).

Only one of these studies proposed an integral valorisation of olive in line with CBE principles, without generation of wastes (Pérez-Jiménez *et al.*, 2015). OP pitted were dried (50 °C in a vacuum oven) followed by milling to a particle size of 0.5 mm (centrifuge mill) and then the powder obtained was extracted with n-hexane. The substantial fraction after drying at 70 °C (to remove traces of the solvent) as characterized identify its key components associated with health properties. Hexane is also removed from the oil. Powder revealed to be a potential food ingredient containing more than 90% of the phenolics present in olive, including HYD, DF, oleic acid and polyalcohols. Additionally, the oil obtained exhibits fatty acids and phenolic compounds profiles similar to those of commercial olive oil, and stones were proposed as calorific fuel. Still, this process involves the use of solvents and a substantial energetic cost with two drying processes.

## 1.6. Conclusion

The implementation of a circular bioeconomy (CBE) approach in the olive oil sector represents one of the most challenging objectives of the modern olive oil industry. Nowadays, the olive oil process's main by-product is OP, due to the higher volumes generated and the need to mitigate its phytotoxicity. The low value uses, such as energy generation, were standard practices well implemented in the sector. However, olive by-products exhibit a great richness in high-value compounds with applicability in several industries such as pharmaceutical, food, feed, biocomposites and construction materials, which have been intensively investigated but still reduced applications in the market. The main reasons for the low applicability of the developed solutions are their complexity, high costs and low profit due to the low market interest.

Olive pomace is a natural source of bioactive compounds and nutrients that can be used as food ingredients or supplements. Besides phenolics recovery, other compounds such as antioxidant dietary fibre and unsaturated fatty acids with health benefits could be obtained from OP to generate new food ingredients. Thus, there is the need to adopt integrated value chain options applying easy/clean technologies that allow recovering several OP fractions that could be explored to obtain food ingredients and other value-added applications, before ending as an energy source. Powdered ingredients have been emerging as new functional ingredients containing different bioactive compounds associated with several health benefits in the same ingredient. Powderization and integrated value chains to produce food ingredients may constitute an option for OP's integral

valorisation following circular bioeconomy principles. To date, bioactive compounds of OP have been studied *in vitro*, but few studies evaluated potential health benefits of OP extracts and even less of powders. Moreover, the bioaccessibility of OP food ingredients has been little examined. Further studies of safety, bioactivity, and bioaccessibility of olive powders should be conducted to complete OP's validation as a functional food ingredient.

# Chapter 2.
# Objectives

The main objective of this thesis was the development of an integral valorisation strategy for Olive pomace (OP) by recovering food-grade compounds of high-value towards a “zero waste” goal.

Olive pomace is a valuable source of compounds, such as dietary fibre and unsaturated fatty acids, which until now have been scarcely studied in comparison to other compounds (e.g. phenolics, which have been intensively investigated as food antioxidants). Dietary fibre and phenolics and unsaturated fatty acids from OP could be used in synergy for the development of new food additives and nutraceuticals that can be applied in existing or new food products.

Besides the few studies that explore the bioactivity and functionality of dietary fibre from OP, the occurrence of association between these biomolecules and phenolics has not been investigated so far. Therefore, exploring OP as a source of dietary fibre and vegetable fatty acids can be an excellent opportunity to obtain new functional ingredients such as antioxidant dietary fibre or new antioxidant powders. These new powdered ingredients possess bioactive and functional properties that have potential to be applied in the development of new food products and help the food industry answer the current demands of consumers for natural, sustainable and healthy products while helping to solve the environmental and economic problem of OP management.

The present work was carried out in partnership with ESB-UCP, University of Minho and the enterprise spin-off BLC3 Evolution, the vehicle for industrialization of BLC3 Association. The entire PhD work was elaborated in agreement with the BLC3 Association pillar strategy of creating new business models by developing new bioproducts/ bioapplications from under-valued features such as OP.

The OP was evaluated as a source of bioactive ingredients to develop new food ingredients encompassing the following specific objectives:

I. Establishment of laboratory procedures for sustainable development of multifunctional powders from OP: integral valorisation approach;
II. *In vitro* characterization of the bioactivity, functionality, and suitability application of the developed powders in the food industry;
III. Qualitative and quantitative compositional analysis of the OP powders obtained;
IV. Study of the interactions between phenolic compounds, polysaccharides and fatty acids throughout *in vitro* gastrointestinal digestion;
V. Characterization of the obtained functional ingredients in terms of specific bioactivities (antihypertensive, antidiabetic and prebiotic activity);
VI. Incorporation of the OP powders into yoghurt to develop an improved functional symbiotic product.

# PART II

## *Olive Pomace Valorisation*

# Chapter 3.
# Total and sustainable valorisation of olive pomace using a fractionation approach


## Abstract

Olive pomace management represents a great concern to the olive oil industry. This work focused on developing a “zero waste” strategy for olive pomace based on a fractionation approach resulting in the obtention of different value-added fractions. The physicochemical composition of edible fractions obtained (liquid and pulp) was analysed. The potential use as a solid biofuel of the non-edible fraction (stones) was evaluated. High amounts of hydroxytyrosol (513.61-625.76 mg/100 g dry weight) were present in the liquid fraction. Pulp fraction demonstrated to be a good source of fibre (53-59% dry weight) with considerable antioxidant activity both from free and bound phenolics. The stones fraction exhibited substantial high heating values (18.65-18.94 MJ/kg). All these results support the value-added of the olive pomace fractions combining the biofuel potential from stones fraction and the functional food ingredients potential both from liquid and pulp fractions. The present methodology seems to be a feasible whole valorisation approach to achieve the circularity in the olive oil sector, prioritising high over low added-value products.


***Keywords:*** olive pomace; valorisation; fractionation; food ingredients; solid biofuel; hydroxytyrosol.

## 3.1. Introduction

The reduction and valorisation of by-products are one of the biggest challenges of the food industry. Nowadays, there is an increasing concern for achieving a more sustainable food production chain, specifically, in reducing the environmental impact of its by-products or the costs associated with its proper treatment (Ducom *et al.*, 2020). Besides the economic and environmental consequences, there is also an ethical and moral dimension within the general concept of global food security, since 805 million people worldwide suffer from hunger (Torres-León *et al.*, 2018).

These negative impacts are even more flagrant in growing industries like the olive oil industry (mainly in the Mediterranean countries) that produce large quantities of by-products and wastes (Ducom *et al.*, 2020; Miranda *et al.*, 2019). Portugal is one of the Mediterranean countries where olive oil production has been rising (with an estimated an increase of 30% in olive oil production in 2019 compared to 2018 campaign) (INE, 2020), which also implies the treatment of more tonnes of waste. Between all the olive industry wastes and by-products, the olive pomace (OP) is the most representative (Nunes *et al.*, 2018). OP is a combination of olive husk and pulp, crushed olive stone and olive mill wastewater with a moisture content of 65% that derives from the modern 2-phase extraction method, in which no water is added (more eco-friendlier than the 3-phase process) (Moreno-Maroto *et al.*, 2019; Rubio-Senent *et al.*, 2012).

OP is phytotoxic and non-biodegradable biomass (Nunes *et al.*, 2019) that is challenging to treat due to its richness in moisture (higher energy demand and thus higher costs during the drying process) and organic compounds (adverse effects on soil) (Salomone and Ioppolo, 2012). Nonetheless, OP is also a significant source of bioactive compounds like fibre (Galanakis, 2011), minerals, polyunsaturated fatty acids and phenolic compounds (Nunes *et al.*, 2018).

Commonly, OP is sent to pomace oil extraction mills in which, after a drying process, is used to extract oil using hexane. This process generates pomace oil but also dried pomace that is mainly used as a solid fuel (Miranda *et al.*, 2019) due to its high calorific power (Mata-Sánchez *et al.*, 2014). However, dried OP contains oil residues and principally low weight particles (pulp and olive stones <1mm), which are considered to be harmful compounds related to uncontrolled emission, corrosion and slagging, compromising the performance of OP as solid biofuel (Mata-Sánchez *et al.*, 2015). In a lesser extent, OP can also be composted to avoid the harmful effects of the organic compounds and used as fertiliser (Salomone and Ioppolo, 2012), or even so as used to generate or cogenerate electricity (AGAPA, 2015). However, these traditional OP treatments were considered a wastage of bioactive compounds with high value-added, as these treatments are not the most sustainable options because of their use of solvents and high $CO_{2\text{-eq}}$ emissions (AGAPA, 2015; Salomone and Ioppolo, 2012). Besides that under the aim of a transition from a non-sustainable linear economy (‘take–make–dispose of’) to a circular bioeconomy (maintain the value of products and materials for as long as possible while minimising resource use and waste generation) of the olive oil sector, higher-value uses should be prioritised over the current energy and compost valorisation (Berbel and Posadillo, 2018).

Bioactive compounds of OP have been described as potent food antioxidants and antimicrobials and can even contribute to preventing chronic diseases such as cardiovascular disease and cancer (Rubio-Senent *et al.*, 2012). Considering this, OP should be first employed as food source ingredients and only after as energy generator (Berbel and Posadillo, 2018).

The adoption of valorisation approaches to increase OP biomass's value is crucial to permit the olive oil sector to enhance its economic and environmental sustainability. Many OP valorisation methods have been studied in the last years, but none of these methods appears to answer the principal problem entirely. OP has been explored as a biomass source for biorefineries to produce second-generation ethanol (Miranda *et al.*, 2019); for agricultural use as compost or as irrigation water (Nunes *et al.*, 2019) and as a component in the manufacture of different materials as lightweight aggregates (Moreno-Maroto *et al.*, 2019). Additionally, more studies about the recovery of value-added products have been explored using conventional solvent-extraction or non-conventional extraction techniques (e.g. microwave-assisted, enzyme-assisted and supercritical fluid extraction) (Roselló-Soto *et al.*, 2015b). Nevertheless, in these previous studies, only a few amounts of OP were used, or merely a small fraction was valorised (often phenolic compounds) leaving the majority of OP left untreated (Pérez-Jiménez *et al.*, 2015). Besides that, some of these approaches are not sustainable (the use of water or solvents) or possess high total operational costs (Roselló-Soto *et al.*, 2015b). Therefore, more comprehensive, sustainable and economically viable valorisation approaches for OP need to be developed and validated.

One of the obstacles for achieving a total OP valorisation scheme is linked to its structural heterogeneity and complexity. OP is a mixture of water, residual skin, pulp, and crushed stone pieces (Ying *et al.*, 2017). OP heterogeneity could be the biggest challenge for its valorisation but could also be an advantage to obtain different value-added products from OP. Therefore, the application of a fractionation process appears as a promising alternative for producing value-added OP fractions without any consumption of water or chemicals and lower energy consumption during the drying process (Delisi *et al.*, 2018).

The fractionation approach could produce a liquid, pulp and stone fractions from crude – OP (C-OP). Stone fraction promises to exhibit the best thermal power generation properties due to its uniform size and high density (Mata-Sánchez *et al.*, 2015). On the other hand, liquid and pulp fraction from OP have potential as food ingredients. It is expected that liquid fraction might be a source of phenolic compounds such as hydroxytyrosol (HYD) and derivatives (Nunes *et al.*, 2019) and that pulp fraction a potential source of antioxidant dietary fibre (Quirós-Sauceda *et al.*, 2014), i.e. a rich source of fibre and phenolics compounds (free and bound to fibre) (Alu'datt *et al.*, 2013). Therefore, the valorisation of these bioactive compounds is required at competitive prices, so that it can be used, for instance, as powdered food ingredients.

In this line, the present work focuses on using a fractionation approach (wet and dry fractionation) as eco-friendly "zero" waste solution to obtain new value-added products from OP. The physiochemical/ bioactive properties and calorific potential of the different obtained OP fractions were assessed to validate their potential as valuable ingredients to the energy and food industry.

Moreover, the nutritional profile and chemical composition (regarding proximate composition, sugars, fibre composition, free and bound phenolics) of OP were also assessed.

## 3.2. Materials and methods

### 3.2.1. Chemicals

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, EDTA, sodium sulphite and sodium lauryl sulfate were purchased from Sigma-Aldrich (Sintra, Portugal). Methanol, acetonitrile and sulphuric acid were purchased from Fischer Scientific Portugal. Folin-Ciocalteu's reagent and potassium persulfate were purchased from Merck (Algés, Portugal). Standards of mannitol, glucose, arabinose, mannose, galactose, xylose, galacturonic acid, trolox, gallic acid, vanillin, quercetin, *p*-coumaric, protocatechuic and caffeic acid and were obtained from Sigma-Aldrich (Sintra, Portugal), whereas HYD, tyrosol, luteolin, luteolin-7-*O*-glucoside were purchased from Extrasynthese (Lyon, France).

### 3.2.2. Olive pomace samples

The C-OP was collected from two olive mills with a continuous biphasic extraction system (OM 1 and OM 2) from Portugal's Inner Centre Region. The main olive cultivar is *Galega Vulgar* (80% of the olive heritage). The samples were homogenised, packed in polyethene flasks, and stored (- 80 ºC) until use.

### 3.2.3. Fractionation of olive pomace

Fractionation of the C-OP samples (OM 1 and OM 2) was carried out to produce liquid, pulp and stones fractions following the scheme evidenced in Figure 3.1. The first step is the wet fractionation by centrifugation (4000 RPM, 10 min) to separate the liquid fraction from the solid, followed by freeze-drying (Telstar Lyo Quest HT 40). The dry fractionation was applied in the freeze-solid by sieving after a previous milling process with a coffee grinder obtaining the pulp fraction (particle size < 1 mm) and the stones fraction (particle size > 1 mm). The stone fraction and edible fractions (liquid and pulp fractions) obtained from olive pomace were vacuum packed and stored in a dark and dry place at room temperature, until use. The C-OP samples were also freeze-dried, milled and sieved as control.

### 3.2.4. Chemical composition determination

#### 3.2.4.1. Proximate composition

The moisture content (AOAC No. 934.06) was determined in the fresh and dried samples. The contents in crude fibre (Weende method), crude protein (Kjeldahl method with conversion factor: 6.25), lipid (AOAC 920.39), crude ash (AOAC No. 942.05) and carbohydrate (calculated by difference from crude fibre, moisture, protein, lipid and ash) were estimated in freeze-dried samples. All

methodologies followed the recommendations of the Official Methods of Analysis (AOAC, 1990). All analysis were done in triplicate. The chemical compounds were expressed as g/100 g dry weight (DW).

#### 3.2.4.2. Detergent fibre

The contents of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the method of Goering and Van Soest (1970). NDF measures all insoluble cell wall material, including hemicelluloses, cellulose, and lignin; and ADF) measures cellulose and lignin.

#### 3.2.4.3. Cellulose, hemicellulose, and lignin

For cellulose (as glucose), hemicellulose (as arabinose, mannose, galactose and xylose) and lignin (soluble and insoluble) determination of C-OP, liquid and pulp fraction samples the methodology of Sluiter *et al.* (2012) was followed. Previous the removal of extractives was carried out using a Soxhlet extraction system (SER 148, Velp) using as solvents ultrapure water and absolute ethanol in two sequential stages (Sluiter *et al.*, 2008). Then the free-extractives samples were submitted to 2-step sequential acid hydrolysis and further determination/ quantification of the cellulose, and hemicellulose content was achieved using high-performance liquid chromatography (HPLC). Structural carbohydrates were determined by HPLC (micro guard column: Aminex Carbo-P, Bio-Rad; carbohydrate analysis column: Aminex HPX-87P heavy metal, 300–7.8 mm, Bio-Rad; flow rate: 0.6 mL/min; detector: refractive index) and were used to calculate the cellulose (as glucose) and hemicellulose (as arabinose, mannose, galactose, and xylose) content. The insoluble lignin content was calculated gravimetrically after hydrolysis residue filtration, and soluble lignin was estimated by UV spectrophotometry at 340 nm. The results were expressed in g/100 g DW.

#### 3.2.4.4. Extractable Pectins

Extractable pectins from the C – OP, liquid and pulp fraction samples were fractionated into water-soluble pectin, chelator-soluble pectin, and hydroxide-soluble pectin and quantified, according to Deng *et al.* (2011). First, 1.0 g of the sample was homogenised in 20 g of deionised water for 10 min. The homogenate was filtrated (Whatman No. 1 filter paper) and the retentate and filtrate collected. Water-soluble pectin was determined as the precipitate that resulted from the addition of 95% ethanol to the filtrate (1:5) and then allowing it to stand overnight in a refrigerator. Chelator-soluble pectin was determined from the water-extracted residue, by boiling the residue with 95% ethanol for 10 min, followed by 3 successive extractions of the resulting residue with 50 mL, 20 mM $Na_2$-EDTA, and pH 8.0. Following each extraction, the suspension was filtered, and the filtrates were combined. The residue obtained from the $Na_2$-EDTA extractions was subsequently extracted with 50 mM NaOH (50 mL) for 15 min at room temperature; the suspension was filtered and the filtrate collected to measure hydroxide-soluble pectin. The referred quantification was based on a colourimetric assay (AOAC 994.13, 2007) using galacturonic acid to determine the calibration curve. Briefly, 250 µL boric acid–sodium chloride solution (content) and 250 µL of the sample (or standard) were mixed with 4 mL of

96% $H_2SO_4$ and incubated at 70 °C for 40 min. Afterwards, 200 µL of dimethylphenol reagent (100 mg of 3, 5-dimethylphenol in 100 mL of glacial acetic acid) was added and mixed, and the absorbance of the resulting solution was measured at 400 and 450 nm, respectively (Deng *et al.*, 2011). Water-soluble, chelator-soluble, and hydroxide-soluble pectins were quantified as galacturonic acid equivalents (GUAE).

#### 3.2.4.5. Soluble sugars

The soluble sugars were estimated in ultra-pure water extractives using the phenol-sulphuric acid method for total carbohydrates (Nielsen, 2010). The sugar content was determined by thoroughly mixing 80 µL of the soluble sugar-containing solution with 2 mL 98% $H_2SO_4$ and 320 µL phenol 5%, incubating during 15 min the reaction mixture at 100 °C, cooling (room temperature), and measuring absorbance at 490 nm. Using a calibration curve obtained with D- (+) -glucose. Results were expressed as g glucose equivalents/100 g DW. Free sugar profiles were determined by HPLC coupled to a refraction index detector using an Aminex 87-H column (Bio-Rad, Berkeley, USA) at 55 °C and 35 mM $H_2SO_4$ as mobile phase (flow rate: 0.5 mL/min) (Campos *et al.*, 2020b). Sugar identification was achieved by comparison of the retention times of sample peaks with glucose and mannitol standards. The results were expressed in g/100 g DW.

### 3.2.5. Structural Characterization

#### 3.2.5.1. Chemical Groups and Bonding Arrangement of Constituents

FTIR spectra of C-OP fraction samples (liquid, pulp and pulp fraction extractives free) were recorded using an IRAffinity-1S, Shimadzu. The spectra were collected through the wavenumber range of 600 $cm^{-1}$ to 4000 $cm^{-1}$, with a resolution of 4 $cm^{-1}$ for 50 scans. The resulting spectra were baseline corrected and analysed using Origin Lab software.

### 3.2.6. Bioactive Characterization

#### 3.2.6.1. Free and bound phenolic compounds

The extracts of free phenolic compounds were obtained according to the Alu'datt *et al.* (2013) with some modifications. Samples (1.5 g) of C-OP, liquid and pulp fraction samples were extracted on an orbital shaker (250 RPM) using methanol (25 mL) for 60 min (twice), followed by centrifugation (4000 RPM, 10 min) and supernatant recuperation. The extraction of bound phenolic compounds was achieved according to Xie *et al.* (2015) with some adaptations. The extraction residue was hydrolysed for 4 h (20 mL of 4 M NaOH, orbital shaker at 250 RPM) followed by acidification to pH 1.5–2.0 using 6 M HCl. After 30 min of centrifugation (8000 RPM), the supernatant was extracted 5 times with ethyl acetate (30 mL). The ethyl acetate was dried using anhydrous $Na_2SO_4$ and evaporated to dryness using a rotary vacuum evaporator at 30 °C. The resulting residue was subsequently dissolved in methanol to a final volume of 10 mL. The free and bound phenolic extracts obtained were stored at −20 °C until use.

The total phenolic content (TPC) of free and bound phenolic extracts was determined according to the Folin-Ciocalteu's method (Oliveira *et al.*, 2016). Briefly, 50 µL of the methanolic extract was mixed with 50 µL Folin-Ciocalteu's reagent, 1 mL of $Na_2CO_3$ (75 g/L) and 1.4 mL of ultra-pure water. The reaction tubes were incubated for 60 min at room temperature in the dark. The absorbance of the reaction mixtures was measured at 750 nm using gallic acid as a standard. All analyses were performed in triplicate. Results were expressed as mg gallic acid equivalents (GAE)/ g DW.

To identify the phenolic compounds, the extracts were analysed in an LC-ESI-UHR-QqTOF-MS system (Bruker Daltonics) following the methodology of Monforte *et al.* (2018) with some modifications in gradient elution program to obtain a good separation of phenolic compounds: 0–5 min (5% B); 5–25 min (15% B); 25-35 min (30%); 35-40 min (95% B), 40-41 (5% B) and 41–42 min (0% B). Identification of main phenolic compounds was based on standard solutions retention time, UV-Vis, and mass spectra, when available. The other peaks were tentatively identified based in the literature, and its elemental composition was confirmed according to accurate mass (within 5 mDa of the assigned elemental composition) and isotope rate calculations designated mSigma (mSigma values of <20). The main phenolic compounds identified were quantified by HPLC (Waters e2695 separation module system interfaced with a Photodiode array UV/Vis detector) using external calibration curves constructed based on their maximum UV signal (280, 320 and 360 nm) following Oliveira *et al.* (2015) procedure. The separation was performed in a reverse-phase column (COSMOSIL 5 C1 8-AR-II packed column – 4.6 mm I.D. × 250 mm; Dartford, UK). Sample and standard solutions were analysed using a gradient program at a flow rate of 1 mL/min: 0–2 min (100% A); 2–28 min (60% A); and 28–30 min (100% A). A water/acetonitrile (95:5 v/v) with 0.2% TFA solution was used as mobile phase A; while mobile phase B was constituted of acetonitrile (100%). Identification of main phenolic compounds (3-HYD, protocatechuic acid, tyrosol, oleuropein, luteolin-7-O-glucoside, caffeic acid, rutin hydrate, p-coumaric acid, luteolin and quercetin) in methanol was performed by comparison of retention times, spectra and peak areas at maximum absorption wavelength. The results of the main phenolic compounds were expressed as mg/100 g DW.

#### 3.2.6.2. Antioxidant activity

The free and bound phenolic extracts were used to evaluate the radical scavenging capacity of C-OP, liquid and pulp fraction samples according to 3 methods: DPPH (Alexandre *et al.*, 2019), ABTS (Cano *et al.*, 2000) and ORAC (Oliveira *et al.*, 2016). In DPPH method, 1.75 mL of a 60 µM $DPPH^{\bullet}$ methanolic solution was added to 250 µL of sample and incubated at 30 °C for one h. The results were obtained by absorbance measurement at 515 nm in a spectrophotometer. $ABTS^{\bullet+}$ was generated through a chemical oxidation reaction with potassium persulfate (Cano *et al.*, 2000). ABTS radical concentration was adjusted with methanol to an initial absorbance of 0.700 ± 0.020 at 734 nm. To 200 µL of this solution of $ABTS^{\bullet+}$, 20 µL of the sample or Trolox or solvent were added, using a 96- well plate. The mixture was incubated (30 °C) for 5 min, and the absorbance at 734 nm was measured with a microplate reader. In ORAC assay the reaction was carried out at 40 °C in 75 mM phosphate buffer (pH 7.4), and the final assay mixture (200 mL) contained fluorescein (70 nM), AAPH (14 mM), and antioxidant (Trolox or sample, at different concentrations). The fluorescence was

recorded during 137 min (104 cycles) in an FLUO star OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUO star Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH, Trolox and fluorescein solutions were diluted in 75 mM phosphate buffer (pH 7.4). Trolox was used as a standard, and a blank control was performed using a sample solvent to all the methods. All values of antioxidant activity (AOX) were performed in triplicate and expressed in μM Trolox-equivalents (TE)/ g DW.

#### 3.2.7. Energy potential

The higher heating values (HHVs) of C-OP, liquid and pulp fraction samples were measured by an automatic adiabatic bomb calorimeter (Parr calorimeter Type 6200). Measurements were made in duplicate (1 g of sample) according to EN 14918 and following (Pontes *et al.*, 2018). The samples of crude olive pomace and its pulp fraction were compacted in pellets before analysis, to prevent uncontrolled combustions. The interior surface of the bomb was washed with distilled water and collected in a beaker. The bomb washings were titrated with a standard sodium carbonate solution (0.0709 N). The pH and moisture content of stones fraction samples were evaluated to validate their quality as solid biofuel.

#### 3.2.8. Evaluation of the potential valorisation of olive pomace using the fractionation approach in the centre region of Portugal: A case study

To illustrate the potential of the proposed approach, a case study was evaluated considered the situation in the centre region of Portugal (third major olive oil producer region in Portugal) using the data from Instituto Nacional de Estatística (INE) (INE, 2019).

#### 3.2.9. Statistical analysis

Data obtained for all parameters were analysed using a one-way ANOVA. Tukey's post hoc test was applied to compare means; differences were considered significant at $p < 0.05$. The Shapiro – Wilk test tested the normality of data distribution. Statistical analyses were carried out using R Software.

## 3.3. Results and Discussion

#### 3.3.1. Fractionation approach

The wet and dry fractionation applied to C-OP samples from OM 1, and OM 2 showed to be reproducible. The yield (% DW) for the different fractions was similar for both C-OP samples. Figure 3.1 presents the proposed process for OP "Zero Waste" valorisation, including the principal mass balances. The first step of wet fractionation (centrifugation) led to two different fractions: sediment and supernatant. The sediment (solid fraction) was the most representative fraction for both C-OP samples (63-53% of C-OP fresh weight). As a result of this first fractionation, the moisture and sugar

concentration of solid fraction was reduced facilitating its drying, and the C-OP phenolics' were recovered mainly in the liquid fraction (supernatant) (Delisi *et al.*, 2018). In the present approach, the wet fractionation (centrifugation) followed by freeze-drying allowed to obtain 0.02 to 0.03 kg of L-OP from 1 kg of the wet OP, showing a yield of 9% (DW) for the both C-OP samples analysed. A higher water loss occurred during the drying of liquid and solid fraction from OM 2 due to their higher moisture (liquid fraction: 95.29 ± 0.81; solid fraction: 68.18 ± 1.86) than liquid and solid fraction obtained from OM1 (liquid fraction: 88.64 ± 0.36; solid fraction: 62.07 ± 0.08).

After the drying step and dry fractionation (milling and sieving), two different fractions were obtained, the stones and pulp fraction. For both C-OP samples, the pulp fraction (0.16 – 0.23 kg from 1 kg of wet OP) had the highest yield (% DW) around 65% and 71% for OM 1 and OM 2 sample, respectively. On the other hand, a yield (% DW) between 20-26% was attained for stones.

Other fractionation approaches have been applied to OP (Delisi *et al.*, 2018; Ying *et al.*, 2017). Delisi *et al.* (2018) obtained an extract rich in phenolic compounds and OP oil from liquid fraction and a solid biofuel from solid fraction. The simple conversion of solid fraction in solid biofuel neglected not only the high value of the compounds like fibre and phenolics presented in this fraction but also compromise its performance as a solid biofuel due to the risk of occurrence of uncontrolled combustion triggered by the presence of fat and small particles (Mata-Sánchez *et al.*, 2015). On the other hand, Ying *et al.* (2017) ignored the stones fraction's energy potential completely, using a considerable amount of water prior centrifugation and discarded considerable amount of OP pulp fraction (not using a dry fractionation step to separate the pulp from stones). Neither of these two studies reported the yields of each of the fractions obtained from OP fractionation. Besides that, the literature reported approaches have not explored the OP whole fractions potential, compromising the quality of the added-value products and the "zero waste" goal. In contrast, the fractionation approach proposed in this work appears to be a promising and sustainable alternative to create different added-value products from OP biomass without any consumption of water nor chemicals, and prioritising firstly higher values applications and lastly the energy valorisation according to circular bioeconomy principles (Venkata Mohan *et al.*, 2019).

### 3.3.2. Proximate composition

The proximate composition of C-OP, liquid and pulp fraction samples is presented in Table 3.1. The liquid (2.67 – 2.73 g/100 g DW) and pulp fraction (0.30 – 0.99 g/100 g DW) exhibited lower moisture content. When comparing the liquid and pulp fraction, the liquid fraction showed to contain a higher amount of carbohydrates (73.34 – 77.17 g/100 g DW) and ashes (11.27 – 10.74 g/100 g DW). An equivalent amount of carbohydrates (82.4% DW) were obtained by Ying *et al.* (2017) to their liquid fraction, however, reported a lower ash content (3.5% DW) that could result from the non-environmental addition of water (Aggoun *et al.*, 2016). On the other hand, pulp fraction was characterised by C-OP's inherent fibre richness (Uribe *et al.*, 2014) exhibiting a high crude fibre content (54-55% DW). Ying *et al.* (2017) reported lower fibre content in their flesh-enriched fraction (42.7% DW), probably because of the discharge of a substantial amount of pulp together with stones and the water addition (extraction of soluble fibres to supernatant). Besides that, pulp fraction

exhibited a significant amount of protein (8-9% DW) and fat (15-21% DW). The significant differences in fat and ash composition of the pulp fractions obtained from OM 1 and OM 2 could be explained by differences in olive varieties and maturation degree (Conde *et al.*, 2008). Comparing the proximate composition from the liquid, and pulp fraction samples obtained from the two crude olive pomace samples used (OM 1 and OM 2), a similar composition was attained.

### 3.3.3. Detergent fibre

The study of the crude olive pomace, liquid, and pulp fraction samples' detergent fibre composition is presented in Table 3.1. Neutral detergent fibre is a good predictor of dietary fibre's nutritional value (Van Soest *et al.*, 1991), especially in insoluble fibre rich samples like OP (Miranda *et al.*, 2019). In the liquid fraction, the neutral detergent fibre content was defined as “not detected”, due to the limitation of neutral detergent fibre method regarding water-soluble fibre measurement. Despite this limitation of the method, pulp fraction exhibited a high amount of neutral detergent fibre (53-59% DW) close to the value of crude fibre reported above and higher than the total dietary fibre reported by Ying *et al.*, (2017) to their flesh-enriched fraction (42.7% DW). This high content of neutral detergent fibre reported to pulp fraction in this study supports its potential application in food formulation. Fibre fortification is growing due to its well-known health-promoting properties (Quirós-Sauceda *et al.*, 2014).

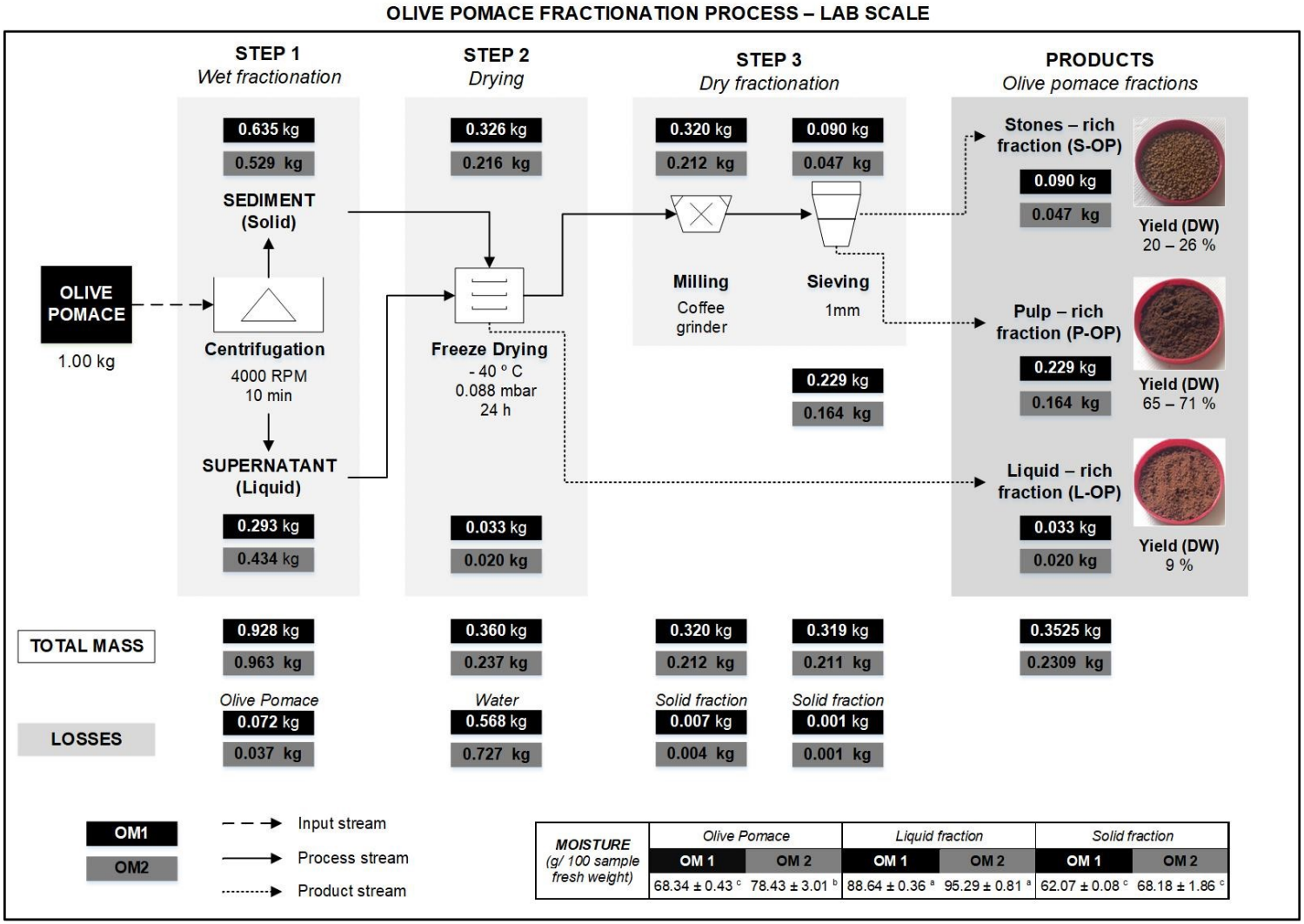



**Figure 3.1. Proposed fractionation process for olive pomace valorisation to achieve maximum value and "Zero Waste": products and yields.**
OM 1 – Olive pomace from olive mill 1; OM 2 – Olive pomace from olive mill 1; C-OP – Crude olive pomace; L-OP – liquid-rich fraction; P-OP – pulp-rich fraction.

**Table 3.1. Chemical composition of crude olive pomace (C-OP) and edible olive pomace fractions (I-OP and P-OP) produced after the wet and dry fractionation process (g/100 g DW).**

| Chemical components | | C-OP | | L-OP | | P-OP | |
|---|---|---|---|---|---|---|---|
| | | OM 1 | OM 2 | OM 1 | OM 2 | OM 1 | OM 2 |
| **PROXIMATE COMPOSTION** (g/100 g DW) | *Moisture* | 0.93 ± 0.01 $^{b}$ | 0.98 ± 0.07 $^{b}$ | 2.66 ± 0.31 $^{a}$ | 2.94 ± 0.01 $^{a}$ | 0.63 ± 0.04 $^{b}$ | 0.91 ± 0.04 $^{b}$ |
| | *Ash* | 4.48 ± 0.09 $^{c}$ | 4.93 ± 0.09 $^{d}$ | 10.74 ± 0.21 $^{b}$ | 11.27 ± 0.18 $^{a}$ | 3.11 ± 0.19 $^{e}$ | 1.97 ± 0.03 $^{f}$ |
| | *Crude Fibre* | 35.90 ± 1.32 $^{b}$ | 31.94 ± 1.44 $^{b}$ | 0.13 ± 0.10 $^{c}$ | 0.09 ± 0.01 $^{c}$ | 54.54 ± 2.63 $^{a}$ | 54.08 ± 2.06 $^{a}$ |
| | *Protein* | 8.75 ± 0.13 $^{a}$ | 8.82 ± 0.15 $^{a}$ | 3.80 ± 0.24 $^{d}$ | 4.41 ± 0.12 $^{c}$ | 7.98 ± 0.18 $^{b}$ | 8.71 ± 0.12 $^{a}$ |
| | *Fat* | 15.61 ± 1.37 $^{b}$ | 20.04 ± 0.58 $^{a}$ | 2.68 ± 0.29 $^{d}$ | 5.56 ± 0.50 $^{c}$ | 14.99 ± 0.41 $^{b}$ | 21.34 ± 0.94 $^{a}$ |
| | *Carbohydrates* | 33.28 ± 2.37 $^{b}$ | 32.31 ± 1.29 $^{b}$ | 77.17 ± 0.88 $^{a}$ | 73.34 ± 1.12 $^{a}$ | 18.77 ± 2.87 $^{c}$ | 11.92 ± 1.89 $^{d}$ |
| **DETERGENT FIBRE** (g/100 g DW) | *NDF* | 46.48 ± 1.47 $^{bc}$ | 40.76 ± 2.76 $^{c}$ | ND | ND | 53.29 ± 0.46 $^{a, b}$ | 59.28 ± 1.98 $^{a}$ |
| | *ADF* | 31.06 ± 0.73 $^{b}$ | 25.34 ± 0.95 $^{c}$ | ND | ND | 33.51 ± 0.88 $^{a, b}$ | 36.66 ± 0.62 $^{a}$ |
| **STRUCTURAL CARBOHYDRATES** (g/100 g DW) | *Cellulose (as glucose)* | 9.55 ± 0.38 $^{ab}$ | 8.60 ± 0.54 $^{b}$ | ND | ND | 10.90 ± 1.26 $^{a}$ | 10.32 ± 0.68 $^{ab}$ |
| | *Hemicellulose* | 11.29 ± 0.50 $^{ab}$ | 10.28 ± 0.25 $^{b}$ | ND | ND | 11.85 ± 0.72 $^{a}$ | 12.40 ± 0.94 $^{a}$ |
| | *Xylose* | 8.03 ± 0.26 $^{b}$ | 6.50 ± 0.21 $^{b}$ | ND | ND | 8.07 ± 0.51 $^{a}$ | 8.35 ± 0.69 $^{a}$ |
| | *Arabinose* | 0.36 ± 0.13 $^{b}$ | 0.83 ± 0.13 $^{a}$ | ND | ND | 0.61 ± 0.11 $^{a, b}$ | 1.70 ± 0.06 $^{a}$ |
| | *Mannose* | 1.02 ± 0.32 $^{a}$ | 1.06 ± 0.20 $^{a}$ | ND | ND | 1.05 ± 0.15 $^{a}$ | 1.26 ± 0.21 $^{a}$ |
| | *Galactose* | 1.88 ± 0.05 $^{a}$ | 1.79 ± 0.05 $^{b}$ | ND | ND | 2.12 ± 0.03 $^{a}$ | 2.07 ± 0.01 $^{a}$ |
| | *Lignin* | 43.95 ± 1.31 $^{a}$ | 42.48 ± 0.56 $^{a}$ | ND | ND | 43.38 ± 0.32 $^{a}$ | 45.72 ± 1.76 $^{a}$ |
| | *Insoluble* | 26.84 ± 0.76 $^{a}$ | 25.06 ± 1.69 $^{a}$ | ND | ND | 23.62 ± 0.94 $^{a}$ | 26.49 ± 1.53 $^{a}$ |
| | *Soluble* | 17.12 ± 0.76 $^{a}$ | 17.42 ± 1.21 $^{a}$ | ND | ND | 19.76 ± 0.63 $^{a}$ | 19.23 ± 0.24 $^{a}$ |
| **PECTINS** (g GUAE/ 100 g DW) | *TSP* | 3.23 ± 0.50 $^{a}$ | 2.92 ± 0.24 $^{a}$ | 0.69 ± 0.28 $^{b}$ | 1.33 ± 0.16 $^{b}$ | 3.37 ± 0.90 $^{a}$ | 2.64 ± 0.15 $^{a}$ |
| | *WSP* | 0.64 ± 0.22 $^{de}$ | 1.00 ± 0.16 $^{b}$ | 0.77 ± 0.12 $^{bc}$ | 1.32 ± 0.16 $^{a}$ | 0.49 ± 0.08 $^{cd}$ | 0.46 ± 0.16 $^{d}$ |
| | *CSP* | 2.50 ± 0.34 $^{ab}$ | 1.86 ± 0.26 $^{b}$ | ND | ND | 2.86 ± 0.81 $^{a}$ | 2.12 ± 0.10 $^{ab}$ |
| | *HSP* | 0.09 ± 0.04 $^{a}$ | 0.17 ± 0.10 $^{a}$ | ND | ND | 0.02 ± 0.01 $^{a}$ | 0.06 ± 0.01 |
| **SOLUBLE SUGARS** (g/100 g DW) | *Total soluble sugars* $^{*1}$ | 6.56 ± 0.36 $^{e}$ | 9.36 ± 0.48 $^{c}$ | 19.01 ± 3.74 $^{b}$ | 28.78 ± 3.40 $^{d}$ | 2.36 ± 0.25 $^{c}$ | 4.41 ± 0.16 $^{c}$ |
| | *Glucose* | 6.85 ± 1.42 $^{b}$ | 6.31 ± 0.61 $^{bc}$ | 19.75 ± 2.00 $^{c}$ | 20.37 ± 1.74 $^{c}$ | 4.00 ± 0.90 $^{bc}$ | 2.67 ± 0.61 $^{c}$ |
| | *Mannitol* | 7.16 ± 1.40 $^{cd}$ | 10.55 ± 1.66 $^{c}$ | 21.10 ± 2.55 $^{c}$ | 32.37 ± 2.65 $^{b}$ | 4.81 ± 1.61 $^{c}$ | 4.08 ± 0.88 $^{d}$ |

OM 1 – olive pomace from olive mill 1; OM 2 – olive pomace from olive mill 2; C-OP – crude olive pomace; L-OP – liquid fraction; P-OP – pulp fraction. NDF – Neutral detergent fibre; ADF – Acid detergent fibre. TSP – Total soluble pectins; WSP – Water-soluble pectins; CSP – Chelator soluble pectins; HSP – Hydroxide soluble pectins. ND – Not detected. $^{*1}$ g Glucose equivalent /100 g sample dry weight. Data were expressed as mean ± SD (n = 3). The different superscripts in the same row represent significant differences between samples ($p < 0.05$)

### 3.3.4. Cellulose, hemicellulose and lignin

As validated by the crude fibre and the detergent fibre method, fibre is the most abundant C-OP and pulp fraction component. Polymerised sugars compose fibre into cellulose and hemicellulose structures, and HPLC can quantify that sugars after acid hydrolysis. The cellulose and hemicellulose content of C-OP samples was not significantly different ($p < 0.05$). Hemicellulose (as the sum of xylose, arabinose, galactose and mannose) was little more abundant in the pulp fraction (12% DW) than cellulose (10-11 DW), being xylose (8%) the main hemicellulose sugar, followed by galactose (2%) (Table 3.1). The pulp fraction richness in xylose turns its potential application in the food industry even more feasible. Xylans have unique properties, like the capacity to interact with bile and cholesterol or activate colon peristalsis and important technological properties (Sedlmeyer, 2011).
Considering that the olive endocarp is mostly lignified (Miranda *et al.*, 2019), the lignin content is high in pulp fraction (42-46%). Lignin is a macromolecule inert for colonic fermentation but can be released when linked to low molecular weight phenolics (bound phenolics as vanillin and caffeic acid). The potential AOX of these phenolics can play an essential role in gut health (Sato *et al.*, 2011). Lignin has also been described as an antiradical scavenger, with antioxidant, antimicrobial and immunological activity (Dong *et al.*, 2011).

### 3.3.5. Pectins quantification

Some studies have reported pectins' extraction from olive by-products (Rubio-Senent *et al.*, 2015b). The extractable pectins of edible fractions (liquid and pulp fraction) were estimated (Table 3.1). The total extractable pectins were not significantly different in the crude olive pomace and pulp fraction samples ($p < 0.05$). Overall, the pulp fraction showed the highest total extractable pectins amount (2.64-3.37 g GUAE/100 g DW), and chelator-soluble pectins were the most significative corresponding to 64-85% of total extractable pectins. On the other hand, in liquid fraction, only water-soluble pectins were detected (0.77-1.32 g GUAE/100 g DW) and in a higher amount than in pulp fraction (0.46-0.49 g GUAE/100 g DW). Only a small amount of hydroxide soluble pectins was detected in pulp fraction. In conclusion, the total extractable pectins were concentrated in the pulp fraction, but the water-soluble pectins were condensed in the liquid fraction from olive pomace. Soluble pectins identified in the liquid fraction were also known to positively affect blood glucose levels, decreasing them (Galanakis, 2011).

### 3.3.6. Soluble sugars

Total soluble sugar content (using glucose as standard) was not significantly different between samples of C-OP and respective fractions, except for the liquid fraction ($p < 0.05$) (Table 3.1). The HPLC analysis of soluble sugars allowed the identification of glucose and mannitol (Table 3.1), revealing the higher concentration of mannitol according to previously reported results for maturated olive fruits (Gómez-González *et al.*, 2010). The liquid fraction revealed significant different amounts of mannitol between samples (OM 1 and OM 2). The higher concentration of mannitol in the liquid

fraction from OM 2 (32.37 ± 2.65 g/100 g DW) than OM 1 (21.10 ± 2.55 g/100 g DW) can be explained by the higher fat content and a possibly higher degree of maturation of its olives. During olive maturation, it is normal to occur an increase of mannitol simultaneously than olive mesocarp accumulates oil (Conde *et al.*, 2008).

In general, the designed fractionation approach led to a concentration of the soluble sugars in liquid fraction, principally mannitol (4-8 times higher than in pulp fraction), as reported previously (Delisi *et al.*, 2018). So, in terms of nutritional composition, the liquid fraction was mostly a source of minerals and carbohydrates (glucose and mannitol). This composition can be an asset as a functional component to formulate sport food products (Babich *et al.*, 2019) or food products with health benefits namely to decrease carbohydrates intake that raises blood glucose levels (Fitch and Keim, 2012). Mannitol as the predominant soluble sugar in the liquid fraction is a non-metabolisable (uptake insulin-independent) and low–energy sweetener (1.6 kcal/g), but also a food preservative (as an antioxidant and as a reducer of the sugar crystallisation) (Ghoreishi and Shahrestani, 2009).

### 3.3.7. Infrared spectroscopy

The major peak assignments in OP fractions measured by FTIR include lipids, polysaccharides, and pectin and cellulose components (Figure 3.2). The unsaturated fatty acids (like linoleic and oleic acid) and triacylglycerols were identified at 3008 $cm^{-1}$ (C-H stretching of olefinic double bonds) and 1744 $cm^{-1}$ (ester peak because of C=O stretching vibration of carbonyl groups of the triacylglycerols), respectively (Uncu *et al.*, 2019). In agreement with the more abundant lipid content of pulp fraction, larger peak areas were detected at 2922 and 2850 $cm^{-1}$ in pulp fraction (methylene absorbance peaks associated with antisymmetric and symmetric stretching vibrations of aliphatic C-H in $-CH_2$ and terminal $-CH_3$ groups, respectively) (Uncu *et al.*, 2019).

The bands at 3300-3400 (vibration of hydrogens in bound OH groups of the type alcohol or phenol) and 2922 $cm^{-1}$ (C-H stretching vibrations of asymmetric aliphatic structures) could be attributed to high levels of less condensed structures such as phenolic compounds and acids (Uncu *et al.*, 2019). These bands were more evident in liquid than in pulp fraction.

The vibration peaks of cellulose and lignin were more evident in pulp fraction, at wavelengths ranging between 1460 and 1315 $cm^{-1}$ [1370 $cm^{-1}$, $CH_2$ bending mode (xyloglucan, cellulose); 1338 $cm^{-1}$, CH deformation from ring vibration (polysaccharides, pectin and cellulose)]. At 1080 and 1024 $cm^{-1}$ two significant peaks in the pulp fraction were identified, typical from xyloglucans (C-O stretching and C-C stretching) and C-O stretching and C-C stretching (pectin, cellulose). Pectin was noticed mainly in peaks between 1600-1100 $cm^{-1}$ [1630 $cm^{-1}$, $COO^-$ asymmetric stretching (pectin ester group); 1240 $cm^{-1}$, C-O stretching (pectin); 1160 $cm^{-1}$ O-C-O symmetric stretching (glycosidic link of cellulose, pectin)]. The presence of soluble sugars was identified between 1160-1045 $cm^{-1}$ in the liquid fraction.

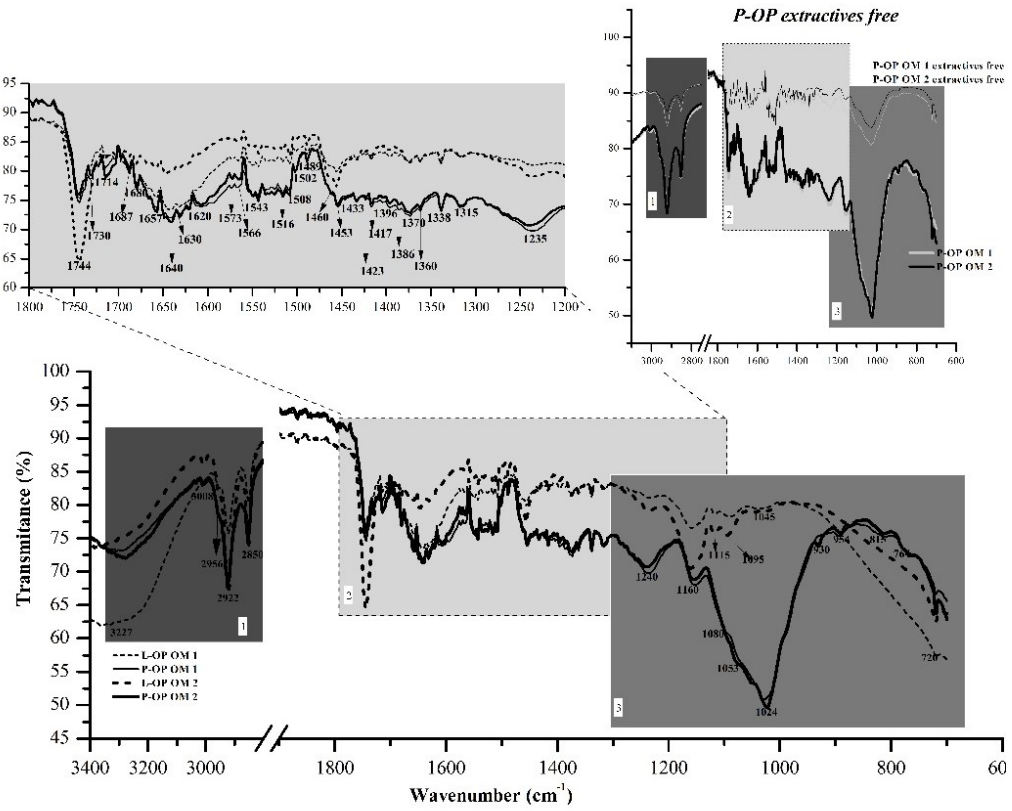



**Figure 3.2. FTIR spectra of the liquid-rich fraction (L-OP) and pulp-rich fraction (P-OP) produced after the wet and dry fractionation process of crude olive pomace (C-OP).**
C-OP – Crude olive pomace; L-OP – liquid-rich fraction; P-OP – pulp-rich fraction.

The differences in peak intensity and some shifts in peak position between liquid and pulp fraction were related to its components' content, type, and structure. The principal spectral variations among the liquid and pulp fraction were principally due to the higher fat content and fibre richness (lignin and xylans composition) of pulp fraction *versus* the higher phenolic and sugar content of liquid fraction obtained from OP. Besides that, the FTIRs results allowed to deduce that pulp fraction could be explored as a source of fibre and unsaturated fatty acids, simultaneously, in food formulation for its beneficial effects on reducing cholesterol triglycerides (Lopez-Huertas, 2010).

Assignments were based on studies from previous work on FTIR analysis of OP (Ducom *et al.*, 2020), olive mill wastewater (Fakharedine *et al.*, 2006), OP fractions (Ying *et al.*, 2017) and olive oil (Uncu *et al.*, 2019).

### 3.3.8. Bioactivity characterization

#### 3.3.8.1. Total phenolic content and antioxidant activity of free and bound phenolics

The TPC and AOX (DPPH, ABTS and ORAC) of crude olive pomace, liquid and pulp fraction samples are presented in Figure 3.3. Total TPC resulted from the sum of the free phenolic compounds with the bound phenolic compounds. TPC in the crude olive pomace samples was between 22.73 – 24.51 mg GAE/ g DW without significant differences ($p > 0.05$). Different TPC results were reported in

previous works for crude olive pomace: higher (Uribe *et al.*, 2014) and lower values (Alu'datt *et al.*, 2013), mainly due to the differences between olive cultivars and oil extraction conditions.

Liquid fraction (21.21-22.67 mg GAE/ g DW) and crude olive pomace exhibited similar values of TPC regarding free phenolic extracts, and pulp fraction (8.32-14.86 mg GAE/ g DW) revealed significantly lower values ($p < 0.05$). On the other hand, TPC of bound phenolic extracts from pulp fraction (2.78-5.13 mg GAE/ g DW) was similar or higher than in crude olive pomace samples (3.20-3-62 mg GAE/ g DW). However, as expected, higher recovery of phenolic compounds was achieved in liquid rather than in pulp fraction.

AOX of crude olive pomace and edible fractions (liquid and pulp fraction) were evaluated using 3 different methods (DPPH, ABTS and ORAC), due to the absence of a single antioxidant determination methodology able to reflect the total AOX of a sample. The model of scavenging stable radicals DPPH is widely used to evaluate antioxidant capacities of natural products, including olive oil samples (Cioffi *et al.*, 2010). DPPH method showed that the proton-removal activity of free phenolic compounds from the liquid fraction (76.03-106.03μM TE/ g DW) was higher than pulp fraction (41.05-79.20 μM TE/ g DW) and analogous to crude olive pomace (79.94-103.15 μM TE/ g DW). ABTS showed the same tendency that DPPH, where free phenolic compounds extract of liquid fraction, showed higher AOX values and similar to crude olive pomace. Nonetheless, bound phenolic extracts of pulp fraction revealed had the double capacity (21.25-26.98 μM TE/ g DW)) to inhibit the free radicals when compared to the liquid fraction (12.34-13.34 μM TE/ g DW) ($p < 0.05$). When comparing ABTS with DPPH, ABTS had higher values, which could be related to methanol use. Water is commonly used as a solvent in ABTS, and its substitution by methanol allows to improve the total AOX evaluation of the complex samples by ABTS (aqueous and organic compounds) (Cano *et al.*, 2000).

In ORAC, liquid fraction (1546.93-1585.46 μM TE/ g DW) also showed the highest AOX value than pulp fraction (454.74-502.80 μM TE/ g DW) or crude olive pomace (641.05-734.81 μM TE/ g DW) ($p < 0.05$). The ORAC values were much higher than the values obtained by DPPH and ABTS. Similar differences were reported in olives' (Gouvinhas *et al.*, 2017) and OP (Uribe *et al.*, 2014) AOX assays. These differences probably arise from the different mechanisms involved during each methodology: single-electron transfer in the case of DPPH/ABTS and hydrogen atom transfer in the ORAC assay case.

The three methodologies' values are in agreement with the TPC results, showing that the phenolic compounds are the main AOX contributor of liquid and pulp fraction. All three AOX methods applied indicate a stronger AOX ($p < 0.05$) of liquid fraction, but the AOX of pulp fraction along with its higher fibre content is by the established concept of an "antioxidant dietary fibre" material (Quirós-Sauceda *et al.*, 2014).

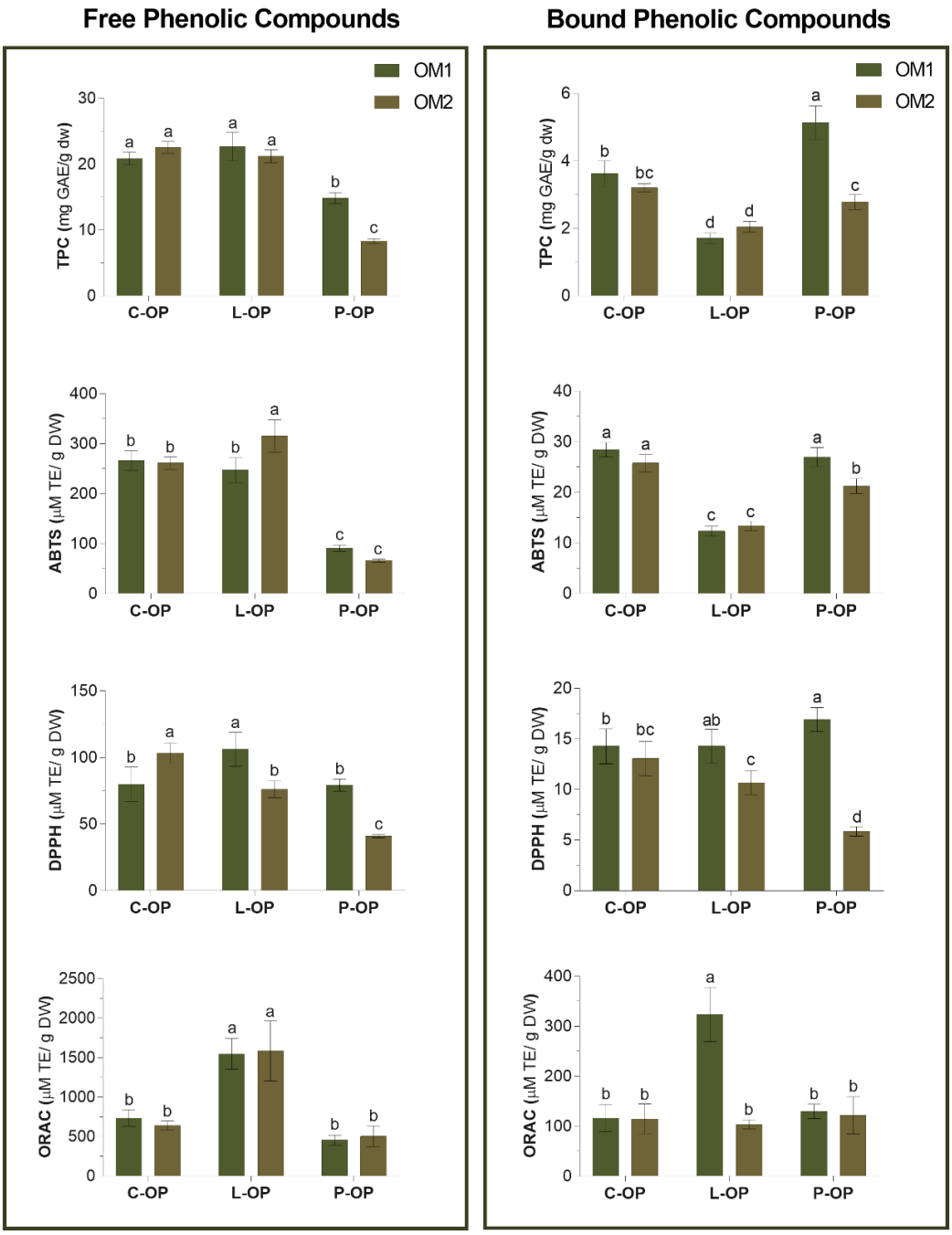



**Figure 3.3. Content of total, free and bounds phenolic compounds and antioxidant activity using DPPH, ABTS and ORAC methods from crude olive pomace (C-OP), the liquid-rich fraction (L-OP) and pulp-rich fraction from olive pomace (P-OP).**
OM 1 – Olive pomace from olive mill 1; OM 2 – Olive pomace from olive mill 1; C-OP – Crude olive pomace; L-OP – liquid-rich fraction; P-OP – pulp-rich fraction. GAE – Gallic acid equivalents; TE – Trolox equivalents. Data were expressed as mean ± SD (n = 3). The different superscripts in the same column were significantly different ($p < 0.05$).

### 3.3.8.2. Identification of phenolic compounds

A detailed analysis of free and bound phenolic compounds is required to elucidate which compounds are responsible by the AOX of the edible fractions (liquid and pulp fraction). Table 3.2 shows the free phenolic and bound phenolic profile of the C-OP, liquid and pulp fraction identified by LC-ESI-UHR-QqTOF-MS.

The HPLC quantification of phenolic compounds based on the available standard is presented in Table 3.3. In agreement with TPC results, the amount of the phenolic compound identified in the free phenolic extract was higher than in the bound phenolic extract for all samples. The main phenolic classes identified in free phenolic extracts were (1) HYD/ tyrosol and its derivatives followed by (2) flavonoids class (largely luteolin), (3) secoiridoids and derivatives class (essentially comselogoside, verbascoside and caffeoyl-6-secologanoside) and (4) phenolic acids (mainly caffeic acid and *p*-coumaric acid). The liquid fraction exhibited a rich composition in HYD and its derivatives (625.76 ± 51.33 and 513.61 ± 27.85 mg/100 g DW in OM 1 and OM2, respectively) owing to the hydrophilic nature of this phenolic class (Klen and Vodopivec, 2012). Previous works also supported centrifugation efficiency to recover these compounds (Delisi *et al.*, 2018). HYD and derivatives are among the principal phenolic compounds present in olive fruits and by-products (Nunes *et al.*, 2018). These phenolics have been characterised to have potent biological activities as an antioxidant, anti-inflammatory and antimicrobial (Nunes *et al.*, 2019; Obied *et al.*, 2007), with promising applications in foods, but also in cosmetics and medicine. Moreover, the European Food Safety Authority (EFSA) has issued a favourable scientific opinion regarding health claims for the dietary consumption of HYD and derivatives (protection of blood lipids from oxidative damage) (EFSA, 2011). As a result, in the last years, HYD application and recovery studies have increased exponentially.

Comselogoside, verbascoside, caffeoyl-6-secologanoside and caffeic acid, phenolic compounds associated with the prevention of cardiovascular diseases, were identified in a slightly higher amount in the liquid than in pulp fraction too.

Luteolin was the predominant phenolic compound of the pulp fraction. The reduced water solubility of luteolin could explain this compound's retention in the pulp fraction (Dang *et al.*, 2014). Studies reported that luteolin has antioxidant, anti-microbial, anti-inflammatory and anti-diabetic activity (Aziz *et al.*, 2018). The pulp fraction was also characterised by a significant amount of bound phenolic compounds, namely vanillin, HYD, protocatechuic acid and caffeic acid. These bound phenolic compounds represented a significant amount of the pulp fraction's total phenolic compounds (18-27%). The highest amount of bound phenolics in pulp fraction could be linked to its higher fibre content and with the possible linkage of vanillin, caffeic and protocatechuic acids to the cell wall (cellulose, lignin and protein) (Calvache *et al.*, 2016). Caffeic acid was detected in a much higher amount in bound form, and protocatechuic acid and vanillin were only noticed in bound form. Caffeic acid has been related to the reduction or complete inhibition of α-glucosidase activity (potential anti-diabetic activity) (Rasouli *et al.*, 2017). Vanillin and protocatechuic acid have been described to have potent AOX and antimicrobial activity (Bezerra *et al.*, 2017; Mudenuti *et al.*, 2018). Other compounds like hydroxybenzoic and ferulic acid were only identified in bound phenolics extracts.

**Table 3.2. Phenolic compounds identified (or tentatively identified) in free (FPC) and bound phenolic extract crude olive pomace (C-OP) and edible olive pomace fractions (L-OP and P-OP) produced after the wet and dry fractionation process.**

| Phenolic Compounds | FPC | BPC | RT (min) | UV-Vis max | Formula | m/z exp | m/z theo | err [mDa] | mSigma | Fragments | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hydroxytyrosol and tyrosol derivatives** | | | | | | | | | | | |
| *Dihydroxytyrosol* | ✓ | ✓ | 1.9 | 279 | $C_8H_{10}O_4$ | 169.0504 | 169.0506 | 0.3 | 5.1 | 151.0397; 123.0448 | (b) |
| *Hydroxytyrosol glucoside* | ✓ | ✓ | 3.9 | 278 | $C_{14}H_{20}O_8$ | 315.1092 | 315.1088 | -0.4 | 16.3 | 315.1090; 153.0555; 123.0450 | (c) |
| *Hydroxytyrosol* | ✓ | ✓ | 4.3 | 281 | $C_8H_{10}O_3$ | 153.0557 | 153.0555 | -0.2 | 0.9 | 153.0554; 123.0450 | (a) |
| *Tyrosol glucoside* | ✓ | × | 8.3 | 275 | $C_{14}H_{20}O_7$ | 299.1144 | 299.1136 | -0.2 | 15.1 | 299.1139; 119.0349; 101.0244, 89.0245 | (c) |
| *Tyrosol* | ✓ | × | 12 | 276 | $C_8H_{10}O_2$ | 137.0609 | 137.0608 | 0.5 | n.a. | 137.0603; 111.0084; 95.0510 | (a) |
| **Secoiridoids and derivatives** | | | | | | | | | | | |
| *Oleoside* | ✓ | ✓ | 6.2 | 270 | $C_{16}H_{22}O_{11}$ | 389.1092 | 389.1089 | -0.3 | 5.6 | 389.1088;183.0664; 165.0560; 121.0656; 345.1195 | (c) |
| *Verbascoside* | ✓ | × | 13,3 | 330 | $C_{29}H_{36}O_{15}$ | 623.1990 | 623.1981 | -0.9 | 8.3 | 623.1983; 161.0244; 461.1665; 162.0276; 135.0451 | (c) |
| *Caffeoyl-6'-secologanoside* | ✓ | × | 14.7 | 326 | $C_{25}H_{28}O_{14}$ | 551.1416 | 551.1406 | 0 | 18.1 | 551.1416; 507.1504; 345.1193; 281.0673; 161.0245 | (c) |
| *Oleuropein* | ✓ | × | 17,1 | 280 | $C_{25}H_{32}O_{13}$ | 539.1761 | 539.1771 | 0.7 | 27.6 | 539.1764; 307.0828; 275.0931; 223.0613; 179.0566 | (a) |
| *Comselogoside* | ✓ | × | 17.5 | 311 | $C_{25}H_{28}O_{13}$ | 535.1462 | 535.1457 | -0.1 | 19.9 | 535.1465; 145.0296; 491.1558; 389.1093; 345.1197 | (c) |
| **Flavonoids** | | | | | | | | | | | |
| *Rutin* | ✓ | ✓ | 10.7 | 355 | $C_{27}H_{30}O_{16}$ | 609.1465 | 609.1461 | -0.1 | 18 | 609.1462; 300.0289 | (a) |
| *Luteolin-7-O-glucoside* | ✓ | ✓ | 11.6 | 352 | $C_{21}H_{20}O_{11}$ | 447.0929 | 447.0933 | 0.8 | 20.5 | 447.0925; 285.0414 | (a) |
| *Luteolin* | ✓ | × | 20.4 | 349 | $C_{15}H_{10}O_6$ | 285.0414 | 285.0405 | -1 | 3.3 | 285.0414; 151.0037 | (a) |
| *Quercetin* | ✓ | × | 20.5 | 342 | $C_{15}H_{10}O_7$ | 301.0362 | 301.0354 | -0.5 | 4.5 | 301.0359; 151.0035; 178.9988; 121.0294 | (a) |
| *Apigenin* | ✓ | × | 25.0 | 339 | $C_{15}H_{10}O_5$ | 269.0461 | 269.0455 | -0.5 | 4.8 | 269.0461; 151.0035 | (a) |
| **Phenolic Acids** | | | | | | | | | | | |
| *Vanillin* | × | ✓ | 4.2 | 279 | $C_8H_8O_3$ | 151.0400 | 151.0401 | 0.3 | 18.7 | 151.0397; 137.0235; 109.0290; 105.0341 | (a) |
| *Hydroxybenzoic acid* | × | ✓ | 5.6 | | $C_7H_6O_3$ | 137.0241 | 137.0244 | 0.4 | 4 | 137.0241; 138.0280 | (b) |
| *Caffeic acid-3-glucoside* | ✓ | ✓ | 5.8 | 277 | $C_{15}H_{18}O_9$ | 341.0876 | 341.0878 | 0.2 | 9.5 | 341.0876; 179.0351; 135.0450 | (c) |
| *Caffeic acid* | ✓ | ✓ | 7.0 | 323 | $C_9H_8O_4$ | 179.0350 | 179.0350 | 0.0 | 7.5 | 179.0350; 135.0448 | (a) |
| *Coumaric acid* | ✓ | ✓ | 9.0 | 309 | $C_9H_8O_3$ | 163.0397 | 163.0401 | 0.3 | 19.6 | 163.0397; 119.0499 | (a) |
| *Ferulic acid* | × | ✓ | 10.4 | 323 | $C_{10}H_{10}O_4$ | 193.0509 | 193.0506 | -0.3 | 18.6 | 193.0504; 178.0268; 134.0370 | (a) |

RT – retention time; ✓ - Compound identified; × - Compound not identified; Ref. – reference, (a) Comparison with standard; (b) Capriotti *et al.*, 2014; (c) Peralbo-Molina *et al.*, 2012.

**Table 3.3. Phenolic composition (mg/100 g DW) in crude olive pomace (C-OP), a liquid-rich fraction (L-OP) and pulp-rich fraction (P-OP) from olive pomace.**

| *Phenolic compound* | | C-OP | | L-OP | | P-OP | |
|---|---|---|---|---|---|---|---|
| | | **OM 1** | **OM 2** | **OM 1** | **OM 2** | **OM 1** | **OM 2** |
| **Hydroxytyrosol** | *Free* | 207.08 ± 13.95 $^{c}$ | 573.43 ± 59.62 $^{a}$ | 81.62 ± 20.98 $^{d}$ | 173.67 ± 13.68 $^{c}$ | 504.73 ± 27.67 $^{b}$ | 26.54 ± 8.35 $^{e}$ |
| | *Bound* | 17.35 ± 6.03 $^{c}$ | 78.51 ± 8.70 $^{a}$ | 43.14 ± 9.85 $^{b}$ | 16.44 ± 4.70 $^{c}$ | 15.99 ± 1.53 $^{c}$ | 11.93 ± 3.69 $^{c}$ |
| **Tyrosol** | *Free* | 51.21 ± 3.01 $^{b}$ | nd | 35.48 ± 1.87 $^{c}$ | 65.89 ± 6.91 $^{a}$ | nd | 20.75 ± 3.48 $^{d}$ |
| **Protocatechuic acid** | *Bound* | 10.30 ± 1.17 $^{b}$ | nd | 15.73 ± 1.32 $^{a}$ | 8.38 ± 1.02 $^{b}$ | nd | 10.09 ± 1.03 $^{b}$ |
| **Caffeic acid** | *Free* | 21.92 ± 1.71 $^{a}$ | 23.63 ± 3.10 $^{a}$ | 14.01 ± 0.58 $^{b}$ | 8.79 ± 1.25 $^{c}$ | 15.14 ± 0.85 $^{b}$ | 0.51 ± 0.12 $^{d}$ |
| | *Bound* | 25.15 ± 2.15 $^{b}$ | 40.71± 5.23 $^{a}$ | 34.76 ± 3.48 $^{a}$ | 16.61 ± 3.25 $^{cd}$ | 23.10 ± 1.49 $^{bc}$ | 13.47 ± 1.87 $^{d}$ |
| **Vanillin** | *Bound* | 1.39 ± 0.23 $^{b}$ | ND | 1.63 ± 0.18 $^{ab}$ | 0.85 ± 0.20 $^{c}$ | nd | 1.79 ± 0.26 $^{a}$ |
| ***p* - Coumaric acid** | *Free* | 7.41 ± 0.58 $^{b}$ | 8.04 ± 1.17 $^{b}$ | 6.46 ± 0.16 $^{b}$ | 8.30 ± 0.97 $^{b}$ | 15.75 ± 1.80 $^{a}$ | 1.40 ± 0.13 $^{c}$ |
| | *Bound* | 8.69 ± 3.50 $^{d}$ | 13.43 ± 2.60 $^{bc}$ | 9.64 ± 0.64 $^{cd}$ | 14.47 ± 2.85 $^{b}$ | 19.47 ± 0.45 $^{a}$ | 15.64 ± 0.80 $^{ab}$ |
| **Rutin** | *Free* | nd | nd | nd | 30.85 ± 2.71 $^{a}$ | nd | 16.49 ± 0.93 $^{b}$ |
| **Luteolin-7-*O*-glucoside** | *Free* | nd | nd | nd | 10.51 ± 0.77 $^{a}$ | nd | 10.31 ± 1.09 $^{a}$ |
| **Luteolin** | *Free* | 18.40 ± 0.51 $^{c}$ | nd | 22.34 ± 0.66 $^{b}$ | 44.47 ± 2.98 $^{a}$ | nd | 45.26 ± 1.47 $^{a}$ |
| **Quercitin** | *Free* | 3.22 ± 0.34 $^{ab}$ | nd | 2.80 ± 0.36 $^{b}$ | 3.45 ± 0.46 $^{a}$ | nd | 0.91 ± 0.24 $^{c}$ |
| **Total** | *Free* | 290.18 ± 15.51 $^{c}$ | 581.47 ± 60.70 $^{a}$ | 152.45 ± 22.0 $^{e}$ | 346.03 ± 24.37 $^{d}$ | 520.47 ± 29.17 $^{b}$ | 127.66 ± 31.43 $^{e}$ |
| | | 77% | 84% | 58% | 84% | 92% | 72% |
| | *Bound* | 62.87 ± 8.26 $^{c}$ | 132.64 ± 0.93 $^{a}$ | 97.17 ± 18.98 $^{b}$ | 56.75 ± 10.99 $^{c}$ | 58.55 ± 2.41 $^{c}$ | 51.41 ± 8.49 $^{c}$ |
| | | 17% | 19% | 37% | 13% | 10% | 28% |

OM 1 – Olive pomace from olive mill 1; OM 2 – Olive pomace from olive mill 1; C-OP – Crude olive pomace; L-OP – liquid-rich fraction; P-OP – pulp-rich fraction. nd – not detected. Data were expressed as mean ± SD (n = 3). The different superscripts in the same row were significantly different ($p < 0.05$).

Overall, liquid fraction allowed the recovery of a considerable amount of HYD and derivatives related to the prevention of cardiovascular diseases and pulp fraction exhibit a significant quantity of free (mainly luteolin) and bound phenolics. Therefore, liquid fraction could be explored by the food industry to achieve new food products with health benefits in the prevention of cardiovascular diseases. Daily consumption of less than 1 g of the liquid fraction would provide the amount of HYD (5 mg) that would be needed to protect low-density lipoprotein (LDL) particles from oxidative damage, according to the health claim approved by the EFSA (EFSA, 2011). Besides the health benefits, liquid fraction exhibited a more potent AOX, supporting its potential application as a food preservative (as an antioxidant and/ or antimicrobial). In the other hand, the pulp fraction fibre, including the lignin, could act as a carrier of the bound phenolics (Sato *et al.*, 2011) that could exert significant gut health benefits including antioxidant and antibacterial activity able to repress pathogenic bacteria in the colon (i.e., *Escherichia coli*, *Clostridium*), and prebiotic-like effects by stimulating the growth of beneficial bacteria (i.e., *Lactobacillus* and *Bifidobacterium* spp.) (Gong *et al.*, 2018). Besides that, P-OP exhibits a significant amount of free phenolics (mainly luteolin and HYD) and significant AOX. So, P-OP could be considered a significant source of "antioxidant dietary fibre", which can be applied as a food ingredient that offers the technological and gut health-promoting benefits associated to fibre and antioxidants (Quirós-Sauceda *et al.*, 2014).

### 3.3.9. Energy content

The energy content obtained for samples following the fractionation ranged between 19-22 MJ/ kg higher heating value (Table 3.4). C-OP showed energy content equivalent to previous works (Mata-Sánchez *et al.*, 2015). The wet fractionation (pulp + stones fraction), did not reveal significant differences in HHV ($p < 0.05$) in comparison to C-OP. However, the separation of pulp fraction from stones fraction significantly influenced the energy content values ($p < 0.05$). As expected, pulp fraction exhibited the higher HHV (~22 MJ/ kg), due to its substantial oil content. However, pulp fraction high composition in oil and low weight could generate uncontrolled combustions and emissions disturbing the combustion and its performance as fuel (Mata-Sánchez *et al.*, 2015). The lowest HHV was shown in stones fraction (~19 MJ/ kg). Nonetheless, these values were similar to those achieved in previous works focused on stones fraction (18.80 MJ/ kg) (Topal *et al.*, 2017). Besides, the HHV obtained to stones fractions were identical to other biomass sources, namely oak wood (18.70 MJ/ kg) and pine bark (18.30 MJ/kg) and to most agricultural residues [e.g. vine shoots (18.30 MJ/ kg), palm kernel (18.67 MJ/ kg)] (Vamvuka and Kakaras, 2011).

The moisture content of stones fraction samples showed a moisture content of 6% (OM1: 5.48 ± 0.01 g/100 g DW; OM2: 5.39 ± 0.08 g/100 g DW) and a pH value of 4.3. This moisture values are in agreement with the values (6.1 g/100 g DW) reported by Topal *et al.* (2017).

HHV values, moisture, and pH were measured to support the stones fraction application as solid biofuel. Besides, its free of impurities' character (oil and low weight particles) guarantees a better and safer combustion performance for this OP fraction.

In the future, other compounds as ash, chlorine, nitrogen, potassium, magnesium and phosphorus needed to be quantified in order to ensure stones fraction quality as solid biofuel (Mata-Sánchez *et al.*, 2015). The possibility of incorporating binders and additives to improve stones fraction stability and combustion characteristics also needs to be surveyed (Christoforou and Fokaides, 2016).

**Table 3.4. Higher Heating Value (HHV) of crude olive pomace (C-OP) and olive pomace fractions produced after the wet and dry fractionation process (MJ/Kg DW).**

| High calorific value (MJ/Kg dry weight) | | | | | | | |
|---|---|---|---|---|---|---|---|
| C-OP | | P + S-OP | | P-OP | | S-OP | |
| OM 1 | OM 2 | OM 1 | OM 2 | OM 1 | OM 2 | OM 1 | OM 2 |
| 20.57 ± 0.03 $^{c, d}$ | 21.67 ± 0.20 $^{b}$ | 20.21 ± 0.05 $^{d}$ | 20.86 ± 0.11 $^{c}$ | 21.52 ± 0.12 $^{b}$ | 22.21 ± 0.01 $^{a}$ | 18.94 ± 0.00 $^{d}$ | 18.65 ± 0.00 $^{d}$ |

OM 1 – Olive pomace from olive mill 1; OM 2 – Olive pomace from olive mill 1; C-OP – Crude olive pomace; L-OP – liquid-rich fraction; P-OP – pulp-rich fraction, P+S-OP – Solid fraction without application of the dry fractionation. Data were expressed as mean ± SD (n = 3). The different superscripts in the same line represent significant differences between samples ($p < 0.05$).

### 3.3.10. Evaluation of the fractionation valorisation approach for olive pomace in the centre region of Portugal: A case study

The present fractionation process can be a promising strategy for the whole valorisation of C-OP at large scale, due to being cheaper and more efficient regarding water and energy usage. To demonstrate the potential of the fractionation process proposed, we have considered Portugal's centre region (third major olive oil producer region in Portugal), which represents around 15% of national olive oil production in 2018 (INE, 2019). According to Instituto Nacional de Estatística (INE, 2019), the production of olives used for olive oil extraction was of 62 280 tons in the centre region. It is estimated that approximately 35-40 kg of OP is produced per 100 kg of olives [64] or according to data of the most significant world producing regions of olive oil (Andalusia) it represents approximately 65% of the initial weight (AGAPA, 2015). So, at least 21 978 tons of C-OP was produced in 2018, only in Portugal's centre region.

Assuming the recovery of 80% of C-OP through the selective collection in the centre region of Portugal, over 17 500 tons of C-OP could be used to produce in average: (1) 460 tons of enriched phenolic-mineral-sugar extract (liquid fraction) with a very significant recovery of HYD (~53%), (2) 3440 tons of antioxidant fibre powder (pulp fraction) and also (3) 1200 tons of stones fraction with an HHV of about 19 MJ/ kg that could be applied as solid biofuel (Figure 3.4). This study presents a highly promising methodology for the OP valorisation; however, a detailed cost-benefit analysis would be necessary to assess the proposed approach's economic feasibility.

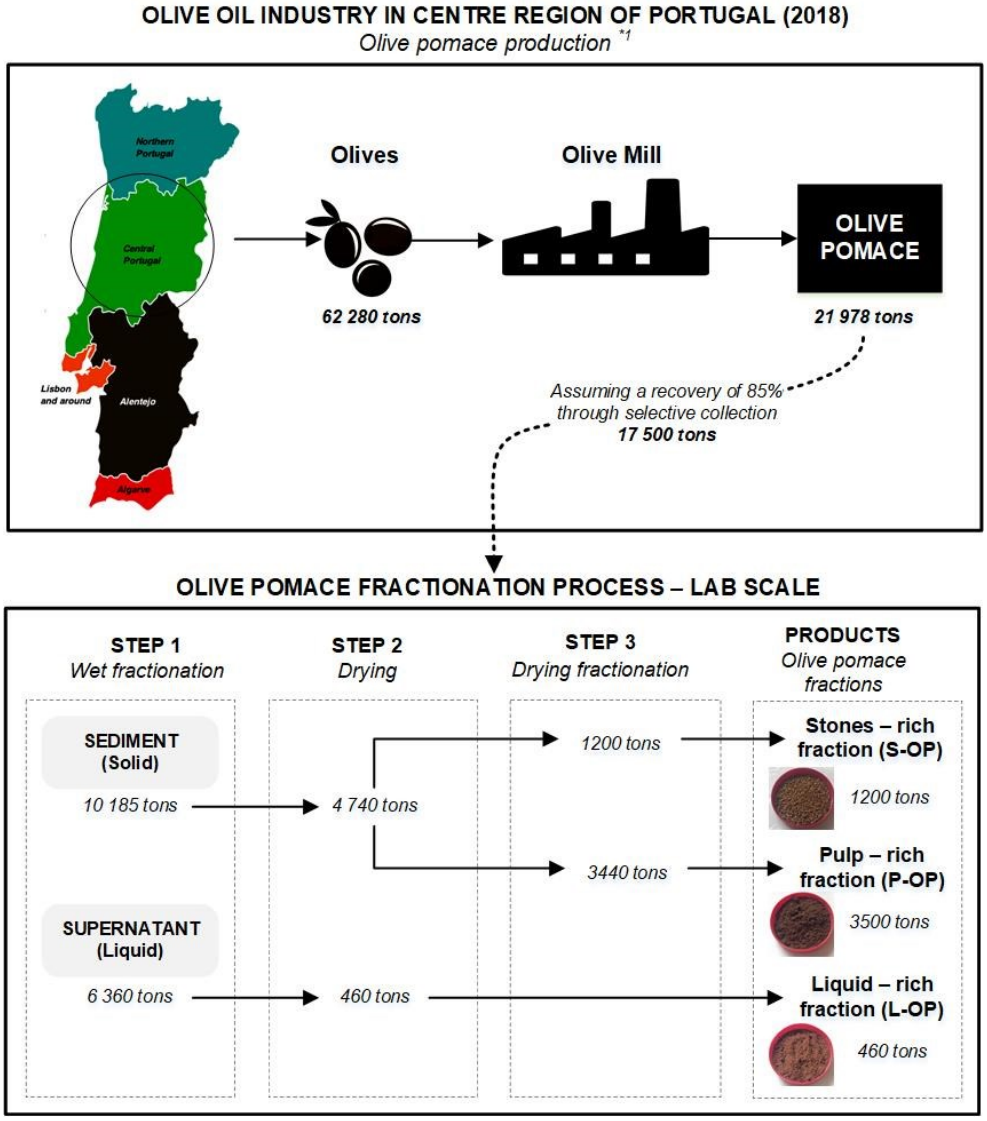



**Figure 3.4. Yield of the fractionation process for olive pomace valorisation of centre region of Portugal according to estimated production of 2018.**

## 3.4. Conclusion

This study demonstrated that a fractionation approach might constitute a new and promising route for converting olive pomace into multiple value-added products, like add-value energy applications without throwing away high-value compounds with food application. The first step (wet fractionation) leads to the production of a liquid fraction (yield of 9% in dry weight) rich in minerals (≈ 11 g/100 g), sugars (mainly mannitol: 21-32 g/100 g), and phenolic compounds (mainly hydroxytyrosol: 514- 626 mg/100 g), but also has the advantage of optimising the drying process of the solid fraction. The dry fractionation allowed attaining a stones fraction (yield of 20-26% in dry weight) with significant calorific power (~19 MJ/ kg) and a pulp fraction (yield of 65-71% in dry weight), which is a relevant source of antioxidant dietary fibre with a neutral detergent fibre content of 53-59 g/100 g and a significant antioxidant activity not only in free phenolics (ORAC: 455–503 μM trolox equivalents/g) but also in bound phenolics (ORAC: 121– 130 μM trolox equivalents/g).

Considering the yield, the chemical composition and the *in vitro* antioxidant activity of both edible fractions, this approach allowed to obtain two sources of food ingredients: an enriched phenolic - mineral-sugar extract (liquid fraction) with potential application as a food preservative or health-promoting food ingredient, and a source of antioxidant dietary fibre (pulp fraction), while impurities (olive pulp and the stone fines) related to emission, corrosion and slagging were removed to obtain a solid biofuel with better performance.

In the future, optimisation studies should be performed in combination with life cycle analysis (LCA) to improve the sustainability and economics of the fractionation approach proposed in this study. To guarantee the application of olive pomace fractions to develop new food ingredients, future studies about the safety, bioactivity and stability/bioaccessibility of its bioactive compounds will be necessary. At a pilot-scale, the centrifugation replacement without refrigeration and freeze-drying (adopted at lab-scale) by mechanical pressing and conventional drying techniques, respectively, could be feasible options to ensure a more affordable process.

# Chapter 4.
# Are olive pomace powders a safe source of bioactives and nutrients?


## Abstract

Olive oil industry generates significant amounts of semi-solid wastes, namely the olive pomace. Olive pomace is a by-product rich in high-value compounds (e.g. dietary fibre, unsaturated fatty acids, phenolics) widely explored to obtain new food ingredients. However, conventional extraction methods frequently use organic solvents, while novel eco-friendly techniques have high operational costs. The development of powdered products without any extraction step has been proposed as a more feasible and sustainable approach. The present study fractionated and valorised the liquid and pulp fraction of olive pomace, obtaining two stable and safe powdered ingredients, namely a liquid-enriched powder (LOPP) and a pulp-enriched powder (POPP). These powders were characterized chemically, and their bioactivity was assessed. LOPP exhibited a significant amount of mannitol (141 g/kg), potassium (54 g/kg) and hydroxytyrosol/derivatives (5 mg/g). POPP exhibited a high amount of dietary fibre (620 g/kg) associated with a significant amount of bound phenolics (7.41 mg GAE/ g fibre DW) with substantial antioxidant activity. POPP also contained an unsaturated fatty acid composition similar to olive oil (76% of total fatty acids) and showed potential as a reasonable protein source (12%). Their functional properties (solubility, water-holding and oil-holding capacity), antioxidant capacity and antimicrobial activity were also assessed, and their biological safety was verified. The development of olive pomace powders to apply in the food industry could be a suitable strategy to add-value to olive pomace and obtain safe multifunctional ingredients with higher health-promoting effects than dietary fibre phenolics itself.


***Keywords:*** olive pomace; powder; hydroxytyrosol; antioxidant dietary fibre; functional ingredient

## 4.1. Introduction

The large quantities of food by-products and waste produced every year are responsible for significant resources depletion and environmental problems. In this sense, the food industry strives to achieve “zero wastes food systems” in line with the recent EU Circular Economy Action Plan (European Commission, 2020) and EU Bioeconomy Strategy (European Commission, 2018).

Olive oil production is currently one of the most polluting agro-food industries (Nunes *et al.*, 2019), producing the commonly known olive pomace (OP). This semi-solid waste is obtained from the two-phase system and represents the sector's highest waste-management challenge (Dermeche *et al.*, 2013). OP is challenging to treat because of its high water and organic compounds content (Rigane *et al.*, 2012). However, OP contains also a substantial amount of compounds as dietary fibre (DF) (Galanakis, 2011), unsaturated fatty acids (UFAs) (Nunes *et al.*, 2018), minerals (Rodrigues *et al.*, 2017) and phenolic compounds (Rigane *et al.*, 2012). Taking into account its richness in high-value compounds for food formulation, OP should be considered a by-product. The term “food by-products” was introduced by EU Commission to advise that “food wastes” represent a loss of valuable biomass and nutrients which could be recovered to develop new co-products with market value (European Commission, 2018, 2008; Kasapidou *et al.*, 2015).

Currently, EU legislation and plans promote the search for new end-uses for food by-products (European Commission, 2018, 2008; Kasapidou *et al.*, 2015). In consequence, the development of new food ingredients from by-products had been emerging, not only as an attempt to achieve more sustainable food production chains but also to attending to the increasing demand of consumers for healthier and more functional foods (Coderoni and Perito, 2020; Galanakis, 2012; Laufenberg *et al.*, 2003; Torres-León *et al.*, 2018). Furthermore, this valorisation strategy could generate economic gains for the food industry, reducing resource depletion and environmental problems from waste mismanagement (Coderoni and Perito, 2020; Torres-León *et al.*, 2018).

In the same framework, the development of powdered ingredients without any extraction has been proposed as a more feasible and low environmental impact approach than the traditional and emerging technologies that involve using organic solvents or possess higher operational costs, respectively (García-Lomillo *et al.*, 2014). Besides that, powdered products have the advantage to retain several functional compounds, namely fatty acids, polysaccharides, minerals and phenolics together under synergic interaction ascribing these products multifunctional properties (Saura-Calixto, 1998).

The OP solid fraction stood out as potential raw material to develop new antioxidant dietary fibre (ADF) powders in our previous work. Furthermore, 1 g of OP liquid fraction showed to retain more than 5 mg of hydroxytyrosol (HYD) and its derivatives which, according to the health claim approved by the EFSA, if daily consumed would “protect low-density lipoprotein (LDL) particles from oxidative damage” (EFSA, 2011). However, until now, the studies substantiating the antioxidant capacity of phenolics present in olive oil have been limited, and no data is available for other food matrices (EFSA, 2011). Nevertheless, other numerous studies have shown the potential of HYD for preventing

other diseases (e.g. cancer, digestive disorders) (Achmon and Fishman, 2014) and as food preservative against fat oxidation and microbial contamination (Martínez *et al.*, 2018; Pazos *et al.*, 2008; Quaglia *et al.*, 2016). Other compounds as simple phenolics (caffeic and *p*-coumaric acid) and phenolics (luteolin) were identified in OP liquid fraction, but dihydroxy phenolics such as HYD and tyrosol were the most significative.

After the validation of the potential of the OP liquid and solid fractions as potential food ingredients, the next step was to be developed new food powdered ingredients from OP. To guarantee the economic feasibility, stability and safety of the OP powders some procedures were taken, including: (1) selection of a more cost-effective drying process taking in consideration the stability of the bioactive compounds and microbiological safety (Ahmad-Qasem *et al.*, 2013; Uribe *et al.*, 2013); (2) addition of food additives to improve their stability (Campos *et al.*, 2017) and (3) remotion of physical hazards as small pieces of stones (Sinrod *et al.*, 2019).

OP powders exhibit a reasonable potential as a source of different nutrients and bioactive compounds (Galanakis, 2011; Nunes *et al.*, 2018; Rigane *et al.*, 2012; Rodrigues *et al.*, 2017) with potential complementary/ synergetic benefits in terms of health benefits (Pérez-Jiménez *et al.*, 2015) and technological properties (antioxidant, antimicrobial and enhancer of aroma/taste). The production and characterisation of OP powders have been scarcely studied (Conterno *et al.*, 2019; Pérez-Jiménez *et al.*, 2015; Speroni *et al.*, 2019; Ying *et al.*, 2017). Nonetheless, their potential application in the food industry as safe and multi-source of compounds has been even more undervalued (Pérez-Jiménez *et al.*, 2015). The identification and evaluation of the antioxidants present in DF from OP have been barely studied (Pérez-Jiménez *et al.*, 2015; Uribe *et al.*, 2015), and the potential toxic character of the OP phenolics has been neglected (Pukalskienė *et al.*, 2018). Thus, a more systematic and comprehensive evaluation of their benefits and possible adverse effects is required, *e.g.*, a study concerning genotoxicity and mutagenicity, that have been scarcely investigated.

So, in the present work, two OP powders from the liquid and solid fractions were proposed as new sustainable co-products with food market value. To understand the potential of these powders and guarantee their safe application in the food industry an evaluation of their nutritional composition, bioactive composition (including DF), bioactivity (antioxidant and antimicrobial); functional properties and safety issues (geno and cytotoxicity) were attained. The cytotoxicity and mutagenicity of OP powders were analysed for the first time.

## 4.2. Materials and methods

### 4.2.1. Reagents

Mannitol, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS diammonium salt (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, methanol, potassium sorbate, amino acids pure standards and sodium carbonate were purchased from Sigma-Aldrich (Sintra, Portugal). Folin-Ciocalteu's reagent and

potassium persulfate were purchased from Merck (Algés, Portugal). Muller Hinton broth (MHB) was attained from Biokar Diagnostics (Beauvais, France). Standards of Trolox, gallic acid, p-coumaric acid, vanillin, protocatechuic acid, caffeic acid and quercetin were obtained from Sigma-Aldrich (Sintra, Portugal). HYD, tyrosol, luteolin were purchased from Extrasynthese (Lyon, France).

### 4.2.2. Preparation of olive pomace powders

OP was collected in an olive mill with the two-phase extraction system from Oliveira do Hospital, Portugal. Various samples of OP were collected shortly after being separated from olive oil and mixed. These homogenous samples were immediately packed in polyethene flasks and frozen at -80 $^{0}$C, where they were kept until use.

OP was fractionated by centrifugation (10,000×g for 10 min). The liquid fraction was freeze-dried (Telstar Lyo Quest HT 40) with 2% of mannitol (as a cryoprotectant to prevent aggregation) and denominated liquid-enriched olive pomace powder (LOPP). The solid fraction was dried at 90 °C until levels of water activity ($a_w$) were below 0.4 (on average 90 min), in order to obtain a stable product (Ahmad-Qasem *et al.*, 2013). Previous drying experiments were performed (50, 70 and 90 °C) and the highest temperature presented the best results regarding the total phenolic compounds (TPC) and antioxidant activity (AOX), in line with previous studies (Ahmad-Qasem *et al.*, 2013; Uribe *et al.*, 2013). The dried solid fraction was milled using a coffee grinder and sieved to isolate all the stones pieces and obtain a potentially food-grade ingredient free of physical hazards (Sinrod *et al.*, 2019). A particle size distribution study using a sieve shaker with a series of sieves (mesh No. 10, 18, 30, 40, 60, 100 and 200) was performed to guarantee the elimination of all the pieces of stones (Table 4.1). The fractions retained until mesh 40 (fractions with pieces of stones) were removed, and the other fractions were combined to obtain the pulp-enriched olive pomace powder (POPP). The POPP represents 51.55% dry weight (DW) of the total solid fraction, and its particle size ranged between 75 - 400 µm. The average mesh most representative was the 100 mesh (75 - 150 µm). The relatively large particle size was chosen to maintain the hydration characteristics and the microtexture of the fibre (Martínez *et al.*, 2012).

**Table 4.1. Particle size distribution of solid olive pomace fraction.**

| OP Solid Fraction | Sieving Mesh Size | Distribution (% DW) | |
|---|---|---|---|
| *Fractions with stones* | Unscreened | 5.12 ± 0.30 | *26.18* |
| | 10 (2000 - 1000 µm) | 21.03 ± 0.78 | |
| | 18 (1000 - 600 µm) | 14.38 ± 0.62 | *22.84* |
| | 30 (600 - 400 µm) | 8.31 ± 0.22 | |
| *POPP* | 40 (400 - 250 µm) | 9.54 ± 0.44 | *51.55* |
| | 60 (250 - 150 µm) | 12.76 ± 1.46 | |
| | 100 (150 - 75 µm) | 22.98 ± 0.52 | |
| | 200 (< 75 µm) | 6.00 ± 0.57 | |

### 4.2.3. Chemical composition of OP powders

#### 4.2.3.1. Proximate composition

All procedures followed the recommendations of the Official Methods of Analysis (AOAC, 1990). The crude protein content was achieved using the Kjeldahl method (conversion factor: 6.25). The lipid content was obtained according to method 920.39. The crude ash content was estimated by incineration (method 942.05). The moisture content was determined following the method 934.06. All measurements were done in triplicate and expressed as g/kg DW.

### 4.2.4. Analysis of chemical components of ash, protein and lipids and carbohydrates

Mineral concentration was carried out following the Amorim *et al.* (2016) methodology using an optical emission spectrometer Model Optima 7000 DV™ ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with a radial configuration. The free sugar and organic acid content were obtained (Deng *et al.*, 2011) and determined by Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to RI and UV detector using Aminex 37-H column (Bio-rad, Berkeley, USA) at 55 °C and 35 mM $H_2SO_4$ as mobile phase (flow rate: 0.5 mL/min). The quantification was achieved using standard calibration curves (0.2 - 2.0 mg/mL). Total amino acids content of each powder was performed using pre-column derivatisation with orthophthalaldehyde (OPA) methodology (Amorim *et al.*, 2016) and quantified using a calibration curve built with amino acids pure standards. The profile of fatty acids was obtained and analysed following the methodology of Pimentel *et al.* (2015) with some modifications regarding the internal standard used (glyceryl tritridecanoine, TG-C13) and the derivatisation process (methanol and sodium methoxide were added to 50 mg of sample in the amounts of 2.26 mL and 240 µL, respectively). All measurements were done in triplicate and expressed as g/kg DW.

### 4.2.5. Bioactive phytochemicals

### 4.2.6. Total phenolic compounds extraction and quantification: Free and Bound phenolics

The free phenolics compounds extract (FPC) were obtained using methanol as solvent (1:10, 1 h of agitation on orbital shaker 200 RPM, two successive extractions). The residue obtained after FPC extraction was hydrolysed with 20 mL of 4 M NaOH at room temperature (Xie *et al.*, 2015). The collected fraction of phenolic compounds were designated as bound phenolic compounds (BPC). The total phenolic content (TPC) of FPC and BPC extracts was determined according to the Folin-Ciocalteau method (Oliveira *et al.*, 2016). Results were expressed as mg gallic acid equivalents (GAE)/100 g DW.

#### 4.2.6.1. Determination of dietary fibre composition

TDF content was estimated using the enzyme-gravimetric method, according to AOAC method 991.43 (1990), with slight modifications. After enzymatic hydrolysis, IDF was recovered by filtration on a fritted crucible Pyrex 30 mL (no. 2) and the supernatant was saved for SDF analysis. The IDF residue was washed twice with 10 mL of distilled water, and the supernatants were combined with the previous SDF supernatant obtained for determining total SDF content. The IDF residue was washed once with 20 mL of 96% ethanol, followed by 20 mL of acetone twice, and then dried overnight at 45 °C. The filtrate was purified by dialysis (molecular weight cut-off of 3,5 kDa) to obtain the SDF. The dialysis was applied to avoid the error caused by precipitating DF with ethanol (Deng *et al.*, 2011). Afterwards, SDF was recovered by dialysate freeze-drying. The results were expressed as TDF, IDF and SDF g/100 g DW.

According to the NREL method, the IDF was submitted to two different acid hydrolysis (Sluiter *et al.*, 2012). The residue is used for the determination of Klason lignin (KL). KL was the weight of residue after drying at 105 °C for 16 h subtracting the ash (ashing for 5 h at 525 °C) and the resistant protein (RP). RP defined as the protein after protease treatment, and acid hydrolysis in IDF was determined by the micro-Kjeldahl method using a nitrogen-to-protein conversion factor of 6.25. The filtrate is used to quantify uronic acids (UA) and neutral sugars (NS). The individual neutral sugars were determined by HPLC (micro guard column: Aminex Carbo-P, Bio-Rad; carbohydrate analysis column: Aminex HPX-87P heavy metal, 300–7.8 mm, Bio-Rad; flow rate: 0.6 mL/min; detector: refractive index), and uronic acids (UA) were determined colourimetrically by adapting the 3-hydroxydiphenyl method of Blumenkrantz & Asboe-Hansen (1973) with *d*-galacturonic acid as standard. IDF was the total content of NS, UA and KL. The SDF preparation was acid hydrolysed in 6% sulfuric acid at 121 ◦C for 1 h (Bravo and Saura-Calixto, 1998). The hydrolysate is used for quantifying UA and NS as described above. The sum of NS and UA was taken as the amount of SDF in OP powders.

The phenolic compounds were released from IDF and SDF fractions using the same hydrolysis process described above. This methodology is in agreement with the procedure applied in other food matrices as whole-grain cereals (Guo and Beta, 2013) to release BPC from IDF and SDF. The BPC extract obtained from IDF and SDF were denominated as IDF-BPC and SDF-BPC, respectively. These extracts were used to measure TPC (as described above), AOX (as described below) and identification of phenolic compounds by LC-ESI-UHR-QqTOF-MS (as described below).

#### 4.2.6.2. Identification of phenolics by LC-ESI-UHR-QqTOF-MS and HPLC system

The phenolic compounds of FPC, BPC, IDF-BPC and SDF-BPC extracts were identified and analysed in an LC-ESI-UHR-QqTOF-MS following the methodology of Monforte *et al.* (2018). Identification of main phenolic compounds was based on the retention time, UV-Vis and mass spectra with those obtained from the standard solutions, when available. The other peaks were tentatively identified comparing the information with available data reported in the literature (Klen and

Vodopivec, 2012; Lozano-Sánchez *et al.*, 2013). The elemental composition for these compounds was confirmed according to accurate mass (5 mDa), and isotope rate calculations designated mSigma (<20) (Bruker Daltonics) (Monforte *et al.*, 2018).

The chromatographic analysis of FPC, BPC, IDF-BPC and SDF-BPC extracts were performed following the methodology described by Oliveira *et al.* (2015). The main phenolic compounds identified (HYD, protocatechuic acid, tyrosol, vanillin, caffeic acid, p-coumaric acid and luteolin) were quantified by HPLC using external calibration curves constructed based on their maximum UV signal. The results were expressed as mg/100 g DW. The HYD glucoside and tyrosol glucoside were respectively expressed as HYD and tyrosol equivalents in mg/100 g DW.

### 4.2.7. Antioxidant activity evaluation

The FPC, BPC, IDF-BPC and SDF-BPC extracts were used to evaluate the AOX of OP powders according to the methods of DPPH (Alexandre *et al.*, 2019), ABTS (Cano *et al.*, 2000) and ORAC (Oliveira and Pintado, 2015) using a microplate reader (Fluostar, Optima; BMG Labtech, Ortenberg, Germany). The radical stock solutions were freshly prepared. All analyses were performed in triplicate and expressed in μM of Trolox-equivalents (TE)/g DW.

### 4.2.8. Screening of antimicrobial activity

#### 4.2.8.1. Bacterial species and test solutions

*Bacillus cereus*, methicilin sensitive *Staphylococcus aureus* (MSSA), *Salmonella enteritidis* and *Escherichia coli* were obtained from American Type Culture Collection (ATCC 2599, ATCC 25923, ATCC 13076 and ATCC 25922 respectively). *Yersinia enterocolitica* was attained from the National Collection of Type Cultures (NCTC 10406), and *Listeria monocytogenes* (13562) was kindly provided by Universidade Católica's Culture Collection. Inocula were grown overnight in MHB (Biokar Diagnostics, Beauvais, France) at 37 °C. OP powders were suspended in MHB and sterilised by filtration through a 0.22 μm filter (Millipore, Billerica, USA).

#### 4.2.8.2. Growth inhibition curves

OP powders solutions between 1 and 3% (w/v) were prepared and inoculated at 1% (v/v) using an overnight inoculum of $10^8$ CFU/mL. Optical density (OD) at 660 nm was assessed for a 24 h period at 37 °C (1 h intervals) using a microplate reader, with the increase in OD was considered a consequence of bacterial growth. A positive control (inoculated MHB without powder) and negative control (medium only) were used (Alexandre *et al.*, 2019). Each condition was assayed in duplicate.

An inhibition percentage was calculated using the following formula:

$$Inhibition\ \% = \frac{OD_{control\ bacteria} - OD_{bacteria}}{OD_{control\ bacteria}} \times 100$$

where $OD_{control\ bacteria}$ and $OD_{bacteria}$ represent the OD (at 650 nm) after 24 h of incubation of the control bacteria without OP powder and in the presence of the OP powder, respectively (Madureira *et al.*, 2015).

#### 4.2.8.3. Minimum inhibitory and bactericidal concentrations

Based on the results of the growth inhibition curves, the minimum inhibitory concentration (MIC) determination was performed only for the OP powders and microorganisms with relevant results. Test solutions ranging from 20 to 40 mg/mL were tested. MIC was executed based on the guidelines standard M07-A9. The minimum bactericidal concentration (MBC) was determined as the lowest concentration tested, which prevented bacterial growth (Fernandes *et al.*, 2008). It was determined by inoculation of 20 µL aliquots of negative wells (absence of turbidity in MIC determination) on Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France), using the plate count technique. All assays were performed in quadruplicate.

### 4.2.9. Functional properties

The powders solubility in water was determined, according to Cano-Chauca *et al.* (2005). The water/oil-holding capacity (WHC/OHC) was determined by centrifugation (Rubio-Senent *et al.*, 2015a). The oil-holding capacity (OHC) was determined under the same conditions as WHC using sunflower oil (0.92 g/mL density) and was expressed as g oil retained/g sample.

### 4.2.10. Toxicity

#### 4.2.10.1. Cytotoxicity

Caucasian colon carcinoma (Caco-2; 8601020 European Collection of Authenticated Cell Cultures) cells were grown in Dulbecco's Modified Eagle's Medium (Lonza, Basel, Switzerland) supplemented with 10% (v $v^{-1}$) heat-inactivated Fetal Bovine Serum (Biowest, France), 1% (v $v^{-1}$) Pen-Strep (Lonza, Basel, Switzerland) and 1% (v $v^{-1}$) of non-essential amino acids 100x (Lonza, Basel, Switzerland). All cells were incubated at 37 °C in a humidified atmosphere with 5% $CO_2$. To evaluate the OP powders impact upon Caco-2 viability, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-caboxanilide (XTT) assay was used (Costa *et al.*, 2019). The cells were exposed to test solutions at concentrations of 20, 10, 5, 2.5 and 1.25 mg/mL. LOPP was freshly prepared, while POPP was previously leached overnight in culture media. All solutions were sterilized through filtering with a 0.22 µm filter. Plain culture media was used as a negative control. All assays were performed in quintuplicate.

#### 4.2.10.2. Mutagenicity

The mutagenic effect was assessed by the Ames test using *Salmonella typhimurium* strain (TA98) according to Maron & Ames, (1983). The OP powders were tested at different concentrations (15 - 1000 µg sample/plate). Positive control (quercetin 20 µg/plate) and solvent control (0.05 mL DMSO/plate) were carried out. For a powder to be considered positive, it had to produce at least a 2-fold increase in the mean revertant colonies per plate over the mean revertant colonies per plate of the positive control. Three plates per two separate experiments were assayed for each concentration tested and for positive and negative controls.

### 4.2.11. Statistical analysis

Data obtained for all parameters were analysed using a t-student and two-way ANOVA. Tukey's post hoc test was applied to compare means; differences were considered significant at $p < 0.05$. The Shapiro-Wilk test evaluated the normality of data distribution. R Software (R Core Team, 2017) was used to carry out the statistical analysis.

## 4.3. Results and Discussion

### 4.3.1. Chemical and structural characterisation of olive pomace powders

The physicochemical composition of each OP powder, as seen in Table 4.2, demonstrates that overall, higher ash, soluble sugar and organic acid content were present in LOPP, while POPP exhibited a higher crude protein and fat content. Similar results were reported regarding OP liquid and pulp fraction (Ying *et al.*, 2017). In agreement with their ash content, OP powders also have significantly different amounts of minerals. Potassium was the predominant mineral in both OP powders, as observed before in olive mill by-products (Uribe *et al.*, 2013). The potassium amount was notably higher in LOPP (54.31 g/kg DW) in comparison to POPP (17.56 g/kg DW), probably due to the water solubility of the minerals. The high amount of potassium, low sodium content and a mixture of other minerals (phosphorous, magnesium and calcium) of LOPP could be explored by the food industry, not only in the mineral fortification of foods (potentially as a taste agent/replacer of sodium chloride) but also to add potential health benefits. For instance, potassium's higher consumption is associated with cardiovascular disease prevention and normal blood pressure maintenance (EFSA, 2010) (Amorim *et al.*, 2016). On the other hand, the mixture of other minerals could be an asset to mask the bitter taste of potassium, allowing LOPP to be used as a taste agent.

**Table 4.2. Chemical composition of olive pomace powders (g/kg DW).**

| | | | *LOPP* | *POPP* |
|---|---|---|---|---|
| **Proximate Composition** | | *Moisture* | 33.71 ± 1.62 [a] | 36.55 ± 2.32 [b] |
| | | *Protein* | 11.17 ± 1.48 [a] | 118.24 ± 1.24 [b] |
| | | *Lipid* | 8.01 ± 0.39 [a] | 119.54 ± 6.99 [b] |
| | | *Ash* | 84.56 ± 6.64[a] | 31.92 ± 0.22 [b] |
| **Minerals** | | *P* | 4.76 ± 0.02 [a] | 1.59 ± 0.18 [b] |
| | | *Mg* | 1.53 ± 0.03 [a] | 0.50 ± 0.07 [b] |
| | | *Ca* | 1.32 ± 0.01 [a] | 1.65 ± 0.21 [a] |
| | | *Na* | 0.17 ± 0.01 | * |
| | | *K* | 54.31 ± 1.05 [a] | 17.56 ± 2.61 [b] |
| **Sugars** | | *Glucose* | 125.69 ± 11.10 [a] | 35.29 ± 2.80 [b] |
| | | *Fructose* | 16.43 ± 1.43 [a] | 4.07 ± 0.57 [b] |
| | | *Mannitol* | 141.11 ± 12.45 [a] | 8.85 ± 0.45 [b] |
| **Organic acids** | | *Lactic* | 4.15 ± 0.18 [a] | 1.18 ± 0.02 [b] |
| | | *Formic* | 10.27 ± 0.72 [a] | 4.55 ± 0.36 [b] |
| | | *Acetic* | 2.62 ± 0.13 [a] | 0.49 ± 0.01 [b] |
| **Amino acids** | ***Non-essential*** | *Aspartic acid* | * | 22.11 ± 2.32 |
| | | *Glutamic acid* | * | 21.63 ± 2.58 |
| | | *Glutamine* | * | 8.98 ± 0.84 |
| | | *Alanine* | * | 5.99 ± 0.64 |
| | | *Arginine* | * | 5.75 ± 0.19 |
| | | *Cysteine* | * | 8.98 ± 0.84 |
| | ***Essential*** | *Histidine* | * | 21.38 ± 1.16 |
| | | *Threonine* | * | 1.60 ± 0.14 |
| | | *Tyrosine* | * | 3.73 ± 0.41 |
| | | *Valine* | * | 7.11 ± 0. 54 |
| | | *Methionine* | * | 2.72 ± 0.05 |
| | | *Tryptophan* | * | 11.87 ± 0.94 |
| | | *Phenylalanine* | * | 11.03 ± 1.32 |
| **Fatty acids** | *Saturated* | *Myristic C14:0* | 0.29 ± 0.03 | ND |
| | | *Palmitic C16:0* | 1.22 ± 0.16 [a] | 33.69 ± 0.39 [b] |
| | | *Estearic C18:0* | 0.24 ± 0.03 [a] | 4.98 ± 0.07 [b] |
| | | *Archidic C20:0* | 0.03 ± 0.00 [a] | 1.10 ± 0.03 [b] |
| | ***Unsaturated*** | *Palmitoleic C16:1 c9* | 0.14 ± 0.02 [a] | 3.75 ± 0.04 [b] |
| | | *Oleic C18:1 c9* | 5.21 ± 0.71 [a] | 160.58 ± 1.91 [b] |
| | | *cis-Vaccenic C18:1 c11* | 0.26 ± 0.04 [a] | 7.82 ± 0.06 [b] |
| | | *Linoleic C18:2 c9c12* | 0.40 ± 0.05 [a] | 14.73 ± 0.17 [b] |
| | | *$\alpha$-linolenic $\alpha$ C18:3 c9c12c15* | 0.07 ± 0.01 [a] | 1.73 ± 0.03 [b] |
| **Total phenolic compounds (TPC)[*1]** | | *FPC* | 30.49 ± 1.42 [a] | 4.48 ± 0.23 [b] |
| | | *BPC* | 4.97 ± 0.36 [a] | 8.48 ± 0.39 [b] |
| **Dietary Fibre** | | *TDF* | 92.03 ± 11.79 [b] | 620.64 ± 5.38 [a] |
| | | *IDF* | ND | 521.72 ± 0.07 |
| | | *SDF* | 92.03 ± 11.79 [a] | 98.92 ± 5.44 [a] |

* < LOD. FPC: Free phenolic compounds extract; BPC: Bound phenolic compounds extract; [*1] - mg GAE/g DW. TDF – Total dietary fibre; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. Results are the means of three determinations ± standard deviation. Different letters in the same line are significantly different, as determined by the t-Student test ($p < 0.05$).

LOPP also exhibited a higher amount of soluble sugars and organic acids than POPP. The HPLC analysis of soluble sugars and organic acids allowed the identification of mannitol and formic acid as the most representative sugar and organic acid, respectively (Table 4.2). The quantification of soluble sugars in OP-derived ingredients was also achieved in previous work (Pérez-Jiménez *et al.*, 2015). Pérez-Jiménez *et al.* (2015) obtained powder with a similar amount of fructose and a smaller amount of glucose or mannitol compared to LOPP, but POPP exhibited a smaller amount of all the soluble sugars quantified. These differences arose mainly due to the liquid-solid fractionation process to obtain LOPP and POPP from OP. LOPP's high mannitol content (141.11 ± 12.45 g/kg DW) could be an asset to its application as a food ingredient. Mannitol has been reported as an antioxidant compound, as well as a food preservative (increase food shelf life by reducing sugar crystallisation) and also as being a non-metabolisable sweetener (uptake independent of insulin) (Ghoreishi and Shahrestani, 2009).

Organic acids have been scarcely studied in OP-derived ingredients. However, LOPP's organic acid composition supports its applicability as a food preservative and taste agent. Formic acid, which was the main organic acid found in LOPP (10.27 ± 0. 72 g/kg g DW) is known for its antimicrobial action (Aquilina *et al.*, 2014). Lactic and acetic acids, detected in smaller amounts (2.62 – 4.15 g/kg DW) are also commonly used as antimicrobial agents, but could also be applied as a flavour enhancer, as reported before in fish sauce (Park *et al.*, 2001).

The relatively high crude protein (118.24 ± 1.24 g/kg g DW) and fat content (119.54 ± 6.99 g/kg g DW) of POPP indicate this powder may be a rich source of amino acids and fatty acids. Similar protein (Ying *et al.*, 2017) and fat content (Crizel *et al.*, 2016) were reported in the literature regarding OP-derived powders. OP has not been *considered* a reputable source of protein (less than 8% DW) (Prandi *et al.*, 2019). However, the dry fractionation approach applied to obtain POPP had a small concentrating effect (Schutyser *et al.*, 2015) in line with Ying *et al.* (2017) results. On the other hand, the OP powder developed by Crizel *et al.* (2016) without fractionation of OP (drying at 55 ºC and discarding particles higher than 125 mm) in comparison to POPP presented only half of POPP's protein, but similar fat content.

The quantitative composition of total amino acid and fatty acids was achieved (Table 4.2). Regarding the amino acid profile and the amino acid pool, POPP maintained the values reported to OP (Dermeche *et al.*, 2013; Prandi *et al.*, 2019). Amino acids Glu (21.63 ± 2.58 g/kg g DW) and Asp (22.11 ± 2.32 g/kg g DW) showed the highest values. These amino acids are sour stimuli and cause umami taste when present in the form of their sodium salt (Amorim *et al.*, 2016), which could be an asset in POPP application in meat products, for example. Concerning fatty acids, POPP exhibits a higher level of oleic acid (corresponding to 70% of total fatty acids) followed by linoleic acid (that corresponds to 6%). A similar relative percentage of these UFAs were reported to olive oil and an OP-derived powder (Pérez-Jiménez *et al.*, 2015). The UFAs composition of POPP is similar to commercial olive oil. Thus, it was expected health benefits like reduction of cholesterol /triglycerides or even obesity prevention (by restoring the gut bacteria composition) from POPP application (Lopez-Huertas, 2010; Mujico *et al.*, 2013).

### 4.3.2. Bioactive phytochemicals composition of OP powders

A complete characterisation of the OP powders phenolic content (TPC) was performed (Table 4.2), including the usually determined FPC fraction, as well as BPC fraction which is commonly ignored (Saura-Calixto, 1998). The LOPP exhibited a significantly higher TPC for FPC (30.49 ± 1.42 mg GAE/ g DW) than POPP (4.48 ± 0.23 mg GAE/g DW) ($p < 0.05$). Regarding BPC, POPP showed the highest TPC value ($p < 0.05$). The TPC value of FPC in LOPP was very similar to the value obtained before for the freeze-dried liquid fraction powder obtained by Ying *et al.* (2017). A freeze-dried pulp fraction was also obtained in the same study, which exhibited a higher TPC value for FPC (≈ 20 mg GAE/ g DW) than POPP. These differences most likely arise from the different drying process employed by Ying *et al.*, (2017) (freeze-drying) and in this study (oven drying). The use of temperature to obtain POPP is probably the main reason for its lower TPC value (Ahmad-Qasem *et al.*, 2013; Uribe *et al.*, 2013). Other OP-derived powder reported in literature prepared using temperature (55 ºC) exhibited a lower TPC (≈ 10 mg GAE/ g DW) in comparison with the one obtained in our study that used a drying temperature of 90 ºC (Crizel *et al.*, 2016). The bound phenolic content was not estimated in either study. Only Pérez-Jiménez *et al.* (2015) evaluated BPC of their OP-derived powder obtained without separation of the liquid and solid fraction of OP and using vacuum drying. The BPC value obtained in the study mentioned above (≈ 16 mg GAE/g DW) is analogous with the sum of BPC of LOPP (4.97 ± 0.36 mg GAE/ g DW) and POPP (8.48 ± 0.39 mg GAE/g DW) attained. POPP's higher BPC value was possibly related to its superior IDF content (Saura-Calixto, 2011). Other studies with vegetable and fruits reported that BPC was mainly associated with IDF (Goñi *et al.*, 2009; Saura-Calixto, 2011).

POPP showed a substantially higher amount of TDF (620.64 ± 5.38 g/kg DW), where IDF (521.72 ± 0.07 g/kg DW) was the main component (Table 4.2). The SDF amount of both OP powders was similar (9-10% DW). POPP exhibited a higher TDF, IDF and SDF content than the values recorded to other OP-derived powders (Crizel *et al.*, 2016; Pérez-Jiménez *et al.*, 2015; Ying *et al.*, 2017). The process used by Ying *et al.* (2017) to remove stones and the higher time of drying (24 h) at lower temperatures (55 ºC) of Crizel *et al.* (2016) seems to affected TDF content negatively. On the other hand, the defatting process applied to OP by Pérez-Jiménez *et al.* (2015) only lowered IDF and TDF content. In brief, POPP could be considered an excellent source of DF and might be applied as fibre fortifier. Indeed, POPP exhibited higher TDF content (62.06 ± 0.54 g/100 g DW) than wheat bran (44%), and oat bran (23.8%) generally applied in fibre-fortification of foods (Crizel *et al.*, 2016).

The determination of the chemical structure (*i.e.*, monosaccharides, lignin, uronic acids, phenolics) is fundamental to understand the several health benefits of fibre. Despite the OP fibre richness, until now the fibre profile of the OP-derived powders has been neglected. The results of a monosaccharide, lignin and uronic acids composition of IDF and SDF among OP powders were shown in Table 4.3. The monosaccharide composition of the SDF fraction of OP powders was very distinct. POPP revealed the highest uronic acid content, which could be explained by the higher concentration of pectins reported in our previous work to OP solid fraction (data not shown). Pectins have essential roles in regulating and protecting the gastrointestinal tract due to its potential prebiotic

effect (Gullón *et al.*, 2013). The SDF monosaccharide profile revealed a higher soluble hemicellulose-rich character for LOPP than POPP. The main component of LOPP was galactose (≈ 43%), followed by arabinose (≈ 33%) and glucose (≈ 19%).

**Table 4.3. Constituents (mg/g fibre DW) of IDF and SDF from olive pomace powders.**

| | | Monosaccharide composition | | | | | | UA[*1] | RP | KL | BPC [*2] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Glu* | *Xyl* | *Gal* | *Arab* | *Man* | *NS* | | | | |
| ***LOPP*** | ***SDF*** | 28.46 ± 3.60 [a] | * | 62.54 ± 10.08 [a] | 48.14 ± 5.60 [a] | 8.07 ± 1.37 | 147.21 | 16.47 ± 2.46 [a] | | | 14.56 ± 1.74 [a] |
| ***POPP*** | ***IDF*** | 167.91 ± 12.56 [b] | 95.06 ± *7.49* | 8.55 ± 1.02 [c] | 33.54 ± 2.17 [b] | * | 305.06 | 49.52 ± *2.87* [b] | 7.46 ± 0.03 | 281.64 ± 9.35 | 7.41 ± 0.62 [b] |
| | ***SDF*** | 14.18 ± 1.90 [c] | * | 25.88 ± 0.53 [c] | 39.07 ± 1.86 [c] | * | 79.13 | 81.25 ± 4.61 [c] | | | 7.63 ± 0.30 [b] |

* < LOD. Glu – Glucose; Xyl – Xylose; Gal - Galactose; Arab – Arabinose; Man – Mannose; NS – Neutral sugars; UA – Uronic acids; RP – Resistant protein; KL – Klason lignin; BPC: Bound phenolic compounds; TDF – Total dietary fibre; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. [*1] - mg GUAE / g fibre dry weight; [*2] - mg GAE/g fibre DW. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as ANOVA ($p < 0.05$) determined.

POPP's IDF profile revealed a higher content of glucose (≈ 55% of total neutral sugars) followed by xylose (≈ 31% of total neutral sugars). Hemicellulose (as the sum of xylose, galactose, and arabinose) was less abundant in the IDF from POPP than cellulose (glucose monomers). Based on the monosaccharide composition results, IDF fraction of POPP was mostly a source of insoluble arabinoxylans and cellulose. However, the main component of IDF of POPP was Klason lignin (≈ 43% of total IDF composition), in line with the previous studies about OP fibre composition (Dermeche *et al.*, 2013). Arabinoxylans are essential functional ingredients in baked products that have been associated with various biological functions, including positive gut microbiota effects (Chen *et al.*, 2019). On the other hand, the lignin as complex macromolecule with linked phenolic compounds (BPC) protects these compounds through the gastrointestinal tract until the colon, where they play a beneficial role in gut health (Sato *et al.*, 2011).

In the past, DF and phenolics were assessed separately as nonrelated compounds. However, phenolics are a constituent of DF with a significant role in its properties (Saura-Calixto, 2011). To best our knowledge, the role of phenolics in DF has never been investigated in OP-derived ingredients. According to the TPC results, the OP powders fibre fractions showed different TPC amounts (Table 4.3). Comparing the BPC extracts of LOPP, SDF-BPC extract displayed tree time's superior TPC levels than BPC extract, which allowed to conclude that BPC of LOPP was mainly linked to fibre. The IDF-BPC and SDF-BPC extract of POPP exhibited similar TPC contents. Comparing the TPC of BPC extracts from POPP and their fibre fractions, it is evident that DF plays a crucial role as a carrier of more than half of total BPC (≈ 54%). As expected, the IDF fraction presented the highest amount of BPC (≈ 45%), but SDF also presents some phenolics linked to them. Therefore, in POPP, it is possible to hypothesise the existence of phenolics linked to other macromolecules besides DF, namely protein and lipids.

In previous studies, the occurrence of interactions between lipids/proteins and phenolics and consequent protection of phenolic through the gastrointestinal tract has been described (Jakobek, 2015). In protein-phenolics association, the core interactions are non-covalent hydrophobic stabilised by hydrogen bonds, mainly with polar amino acids (*e.g*. aspartic acid, glutamic acid, cysteine) (Ozdal *et al.*, 2019). As highlighted above, the POPP exhibited a high content in polar amino acids, which could establish hydrogen bonds with the phenolics. Nevertheless, in the case of the lipids, not only the lipids could protect the phenolics, but phenolics could also protect lipids from peroxidation (Gorelik *et al.*, 2013). The lipid-phenolic interaction could be an asset to POPP's lipids stability.

Another compound that remains associated with IDF is protein. The amount of RP of POPP's IDF fraction was measured (Table 4.3). The RP content obtained was small (1%) compared to other plant foods (Goñi *et al.*, 2009).

Regarding the identification and quantification of phenolic compounds of OP powders and its fibre fractions, the LC-ESI-UHR-QqTOF-MS and HPLC results were reported in Table 4.4 and Figure 4.1, respectively. The analysis allowed to identify and quantify the phenolic classes present in the OP powders. Regarding specific phenolic classes, mainly simple or dihydroxy phenolics were identified in FPC and BPC fractions of OP powders, more specifically HYD and tyrosol.

HYD stands out as an abundant phenolic in olive by-products with higher added-value (Nunes *et al.*, 2019; Rigane *et al.*, 2012). Additionally, one derivative of HYD was also identified, namely HYD glucoside. This phenolic was identified in all extracts, except for extracts from POPP fibre. HYD glucoside is a polar compound with a considerable size and some glycosylation degree (Romero *et al.*, 2002). Therefore, it is reasonable that it could be linked to POPP protein due to its high molecular weight (Ozdal *et al.*, 2019). Another monoglycoside identified in a substantial amount in FPC, and BPC extracts was tyrosol glucoside. Other compounds like loganin, verbascoside, caffeoyl-6-secologanoside, quercetin and apigenin were only identified in FPC extract of both OP powders, and p-coumaroyl-D-glucose, and comsegoloside was just detected in FPC extract of LOPP. On the other hand, protocatechuic acid and elenolic acid derivatives (a decarboxylated form of hydroxy-elenolic acid) (Lozano-Sánchez *et al.*, 2013) were exclusively found in BPC extracts. Hydroxybenzoic acids such as protocatechuic acid are typically found linked to structural components as cellulose and lignin, which are the main components of the abundant IDF fraction of POPP (Calvache *et al.*, 2016). All the compounds reported were identified before in olive oil and olive by-products (Klen and Vodopivec, 2012; Lozano-Sánchez *et al.*, 2013).

The HPLC quantification of the principal components of FPC and BPC extract from OP powders were assessed (Figure 4.1). Concerning the content of HYD, tyrosol and derivatives (FPC: 655.96 mg/100 g DW and BPC: 79.00 mg/100 g DW) in LOPP, it was substantial, and thus, it could potentially allow obtaining the benefits of the health claim "protection of LDL particles from oxidative damage" with daily consumption of less than 1 g.

As expected, the content of HYD glucoside and hydroxycinnamic acids (caffeic and p-coumaric acid) was higher in the BPC than in FPC of POPP due to their linkage to cellulose, lignin and protein through ester linkages (Calvache *et al.*, 2016). This considerable amount of BPC phenolics linked to

fibre, cannot be absorbed in the small intestine but could be released and metabolised by bacterial enzymes of the gut flora to generate protective health effects, including prebiotic effects, antimicrobial action against pathogens and AOX from phenolic metabolites (Conterno *et al.*, 2019). Therefore, POPP's potential gut health benefits as an ADF source need to be assessed with further detail in future studies.

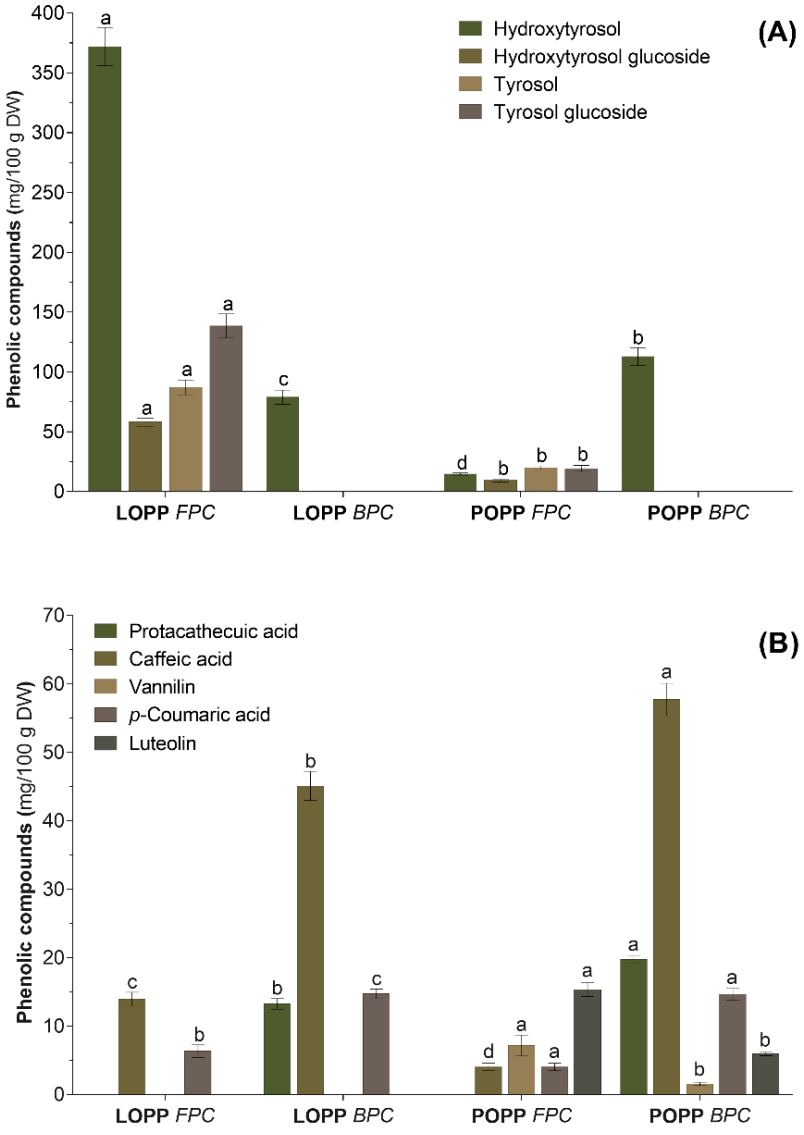



**Figure 4.1. Phenolic profile (mg/100 g sample dry weight) of OP powders: (A) Hydroxytyrosol and derivatives and (B) Other phenolic compounds.**

FPC: Free phenolic compounds extract; BPC: Bound phenolic compounds extract. IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as ANOVA ($p < 0.05$) determined.

**Table 4.4. Retention time, maximum wavelength absorbance and MSn fragmentations profiles of phenolic compounds identified in olive pomace powders in free phenolics (FPC), bound phenolics (BPC) and bound phenolics of dietary fibre (SDF-LOPP; IDF-POPP and SDF-POPP).**

| Compound | Molecular formula | RT (min) | $\lambda_{max}$ | m/z calcd | m/z exptl | Err [mDa] | mSigma | Major fragments ESI negative MS/MS ions | Extract | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | FPC | BPC |
| **Protocatechuic acid** [*2] | $C_7H_8O_4$ | 6.6 | 260/294 | 153.0109 | 153.0105 | 0.5 | 6.6 | 109.0237 | | POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Hydroxytyrosol glucoside** [*2,3] | $C_{14}H_{20}O_8$ | 7.1 | 279 | 315.1088 | 315.1085 | -0.2 | 4 | 315.1092; 153.0556; 123.0451 | LOPP; POPP | LOPP; POPP; SDF-LOPP |
| **Hydroxylated product of the dialdehydic form of decarboxymethyl-elenolic acid** [*3] | $C_9H_{12}O_5$ | 7.2 | 197/279 | 199.0615 | 199.0612 | -0.3 | 9.6 | 198.5356; 111.0814; 123.0453; 97.0296 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Vanillin** [*1] | $C_8H_8O_3$ | 7.3 | 230 | 151.0401 | 151.0399 | 0.1 | 5.6 | 123.0450; 109.0291; 151.0401 | POPP | POPP; SDF-LOPP;IDF-POPP; SDF-POPP |
| **Hydrated product of the dialdehydic form of decarboxymethyl-elenolic acid** [*3] | $C_9H_{14}O_5$ | 7.3 | 197/281 | 201.077 | 201.0768 | -0.2 | 4 | 201.0405; 153.0553; 123.0449; 95.0502 | LOPP; POPP | LOPP; POPP |
| **Hydroxytyrosol** [*1] | $C_8H_{10}O_3$ | 7.4 | 280 | 153.0556 | 153.0557 | 0.1 | 5.7 | 153.0452; 123.0457 | LOPP; POPP | LOPP; POPP; SDF-LOPP;IDF-POPP; SDF-POPP |
| **Loganin** [*2,3] | $C_{17}H_{26}O_{10}$ | 7.6 | 281 | 389.1455 | 389.1453 | -0.2 | 8.9 | 151.0763; 113.0244; 101.0244 | LOPP; POPP | |
| **Oleoside derivative isomer** [*3] | $C_{17}H_{28}O_{11}$ | 7.7 | 228 | 407.1557 | 407.1559 | -0.5 | 11.7 | 151.0760; 119.0346 | LOPP; POPP | LOPP; SDF-LOPP |
| **Decarboxylated form of hydroxy-elenolic acid** [*3] | $C_{10}H_{14}O_5$ | 7.9 | 197/280 | 213.0765 | 213.0768 | 0.1 | 5.6 | 213.0920; 137.0601; 121.0665;111.0085 | | LOPP; POPP; SDF-LOPP; SDF-POPP |
| **Tyrosol glucoside** [*2,3] | $C_{14}H_{20}O_7$ | 8.1 | 227/276 | 299.1139 | 299.1136 | -0.3 | 3.2 | 119.0505; 137.0244; 135.0299 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Oleoside** [*2,3] | $C_{16}H_{22}O_{11}$ | 8.4 | 277 | 389.1093 | 389.1089 | -0.4 | 4.3 | 389.1088; 183.0664; 165.0557 | LOPP; POPP | LOPP; POPP;SDF-LOPP; IDF-POPP; SDF-POPP |
| **p-Coumaroyl-D-glucose** [*2] | $C_{15}H_{18}O_8$ | 8.7 | 195/280 | 325.0925 | 325.0929 | 0.4 | 0.9 | 326.0976; 163.0402; 119.0501 | LOPP | |
| **Caffeic acid** [*1] | $C_9H_8O_4$ | 9.3 | 323 | 179.0350 | 179.0350 | -0.1 | 7.9 | 179.0350; 135.0448 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Oleuropein aglycone isomer** [*2] | $C_{16}H_{26}O_{10}$ | 9.4 | 377 | 377.1457 | 377.1453 | -0.4 | 7.3 | 378.1484; 197.0823; 153.0921;119.0350 | LOPP; POPP | LOPP; POPP; SDF-LOPP |
| **10-Hydroxyloganin** [*3] | $C_{17}H_{26}O_{11}$ | 9.5 | 197 /278 | 405.1402 | 405.1406 | -0.4 | 5.7 | 405.1402; 165.0557; 139.0035; 123.045 | POPP | |
| **Tyrosol** [*1] | $C_8H_{10}O_2$ | 9.8 | 277 | 137.0608 | 137.0608 | 0.5 | 4.5 | 111.0084; 95.0510 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Aldehydic form of decarboxymethyl-elenolic acid** [*3] | $C_{10}H_{16}O_5$ | 10.1 | 198/283 | 215.0927 | 215.0925 | -0.2 | 5 | 215.0923; 171.1025; 153.0918; 141.0917; 125.0696 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Coumaric acid** [*1] | $C_9H_8O_3$ | 10.6 | 309 | 163.0403 | 163.0401 | -0.2 | 10.5 | 163.0397; 119.0499 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Verbascoside** [*2] | $C_{29}H_{36}O_{15}$ | 10.7 | 280/330 | 623.1988 | 623.1981 | -0.7 | 2.4 | 623.1983; 161.0244; 461.1665 | LOPP | |
| **Caffeoyl-6-secologanoside** [*2] | $C_{25}H_{28}O_{14}$ | 10.8 | 221/326 | 551.1407 | 551.1406 | 0 | 18.1 | 551.1416; 161.0245; 507.1504 | LOPP; POPP | |
| **Hydroxylated form of elenolic acid** [*3] | $C_{11}H_{14}O_7$ | 10.9 | 280/320 | 257.0666 | 257.0667 | 0.1 | 2.7 | 257.1271; 181.0505; 137.0603; 109.0655; 95.0498 | POPP | LOPP; POPP; SDF-LOPP |
| **Luteolin- 7 – O- glucoside** [*1] | $C_{21}H_{20}O_{11}$ | 12.0 | 237/266/352 | 447.0998 | 447.099 | 0.8 | 19.4 | 285.0405; 447.0931 | POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Elenolic acid** [*3] | $C_{11}H_{14}O_6$ | 12.5 | 197/288 | 241.0720 | 241.0718 | -0.2 | 0.5 | 241.0737; 139.0035; 127.0398; 111.0086; 95.0551 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Comsegoloside** [*2] | $C_{25}H_{28}O_{13}$ | 12.6 | 223/311 | 535.1462 | 535.1457 | -0.5 | 6.4 | 535.1465; 145.0296; 491.1558 | LOPP; POPP | |
| **Luteolin** [*1] | $C_{15}H_{10}O_6$ | 15.9 | 268/349 | 285.0406 | 285.0405 | -0.1 | 0.8 | 285.0414; 151.0037 | LOPP; POPP | LOPP; POPP; SDF-LOPP; IDF-POPP; SDF-POPP |
| **Quercetin** [*1] | $C_{15}H_{10}O_7$ | 15.0 | | 301.0357 | 301.0354 | -0.3 | 3.4 | 301.0359; 151.0035; 178.9988 | LOPP; POPP | |
| **Apigenin** [*1] | $C_{15}H_{10}O_5$ | 18.0 | 268, 338 | 269.0460 | 269.0455 | -0.5 | 4.8 | 269.0461; 151.0035 | LOPP; POPP | |

[*1] Hydroxytyrosol, tyrosol, luteolin-7-O-glucoside, luteolin, quercetin, apigenin, vanillin, caffeic acid and coumaric acid were identified by comparison with the standards. The other compounds were tentatively identified by LC-ESI-UHR-QqTOF-MS based on accurate mass, isotope rate calculations designated mSigma and literature data [*2] Jerman Klen & Mozetič Vodopivec, 2012; [*3] Lozano-Sánchez *et al.*, 2013.

### 4.3.3. Bioactivity of OP powders

Regarding the AOX, LOPP showed the highest AOX value by ABTS, DPPH and ORAC (Figure 4.2) according to its higher TPC. In LOPP, the higher TPC of FPC extracts than BPC extracts could explain the differences in the AOX of FPC and BPC extracts. LOPP exhibited a DPPH value lower than the reported in the literature to fresh OP (≈ 100 μmol TE $g^{-1}$ DW), but the ORAC value attained was very similar (≈ 950 μmol TE $g^{-1}$ DW) (Uribe *et al.*, 2014). The lower DPPH value could be linked to the lower lipophilic nature of antioxidants present in LOPP, retained in POPP. DPPH and ABTS assays are examples of electron transfer methods, while ORAC is a hydrogen atom transfer (HAT) method. However, DPPH is more efficient to measure AOX of less polar compounds than ABTS and ORAC, due to its solubilisation only in organic media (Arnao, 2000).

Concerning ABTS, in literature, an OP-derived powder used to fortify cereal foods reported a lower ABTS value (≈ 79 μmol TE $g^{-1}$ DW) than LOPP but higher than POPP. The higher ABTS value of LOPP arises probably from the lower drying temperature (35 ºC) used, but the no application of OP fractionation explain the lower ABTS value of POPP (Cedola *et al.*, 2020). Once again, to the best of our knowledge, BPC fraction of OP food ingredients were neglected in most studies published regarding this topic.

POPP exhibited a higher AOX by ABTS, DPPH and ORAC to BPC extract, due to its higher TPC. Similar results were observed for rice bran and other rich insoluble food sources (Zhao *et al.*, 2018). Additionally, the AOX of the FPC fraction of POPP by ORAC and DPPH was higher than results reported to other vegetable by-products powders, namely fruit (passion fruit, pineapple and guava) and vegetable (asparagus) (Martínez *et al.*, 2012).

In our study, the AOX of BPC extracts from IDF and SDF fractions were also evaluated. Consistent with TPC results, regarding the BPC extracts, the fibre fraction extract from LOPP exhibited a higher AOX. This higher value validates the importance of fibre as a carrier of antioxidant compounds in LOPP. In POPP, the AOX values obtained together with its higher fibre content allowed to claim its character as an ADF source since the AOX of POPP fibre fractions corresponded to more than half (50-60%) of the total AOX of BPC.

Concerning antimicrobial activity, growth inhibition curves were made for selected microorganisms, in the presence of OP powders at a concentration of 1, 2 e 3% (w/v), as measured by turbidity at 660 nm. LOPP (Figure 4.3) and POPP (Figure 4.4) were capable of inhibiting; at some level, all microorganisms tested. Only the growth of Gram-positive MSSA and *L. monocytogenes* was entirely inhibited by 3% LOPP (MIC), while all other concentrations tested (*i.e*. sub – MIC concentrations) were capable of reducing microbial growth, while causing an increase of lag phase duration. In the case of *B. cereus*, the growth was completely inhibited by 16 h. Gram-positive microorganisms were more susceptible to LOPP. The growth of Gram-negative microorganisms tested (*E. coli*, *S. enteritidis*, *Y. enterocolitica*) was delayed to half at 2 and 3% of LOPP and suffered a delay on the log phase. POPP (2 and 3%) induced a reduction of the growth and a delay of the log phase of all the microorganisms tested, except for *B. cereus*.

To determine the MBC for LOPP concerning MSSA and *L. monocytogenes*, concentrations 2.5, 3, 3.5 and 4% (w/v) were plated. The 4 and 3% LOPP concentration killed 99% of MSSA and *L. monocytogenes*, respectively, and thus, LOPP had a bactericidal effect.

The higher antimicrobial activity of LOPP may be attributed to the presence of higher amounts of several components, namely minerals (*e.g*. potassium) and some organic acids such as formic, lactic and acetic acid, besides the higher concentration of phenolics like HYD and its derivatives with well-documented antimicrobial activity (Obied *et al.*, 2007). These values of antimicrobial activity support the potential application of OP powders not only as functional ingredients but also as preservatives.

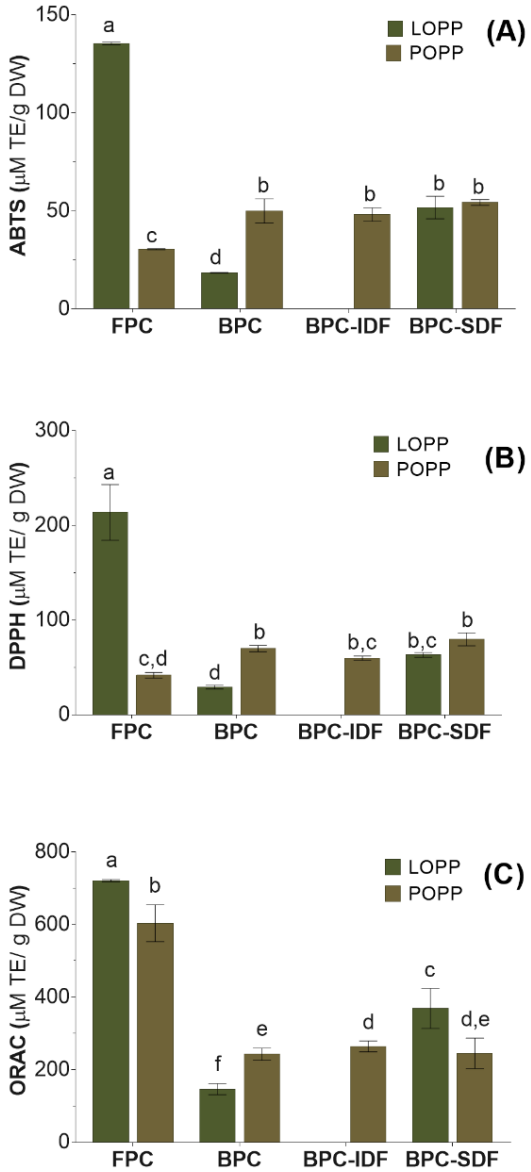



**Figure 4.2. Antioxidant activity using (A) ABTS, (B) DPPH and (C) ORAC methods of free and bounds phenolic compounds of OP powders and its fibre fractions.**

FPC: Free phenolic compounds extract; BPC: Bound phenolic compounds extract. IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as ANOVA ($p < 0.05$) determined.

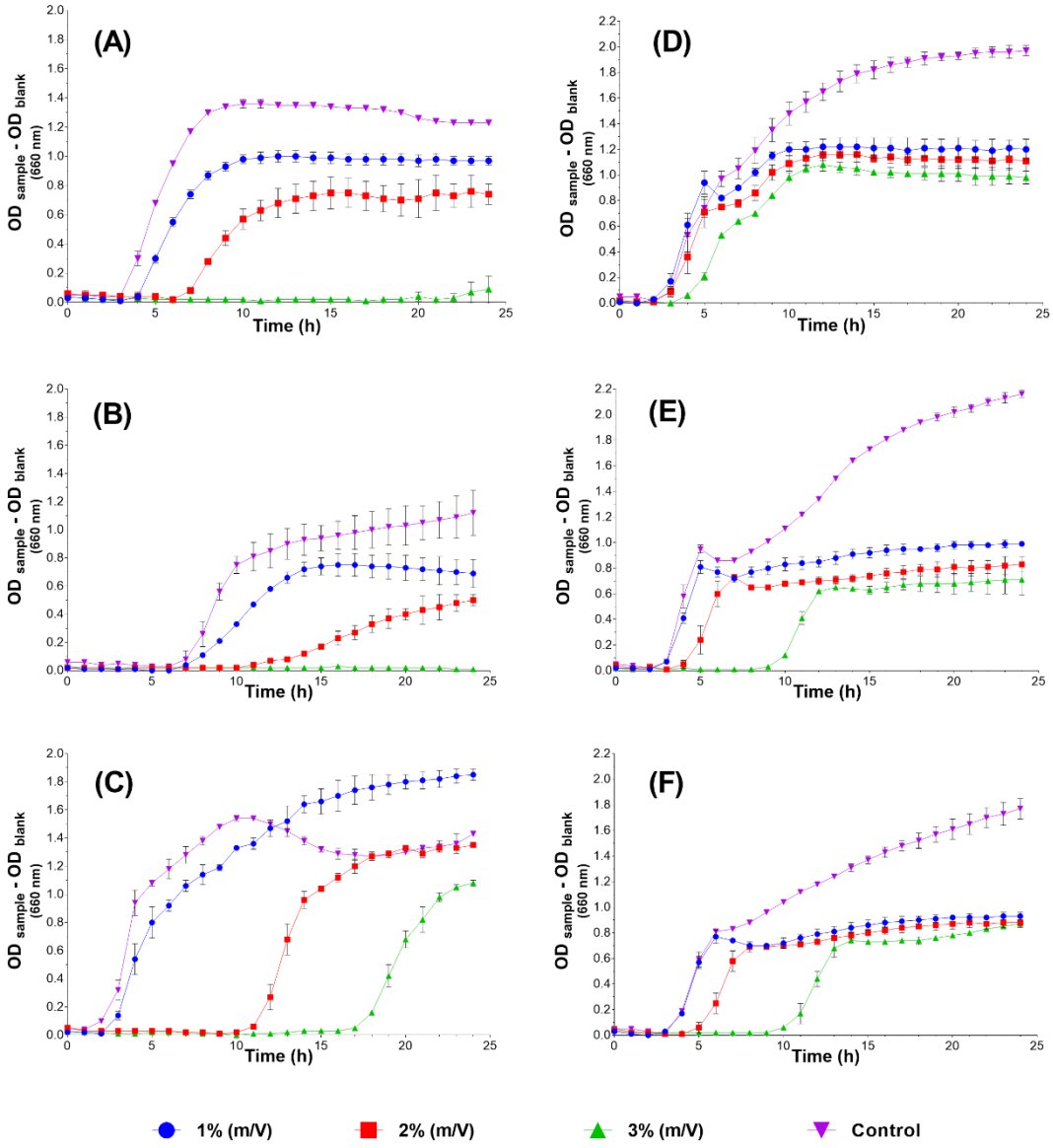



**Figure 4.3. Time inhibition curves drawn at different LOPP concentrations (1, 2 e 3%).**
**(A)** MSSA; (B) *Listeria monocytogenes*; (C) *Bacillus cereus*; (D) *Escherichia coli*; (E) *Yersinia enterocolitica*; (F) *Salmonella enteritidis*. Results are the means of three determinations ± standard deviation.

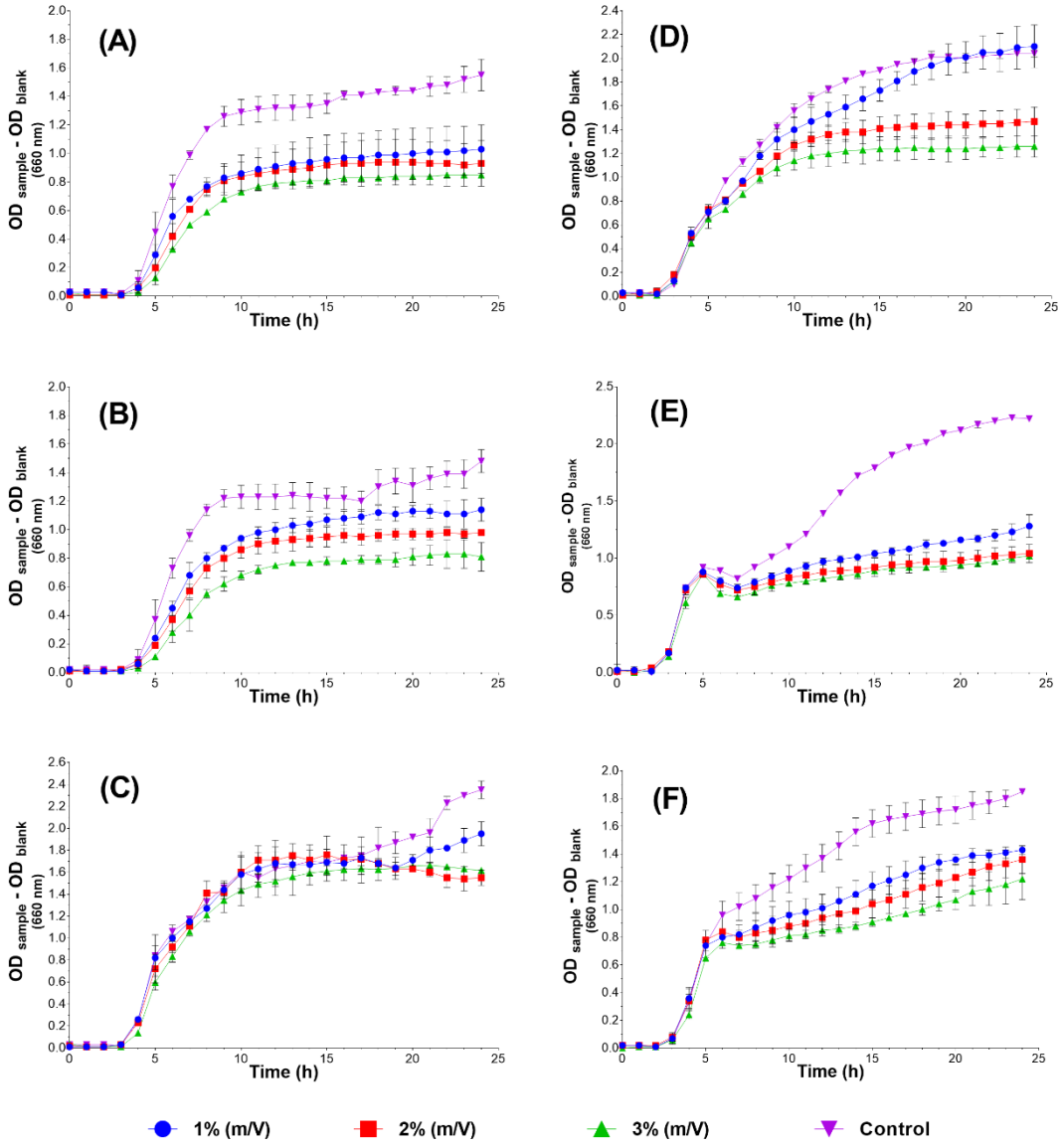



**Figure 4.4. Time inhibition curves drawn at different POPP concentrations (1, 2 e 3%).**

(A) MSSA; (B) *Listeria monocytogenes*; (C) *Bacillus cereus*; (D) *Escherichia coli*; (E) *Yersinia enterocolitica*; (F) *Salmonella enteritidis*. Results are the means of three determinations ± standard deviation.

### 4.3.4. Functional properties of olive pomace powders

To determine the OP powders functionality in a food matrix, it is essential to know its functional properties, *i.e*., solubility, water (WHC) and oil holding capacities (OHC) (Table 4.5). LOPP exhibited a solubility of 93.22%, due to its rich content in soluble sugars, ash and SDF. Contrarily, POPP revealed a lower solubility (18.62%), owing to its richness in IDF. The WHC and OHC of POPP were 1.86 g water/g DW and 2.67 ± 0.23 g oil/g, respectively. Crizel *et al.* (2016) reported higher WHC and similar OHC values for its OP powder. The higher WHC value can be attributed to POPP'S higher IDF amount (Crizel *et al.*, 2016). Other co-products exhibited lower and similar values of WHC: passion fruit albedo by-product (1.80 g/g) and date by-product paste (1.33 g/g) (López-Vargas *et al.*, 2013).

OHC is associated with the polysaccharides' chemical and physical structures (Crizel *et al.*, 2016). LOPP showed similar OHC to POPP since the SDF content of both powders was similar. OHC values of OP powders were higher than other plants fibre: apple (0.60–1.45 g/g) and grapefruit (1.20–1.52 g/g), as well as other by-products: mango (1.60 g/g), passion fruit (0.90 g/g) and guava (0.70 g/g) by-products (Martínez *et al.*, 2012). The higher IDF ratio of POPP explains the results obtained. POPP modification (grinding, chemical and enzymatic hydrolysis) could be applied to enhance its SDF content and consequently, its functional and health properties (Niu *et al.*, 2018).

**Table 4.5. Functional properties of olive pomace powders (LOPP and POPP).**

| | LOPP | POPP | Standard |
|---|---|---|---|
| *Solubility (%)* | 93.22 ± 1.01 [a] | 18.62 ± 0.09 [b] | - |
| *WHC (g water/g DW)* | - | 1.86 ± 0.05 [a] | 1.23 ± 0.01 [b] |
| *OHC (g oil/g DW)* | 2.78 ± 0.20 [a] | 2.67 ± 0.23 [a] | 2.41 ± 0.19 [b] |

Standard: Cellulose; WHC: Water holding capacity; OHC: Oil holding capacity. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by the t-Student test ($p < 0.05$).

### 4.3.5. Safety tests

The cytotoxicity and mutagenicity of OP powders were studied for the first time, to the authors' knowledge. The cell viability was evaluated using the XTT cell proliferation assay upon Caco-2 intestinal cells. In this assay, the XTT is reduced by mitochondrial dehydrogenases in metabolically viable cells. XTT assay is widely utilised in cell proliferation as well as drug sensitivity tests (Jiang *et al.*, 2016). The OP powders presented no cytotoxicity at the assayed concentration range (Figure 4.5). The Ames assay was used to evaluate the mutagenicity. The different concentration of OP powders added to *S. typhimurium* culture did not influence their viability (Table 4.6). None of the dilutions was mutagenic in strain TA98 under the conditions used in this assay, which indicates the inexistence of mutagens that cause frameshift mutations. The absence of cytotoxicity and mutagenicity for OP powders indicates that the present components did not induce cells and DNA damage, and for that reason, they can be considered safe at the concentrations tested.

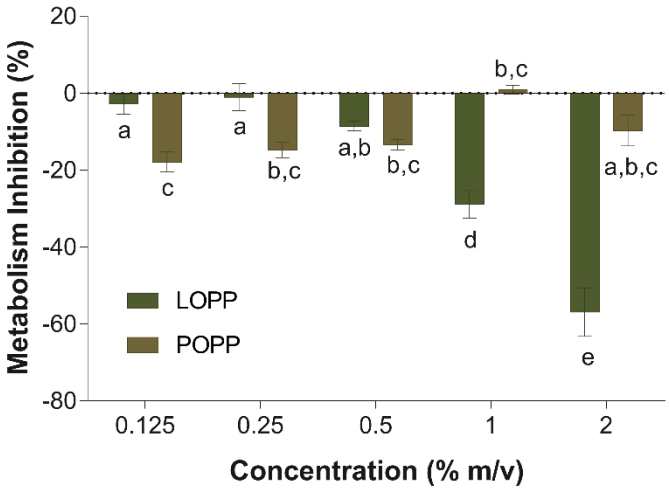



**Figure 4.5. Metabolism of Caco-2 intestinal cells upon the presence of OP powder at concentrations of 2, 1, 0.5, 0.25 and 0.125% (w/v).**
Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by the t-Student test ($p < 0.05$).

**Table 4.6. The numbers of revertant colonies induced by olive pomace powders (LOPP and POPP) in Salmonella typhimurium TA98 strain with metabolic activation (S9 mix).**

| Dose (µg/plate) | Revertant colonies/plate | | Mutagenicity index [a] | |
|---|---|---|---|---|
| | LOPP | POPP | LOPP | POPP |
| 10 | 20.3 ± 8.6 | 21.7 ± 3.5 | 0.12 | 0.13 |
| 25 | 18.0 ± 2.7 | 20.3 ± 5.0 | 0.11 | 0.12 |
| 50 | 20.3 ± 2.5 | 18.0 ± 4.4 | 0.12 | 0.11 |
| 100 | 18.3 ± 6.4 | 17.7 ± 5.7 | 0.11 | 0.11 |
| 250 | 15.3 ± 5.5 | 18.3 ± 2.5 | 0.09 | 0.11 |
| 500 | 14.5 ± 2.1 | 12.5 ± 2.1 | 0.09 | 0.07 |
| 1000 | 25.0 ± 2.8 | 27.5± 9.1 | 0.15 | 0.16 |
| **Blank control** | 18.0 ± 2.5 | | | |
| **Control** (Quercetin) *20 µg/plate* | 168.0 ± 54.0 | | | |

Results are the means of three determinations ± standard deviation. [a] MI, mutagenicity index: number of revertant colonies per treated plate/number of revertant colonies per quercetin control plate.

## 4.4. Conclusion

In conclusion, OP powders demonstrated potential to be multifunctional ingredients with application in food formulation. LOPP could be used simultaneously as a source of mannitol, potassium, hydroxytyrosol, and POPP as a source of antioxidant dietary fibre and oleic/ linoleic acid. The high antioxidant and antimicrobial properties exhibited by LOPP could be explored not only as source potential health benefits but also as a potential food preservative. On the other hand, the richness of POPP in antioxidants mainly carried by fibre and its simultaneous richness in unsaturated fatty acids might give rise to additive or synergic effects as gut health benefits, but also lower lipid peroxidation. Olive pomace powders biological safeness was confirmed, and they also demonstrated adequate functional properties for food applications. This study also brings new insights into the research to develop sustainable olive oil production, thus helping to spread the circular bioeconomy concept in the sector. Further experiments will include its incorporation in food matrixes, as well as the validation of its biological activity after ingestion.

# PART III

# *Bioactivity Characterization*

# Chapter 5.
# Simulated digestion of olive pomace water-soluble ingredient: Relationship between the compounds bioaccessibility and their potential health benefits

## Abstract

Olive pomace is a semisolid by-product with a great potential as a source of bioactive compounds. Using its soluble fraction, a liquid-enriched powder (LOPP) was obtained, exhibiting a rich composition in sugars, phenolics and minerals, with potential antioxidant, antihypertensive and antidiabetic health benefits. The gastrointestinal tract's effect on its bioactive composition and bioactivities was examined to validate LOPP potential as a functional ingredient. Phenolics and minerals were the most affected compounds. However, a significant bioaccessibility of potassium and hydroxytyrosol was verified ($\geq$ 57%). Therefore, the LOPP bioactivities were only moderately affected (losses around 50%). For example, 57.82 ± 1.27% of the recovered antioxidant activity by ORAC was serum - available. From an initial $\alpha$-glucosidase inhibition activity of 87.11 ± 1.04%, at least 50% of the initial potential was kept (43.82 ± 1.14%). Regarding initial ACE inhibitory activity (91.98 ± 3.24%), significant antihypertensive activity was still retained in the serum-available fraction (43.4 ± 3.65%). The colon-available fraction also exhibited an abundant composition in phenolics and minerals. LOPP showed to be a potential functional ingredient not only with potential benefits in preventing cardiovascular diseases but also in gut health.

**Keywords:** Bioeconomy, *In vitro* digestion, Minerals, Phenolics, Antioxidant, Antihypertensive, Antidiabetic, Bioaccessibility index.

## 5.1. Introduction

Currently, sustainable treatment of olive pomace (OP) is one of the biggest challenges of the olive oil industry, despite its high nutritional value (Nunes *et al.*, 2019). As a solution, the development of OP-based ingredients has been studied to add-value and reduce its environmental impact. Powdered ingredients have been proposed as a more feasible, stable and sustainable solution. A liquid-enriched olive pomace powder (LOPP) has been obtained (data not shown). Our previous study has demonstrated the great potential and safety of LOPP as a potential food ingredient to be applied in functional food formulation due to (1) its rich composition in sugars (mainly mannitol), minerals (mainly potassium) and phenolics (mainly hydroxytyrosol (HYD) and its derivatives); and (2) its antioxidant and antimicrobial properties (data not shown). Considering its phenolic composition and antioxidant properties, LOPP could be a functional ingredient with health benefits in cardiovascular prevention, as an antihypertensive and antioxidant ingredient (Romero *et al.*, 2016; Valls *et al.*, 2015), and also as an antidiabetic agent (Dekdouk *et al.*, 2015; Figueiredo-González *et al.*, 2019, 2018; Hadrich *et al.*, 2015). These health benefits have been mostly associated with phenolic compounds with high antioxidant capacity. However, sugars, more specifically mannitol (Chukwuma *et al.*, 2019; Endringer *et al.*, 2014; Hagiwara *et al.*, 2005), and minerals (Avcı *et al.*, 2019; Olson and Kobayashi, 1992), could also have an important role.

HYD and its derivatives (tyrosol, oleuropein, HYD glucoside, etc.) were the principal phenolics identified in LOPP. These phenolics have been associated with potent antioxidant, anti-inflammatory and antimicrobial activities (Nunes *et al.*, 2019). Moreover, HYD and its derivatives have been approved with the claim “Olive oil phenolics contribute to the protection of blood lipids from oxidative stress” by the European Food Safety Authority (EFSA) for olive oil that contains at least 5 mg of HYD and its derivatives per 20 g of olive oil. Besides, bioaccessibility studies have shown that HYD from olive can be absorbed efficiently in humans (de Bock *et al.*, 2013).

Cardiovascular disease and diabetes mellitus are the most prevalent diseases of the 21st century, being hypertension a primary risk factor of cardiovascular disease (Alcaide-Hidalgo *et al.*, 2020), and hyperglycaemia one of the characteristics of diabetes mellitus with main impact in the disease (Hadrich *et al.*, 2015). Hypertension and hyperglycaemia are commonly treated with drugs to inhibit the enzymatic activity of angiotensin-converting (ACE) and α-glucosidase enzymes, respectively. The ACE inhibition may help reduce the formation of angiotensin II, a vasoconstrictor and a ROS initiator (Covas *et al.*, 2015). On the other hand, the inhibition of hydrolytic enzyme α-glucosidase may help minimise postprandial hyperglycemia and delay the absorption of glucose (Hadrich *et al.*, 2015).

Several studies have explored natural products in search of ACE and α-glucosidase inhibitors. Peptides and triterpenes have been described as strong potential inhibitors of ACE. Recent studies have revealed that various phenolic compounds from different plants possess ACE (Al Shukor *et al.*, 2013) and α-glucosidase inhibitory activity (Bhaskarachary and Joshi, 2018), which varies significantly according to their chemical structures. As some studies showed, olive oil phenolics were responsible for the anti-hypertensive effect in hypertensive rats (Romero *et al.*, 2016), but, no

previous reports show ACE inhibitory activity of olive oil phenolics (Alcaide-Hidalgo *et al.*, 2020). Olive oil phenolics have also been related to the anti-diabetic effect by inhibiting α-glucosidase (Bhaskarachary and Joshi, 2018). Figueiredo-González *et al.* (2019), Figueiredo-González *et al.* (2018) and Nadour *et al.* (2015) demonstrated a close relationship between the higher amount of olive phenolic content with most effective α-glucosidase inhibition activity. Indeed, HYD has been suggested as an effective α-glucosidase inhibitor (Hadrich *et al.*, 2015). Other compounds from LOPP that could also act as ACE and α-glucosidase inhibitors are soluble sugars as mannitol (Chukwuma *et al.*, 2019; Endringer *et al.*, 2014). Minerals, like calcium, naturally present in LOPP, have also been reported as potential inhibitors of α-glucosidase (Avcı *et al.*, 2019).

LOPP composition and demonstration of its bioactive properties support this ingredient's potential application as a functional ingredient with antioxidant capacity and health benefits such as antihypertensive and antidiabetic activities. Nevertheless, to guarantee LOPP health benefits, the digestion impact on phenolics, minerals and sugars needs to be evaluated to guarantee bioactives bioaccessibility and related health benefits. Several studies have developed new functional ingredients from OP (Nunes *et al.*, 2016), namely bioactive extracts used as a source of health-protecting effects (Di Nunzio *et al.*, 2018) or food preservatives (Lafka *et al.*, 2011) and powders, used on the formulation of bakery products (Cedola *et al.*, 2020), snacks (Ying *et al.*, 2017) or pasta (Simonato *et al.*, 2019). Besides, these OP functional ingredients were obtained, applying more elaborate processes or using water/organic solvents, while LOPP was obtained using a simple fractionation process followed by a drying step. However, only a few of these studies considered the gastrointestinal tract's impact on potential bioactivities of OP functional ingredients (Conterno *et al.*, 2019; Di Nunzio *et al.*, 2020; Rubió *et al.*, 2014).

The *in vitro* simulation of gastrointestinal digestion (SGD) has been a valuable tool to analyse changes, bioaccessibility and digestibility of bioactive compounds from functional foods. Several differences are observed between *in vitro* models and *in vivo* studies. Nevertheless, SGD models remain a useful and less expensive alternative to animal and human models for rapid screening of food ingredients; furthermore, *in vitro* techniques are ethically superior than *in vivo* techniques aligned with EU directives (Hur *et al.*, 2011; Minekus *et al.*, 2014).

The present study attempted to investigate (1) the bioaccessibility of sugars, minerals, phenolics, dietary fibre throughout SGD and (2) to demonstrate the potential antioxidant, antihypertensive and antidiabetic activities after SGD. This is the first study that demonstrates the stability and bioaccessibility of olive pomace liquid fraction (LOPP bioactive compounds) that constitute critical information because only bioavailable compounds can exert their biological function.

## 5.2. Materials and methods

### 5.2.1. Chemicals and reagents

The ABTS diammonium salt (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pancreatin, pepsin, formic acid, potassium sorbate, sodium carbonate,

trifluoroacetic acid (TFA), peptidyl-dipeptidase, α- glucosidase and all reagents of ACE and α-glucosidase inhibitory activity assay were purchased from Sigma-Aldrich (Sintra, Portugal). Acetonitrile and methanol were purchased from Fischer Scientific (Oeiras, Portugal). Folin-Ciocalteu's reagent and potassium persulfate were purchased from Merck (Algés, Portugal). Bile salts are from Oxoid™ (Hampshire, UK). Standards of Trolox, gallic acid, p-coumaric acid, vanillin, protocatechuic acid, caffeic acid and quercetin were obtained from Sigma-Aldrich (Sintra, Portugal), whereas HYD, tyrosol, luteolin were purchased from Extrasynthese (Lyon, France).

### 5.2.2. Preparation of olive pomace water-soluble ingredient

The OP was collected from an olive mill in Oliveira do Hospital, Portugal, mainly composed of olive cultivar Galega Vulgar (80% of the olive heritage). Various samples were taken and transported to the laboratory, where they were mixed to homogenize them. The homogenous sample was packed in polyethene flasks and kept in a freezer at - 80 $^{0}$C until use.

OP was fractionated by centrifugation (10,000 ×g for 10 min). The liquid fraction was freeze-dried (Telstar Lyo Quest HT 40) with 2% of mannitol (as a cryoprotectant and to prevent aggregation), and the powder obtained was denominated liquid-enriched olive pomace powder (LOPP).

### 5.2.3. *In vitro* digestion

LOPP *in vitro* simulation of gastrointestinal digestion (SGD) (Figure 5.1) was performed according to the method described by Madureira *et al.* (2011) and Costa *et al.*, (2019) with dialyses process to simulate the intestinal and serum absorption (Gullon *et al.*, 2015; Lucas-Gonzalez *et al.*, 2016). Mouth digestion was conducted with 0.6 mL of α–amylase solution (100 U/mL) and incubation took place for 1 min, at 37 °C and 200 rpm. For gastric digestion, the pH was adjusted to 2.0 with concentrated HCl (1 mol/L), and the mixture was incubated with pepsin (25 mg/mL) (from porcine stomach mucosa, pepsin A), at a rate of 0.05 mL/mL of sample, in a shaking bath, for 60 min at 37 °C. Small intestinal digestion was performed by adjusting pH to 6.0 with $NaHCO_3$ (1 mol/L), before the addition of pancreatin (from porcine pancreas, 2 g/L) and bile salts (12 g/L), at a ratio of 0.25 mL/mL of sample, and further incubation of the mixture for an additional 120 min at 37 °C.

To screen the release of individual phenolics from LOPP (1 g) at different stages of digestion, samples were collected from the simulated mouth (ca. 4 mL), gastric digest (ca. 4 mL), and intestinal digest (ca. 4 mL) and used to make extracts to perform phenolics analysis further.

In the last phase of intestinal digestion, a segment (10 cm) of dialysis tubing (3.5 kDa molecular weight cut-off (Costa *et al.*, 2019; Gullon *et al.*, 2015)) filled with $NaHCO_3$ (1 M) was placed inside of screw-topped bottles filled with digested samples and were incubated for 2 h in a shaking water bath, at 37 °C and 50 rpm. The dialysis process aimed at mimetising as close as possible the biologic process (Hur *et al.*, 2011) and simulating, at least, the passage by duodenum and jejunum (Cieplak *et al.*, 2018).

At the end of the incubation process, the solution left outside the dialysis tubing (OUT) represented the non-absorbable sample (colon-available), and the solution that managed to diffuse into the dialysis tubing (IN) represents the sample that is available for absorption (serum-available).

All enzymes solutions were freshly prepared and filter-sterilised using a 0.22 µm-membrane filter. All solutions were maintained in an ice bath during the entire gastrointestinal digestion process before gradual addition (when appropriate). Three replicates of the SGD were made. Two replicates of blanks were prepared without sample and treated using the same conditions. All stages of digestion were lyophilised and stored for analysis of the compounds' composition and potential bioactivities. Blanks are used to correct the concentration of the compounds of the digested samples.

Several protocols of static *in vitro* simulation of digestion can be found in the literature (Brodkorb *et al.*, 2019). All these protocols exhibit different conditions (e.g., pH, duration of each step, the ratio of enzymes to the substrate), making the comparison between studies very difficult. It was recently implemented a standardized static digestion model suitable for foods under the INFOGEST Cost Action (Brodkorb *et al.*, 2019; Minekus *et al.*, 2014). Similarly, to the method adopted in this work, this INFOGEST protocol is also divided in the oral phase, gastric phase and intestinal phase. The main differences between the two methods are in the: execution of the enzyme activity assays, the composition of the simulated fluids for each digestion phase (use of an electrolyte stock solutions), a longer time of gastric digestion (1 h more), different pH on intestinal phase (pH 6 instead pH 7), a higher ratio of sample to digestive fluids (1:10 instead 1:1) and the ratio of enzymes to sample. The different digestion conditions between the protocol adopted in this work compared to the INFOGEST protocol could lead to different results. In future works, the INFOGEST protocol should be applied to harmonize the experimental conditions of the static *in vitro* simulation of gastrointestinal food digestion.

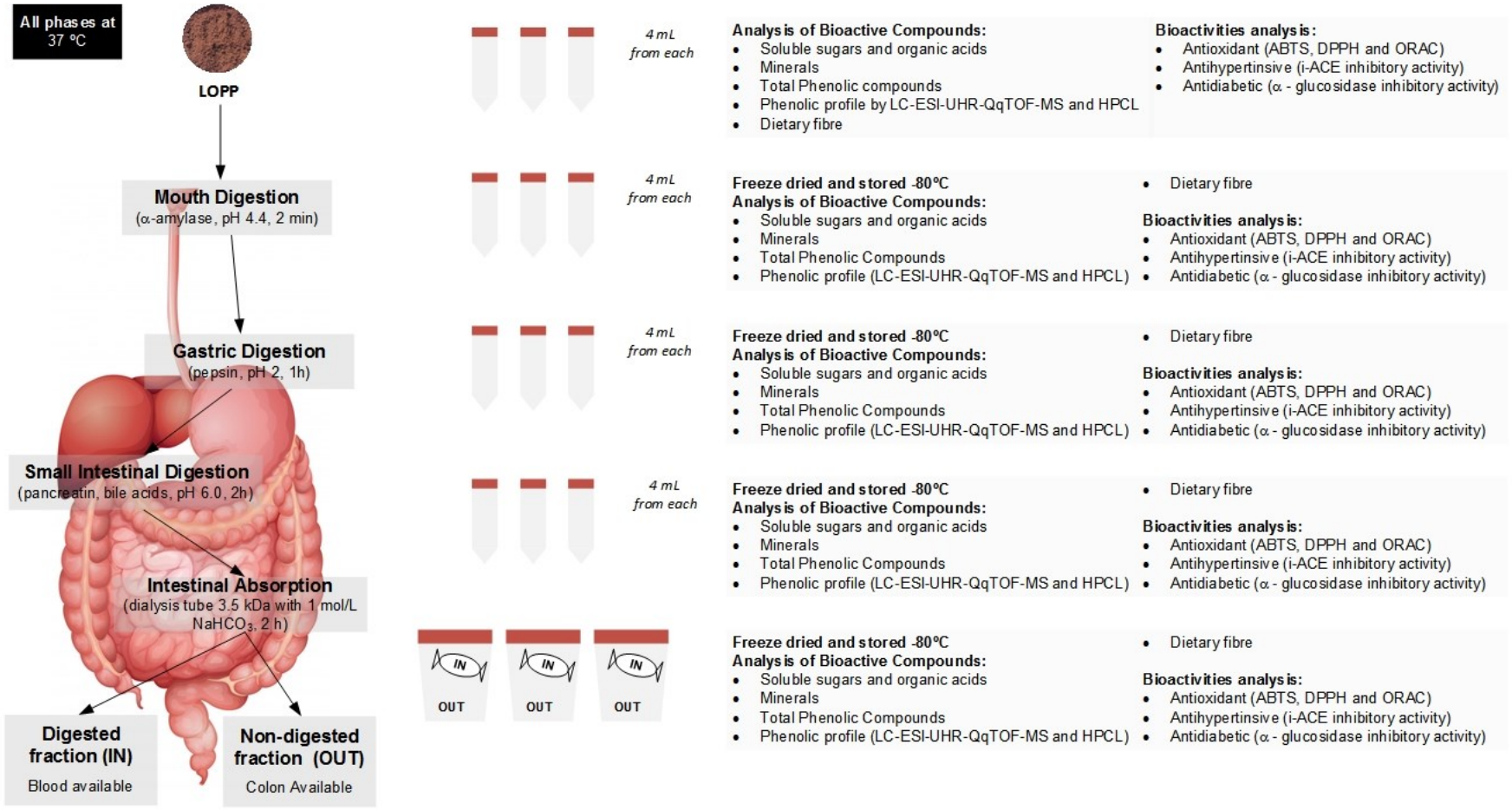



**Figure 5.1. Graphic representation of the static in vitro gastrointestinal digestion procedure carried out with LOPP.**

### 5.2.4. Stability and bioaccessibility of compounds through *in vitro* gastrointestinal digestion

#### 5.2.4.1. Recovery and bioaccessibility Index

The results of each extract determination (on the sample, after mouth, gastric and intestinal digestion) were reported to the 100 g of dry weight (DW) of LOPP.

Recovery index (RI%) and bioaccessibility index (BI%) were studied to evaluate the matrix composition's effect on the digestion of the main nutritional/bioactive components of LOPP compounds and not only of phenolic groups. Before digestion, the values for LOPP were assumed as 100% of all bioactive compounds and bioactivities of the sample (Gullon *et al.*, 2015; Lucas-Gonzalez *et al.*, 2016). At this point, it is essential to define the terms “bioavailability” and “bioaccessibility carefully”. Bioavailability expresses the fraction of ingested bioactive compound or nutrient that reaches the systemic circulation and finally utilised. Before becoming bioavailable, bioactives must be released from the food matrix and modified in the gastrointestinal tract. Thus, bioavailability includes the term bioaccessibility**.** Bioaccessibility is described as the amount of a compound that is released from its matrix in the digestive tract, becoming available for bloodstream absorption (Galanakis, 2017).

The percentage of recovery allows the determination of the amount of each main compound, on the tested food, after oral, gastric and intestinal digestion, according to:

$$Recovery\ index\ (\%) = (BC_{DF}/BC_{TF}) \times 100$$

Where: $BC_{DF}$ is the bioactive content (mg) in the digested, and $BC_{TF}$ is the bioactive content (mg) quantified in the test matrix.

The bioaccessibility is defined as the percentage of the bioactive compound that is solubilised after intestinal dialysis step. Thus, this index defines the proportion of the bioactive compound that could become available for absorption into the blood system:

$$Bioaccessibility\ index\ (\%) = (BC_{S}/BC_{DFE}) \times 100$$

where: $BC_S$ is the bioactive content (mg) in the digested sample after the duodenal dialysis step (IN) and $BC_{DF}$ is the bioactive content (mg) in the digested sample after the duodenal step (IN + OUT) – end of digestion.

#### 5.2.4.2. Sugars and organic acids

Free sugar and organic acid profiles were determined using the lyophilised samples dissolved in water at a concentration comprised between 50 and 70 mg/mL. The analyses were performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to IR and UV detector using Aminex 37-H column (Bio-Rad, Berkeley, USA) at 55 °C and 35 mM $H_2SO_4$ as mobile phase (flow rate: 0.5 mL/min). Both identification and quantification were achieved by comparing the relative

retention times of sample peaks with standards, using a calibration curve in the range of concentrations of 0.2-2.0 mg/mL.

#### 5.2.4.3. Minerals

Mineral concentration was carried out following the methodology of Amorim *et al.* (2016), using an optical emission spectrometer Model Optima 7000 DV™ ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with a radial configuration and an amount of lyophilised digested samples of 250 mg.

#### 5.2.4.4. Phenolic compounds

Lyophilised samples were dissolved in methanol (between 20 and 200 mg/mL) to perform phenolics analysis. Total phenolic content (TPC) of FPC and BPC extracts was determined according to the Folin-Ciocalteu's method (Oliveira *et al.*, 2016). Results were expressed as mg gallic acid equivalents (GAE)/100 g DW.

The chromatographic analysis was performed using a Waters e2695 separation module system interfaced with a Photodiode array UV/Vis detector (PDA 190-600 nm). The separation was conducted in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm; Dartford, UK). The flow rate, gradient program and mobile phases used following the Oliveira *et al.* (2015). Compound detection was performed at specific wavelengths: 280, 320 and 360 nm, while data acquisition and analysis were accomplished using Software Empower 3. Identification of main phenolic compounds in methanol was performed by comparison of retention times, spectra, and peak areas at maximum absorption wavelength.

The complete profile of phenolic compounds and its derivatives trough SGD was also analysed in an LC-ESI-UHR-QqTOF-MS following the methodology of Monforte *et al.* (2018) with some modifications in the gradient elution program, in order to obtain a good separation of phenolic compounds. Mobile phases were 0.1% aqueous formic acid (A) and acetonitrile with 0.1 % formic acid (B). The gradient elution program used was the following: 0–5 min (5% B); 5–25 min (15% B); 25-35 min (30%); 35-40 min (95% B), 40-41 (5% B) and 41–42 min (0% B). Identification of main phenolic compounds was based on the retention time, UV-Vis and mass spectra with those obtained from the standard solutions, when available. The other peaks were tentatively identified comparing the information with available data reported in the literature. The elemental composition for these compounds was confirmed according to accurate mass (5 mDa), and isotope rate calculations designated mSigma (<20) (Bruker Daltonics).

The UV-visible absorption spectra acquired with DAD detector was used to identify and classify the phenolic compounds. DAD is currently the most widely available and commonly used technique for routine qualitative and quantitative analyses of these metabolites. The combination of these data

with mass spectra (MS) data and information from the respective literature or comparison with standard compounds can be used for tentative identification of each peak in a chromatogram.

The main phenolic compounds identified (HYD, protocatechuic acid, tyrosol, vanillin, caffeic and p-coumaric acid) were quantified by HPLC using external calibration curves created based on their maximum UV signal. The results were expressed as mg/100 g DW. Some compounds were expressed as equivalents of its basic constituent: HYD glucoside was expressed as HYD, tyrosol glucoside as tyrosol, verbascoside and caffeoyl-6'-secologanoside were expressed as caffeic acid equivalents (Klen and Vodopivec, 2012).

#### 5.2.4.5. Fibre composition

The digested sample was filtered using a sintered glass crucible (no. 2). The insoluble dietary fibre (IDF) was retained in the crucible, and the supernatant was saved for soluble dietary fibre (SDF) analysis. IDF was washed twice with 10 mL of distilled water, and the supernatants were combined for determining SDF. IDF residue was washed once with 20 mL of 96% ethanol, followed by 20 mL of acetone twice, and then dried overnight at 100 °C (ash and protein were corrected during this step). The filtrate was purified by dialysis (dialysis tube with a molecular weight cut-off of 3.5 kDa) to obtain the SDF. The dialysis was applied to avoid the error caused by dietary fibre precipitation with ethanol (Deng *et al.*, 2011; Mañas *et al.*, 1994). Afterwards, SDF was recovered by dialysate freeze-drying. The results were expressed as total dietary fibre (TDF), IDF and SDF g/100 g DW.

Sugar composition of SDF was achieved by acid hydrolysis (6% sulfuric acid) at 121 ◦C for 1 h (29). The hydrolysate is used for quantifying uronic acids (UA) and neutral sugars (NS). NS were determined by HPLC (micro guard column: Aminex Carbo-P, Bio-Rad; carbohydrate analysis column: Aminex HPX-87P heavy metal, 300–7.8 mm, Bio-Rad; flow rate: 0.6 mL/min; detector: refractive index), and UA was determined colourimetrically by adapting the 3-hydroxydiphenyl method of Blumenkrantz & Asboe-Hansen (1973) with d-galacturonic acid as standard. The sum of NS and UA was taken as the amount of SDF in LOPP after the SGD.

Phenolic compounds were released from SDF using a hydrolysis process with 4 mol/L NaOH at room temperature, according to Xie *et al.* (2015). The extract obtained was dissolved in methanol and named "SDF bound phenolic compounds" (SDF-BPC). Aliquots of the hydrolysates were used for spectrophotometric measurement using Folin-Ciocalteu's and antioxidant activity (AOX) methods, and to identify the phenolic compounds by HPLC and LC-ESI-UHR-QqTOF-MS.

### 5.2.5. Effect of *in vitro* gastrointestinal digestion on bioactivities

#### 5.2.5.1. Antioxidant activity: ABTS, DPPH e ORAC

The AOX of LOPP during the SGD was achieved according to the methods of DPPH (Alexandre *et al.*, 2019), ABTS (Cano *et al.*, 2000) and ORAC (Oliveira and Pintado, 2015) using a multi-detection plate reader (Synergy H1, Vermont, USA). The radical stock solutions were freshly prepared.

Lyophilised samples were dissolved in methanol to obtain a concentration comprised between 20 and 200 mg/mL. All analyses were performed in triplicate and expressed in mM of Trolox-equivalents (TE)/g DW.

#### 5.2.5.2. Antihypertensive activity (ACE-inhibitory activity)

The *in vitro* ACE-inhibitory activity was measured using the fluorometric assay of Sentandreu and Toldra (2006) with some modifications. A total of 40 µL of ultrapure water or ACE working solution was added to each microplate well, then adjusted to 80 µL by adding ultrapure water to a blank (BLK), control (CTL) or samples (SPL). The enzyme reaction was started with the addition of 160 µL of the substrate solution, and the mixture was incubated at 37 °C. The fluorescence generated was measured during 30 min using a multi-detection plate reader (Synergy H1, Vermont, USA). The assay was performed in a black 96-well microplate (Nunc, Denmark). Excitation and emission wavelengths used were 350 and 420 nm, respectively.

The inhibitory activity was expressed as inhibited % of the maximum ACE activity. The equation applied to calculate de percentage of ACE-inhibitory was the following:

$$iACE\ inhibition\ (\%) = [(F_{CTL} - F_{BLK}) - (F_{SPL} - F_{SPLB})] \times [100/(F_{CTL} - F_{BLK})]$$

#### 5.2.5.3. Antidiabetic activity (α-glucosidase inhibitory activity)

The α-glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon *et al.* (2006). The sample (50 µL), was mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL) and pre-incubated at 25 ºC for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each line and absorbance was read. Afterwards, substrate was added, and the absorbance was reread. The reaction mixtures were incubated at 25 ºC for 5 min, and the absorbance readings were recorded at 405 nm by a multi-detection plate reader (Synergy H1, Vermont, USA). As a negative control, 50 µL of buffer solution was used to replace the sample. Acarbose was used as a positive control at the concentration of 10 mg/mL. A blank without the enzyme (used 100 µL of 0.1 mol/L phosphate buffer instead) was performed for each sample.

The α-Glucosidase inhibitory activity was expressed as inhibition (%) and calculated as follows:

$$\alpha\text{- Glucosidase inhibition (\%)} = [(\Delta Abs\ control - \Delta Abs\ sample) / \Delta Abs\ control] \times 100$$

Where Δ*Abs control* is the variation of absorbance of the control and Δ*Abs sample* is the variation of the samples' absorbance.

### 5.2.6. Statistical analysis

R software was used to carry out statistical analyses. All experiments were carried out in triplicates, and data were reported as mean ± standard deviation. Shapiro - Wilk test tested the normality of data distribution. The differences of mean values among a concentration of bioactive compounds or bioactivities obtained in the different steps of the *in vitro* SGD were analysed by one-way analysis of variance (ANOVA). The Tukey's *post hoc* test was applied for comparisons of means, and differences were considered significant at $p < 0.05$. Correlation analysis was performed between bioactive compounds contents and bioactivities of LOPP using Pearson correlation analysis.

## 5.3. Results and Discussion

### 5.3.1. Stability and bioaccessibility of compounds throughout *in vitro* gastrointestinal digestion

#### 5.3.1.1. Soluble sugars and organic acids

Soluble sugars and organic acids concentration through SGD is described in Figure 5.2. Observing this figure is it possible to see that one of the main components detected in LOPP were soluble sugars. Sugars identified in LOPP were mannitol > glucose > fructose. Lactic and acetic acid were detected in LOPP, but the most significant organic acid identified was formic acid.

The predominant sugar present in LOPP, mannitol, is a polyol widely used as a reduced-calorie sweetener (Acceptable Dietary Intake of 0–50 mg/kg), but can also exert other technological functions in food, such as a preservative preventing oxidative damage of food components, and increasing the food shelf life by reducing sugar crystallisation (Deis and Kearsley, 2012). Regarding health benefits, mannitol requires no insulin for its metabolism, making it a potential glycaemic control agent, especially for diabetic individuals. Recently, mannitol was suggested as a dietary supplement in controlling postprandial blood glucose by inhibition of α-glucosidase activity, α-amylase activity, glucose absorption, etc. (Chukwuma *et al.*, 2019).

Formic acid as an organic acid reduces pH value in the stomach, enhancing pepsin activity and consequently, increasing digestibility of nitrogen, phosphorus and minerals (Ragaa and Korany, 2016). Besides that, formic acid has a key role in the colonic acetate production by acetogenic bacteria through the Wood–Ljungdahl pathway (Ríos-Covián *et al.*, 2016). Therefore, assessing sugar and formic acid bioaccessibility during physiological stages allows identifying valuable indicators for the evaluation of their potential health properties.

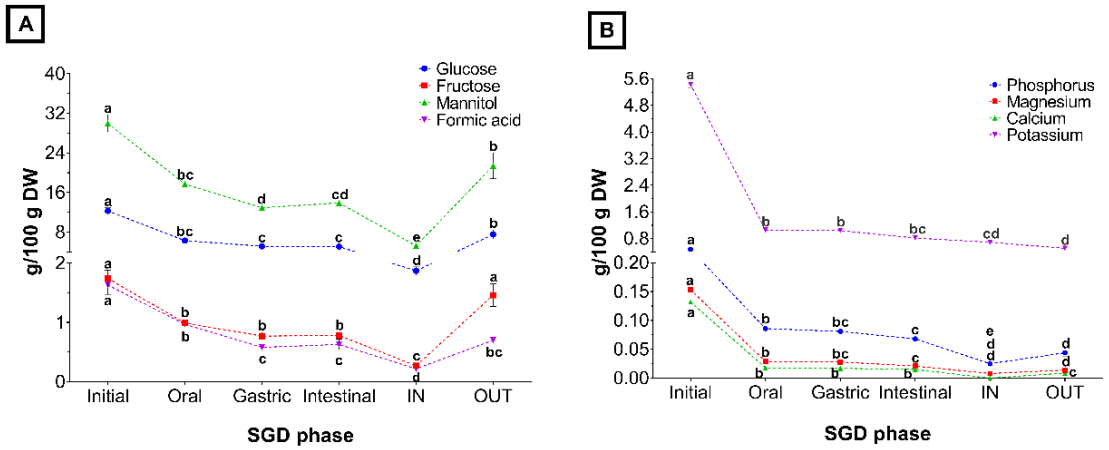



**Figure 5.2.The concentration of LOPP (A) soluble sugars/organic acids and (B) minerals (g/100 g DW) after each phase (oral, gastric, intestinal, after dialysis IN and OUT) of simulated gastrointestinal digestion (SGD).**
Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

Note: The initial amount before digestion (BCTF) and the amounts detected in the digested sample for each digestion step (BCDF) expressed in this figure were used to calculate the Recovery Index (RI%) for each sugar, organic acid and mineral enunciated in Table 5.1. On the other hand, to calculate the Bioaccessibility Index (BI%) of each sugar, organic acid and mineral, the BCS amount detected in the digested sample after the duodenal dialysis step (IN) and BCDFE content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

The RI of soluble sugars and organic acids through SGD is described in Table 5.1. The recovery of soluble sugars and formic acid from LOPP showed similar behaviour throughout the SGD. RI was between 59% and 62% in the mouth, then in stomach ranged between 36-47% and in the intestine, the values remained similar (RI = 39-47%). As an effect of SGD, the concentration of sugars and organic acids was reduced to half in the mouth, followed by a decreasing even higher in stomach step. On the mouth, the surface area increases and multiple interactions can occur between sugars, minerals and phenolic due to LOPP composition (Lucas-Gonzalez *et al.*, 2016; Velderrain-Rodríguez *et al.*, 2014). The highest reduction of sugars and organic acids had place in the stomach as a result of acidic conditions (pH=2) and improvement of pepsin activity by formic acid (Ragaa and Korany, 2016).

A significant increase in RI values was observed after the simulation of intestinal absorption phase. This sugar increase might be related to the breakdown of α-(1→4)-glycosidic bonds and other linkages by the action of amylase present in pancreatin enzyme and the consequent liberation of monosaccharides (Yun *et al.*, 2019). The isomerisation of some of the glucose into fructose could justify the higher RI of fructose. On the other hand, the oxidation processes occurrence might explain the increase of formic acid during this SGD phase. Oxidation processes commonly produce formic acid in olive oil (Lemos *et al.*, 2015).

Regarding the accessibility of the serum available fraction (IN), the uptake was between 14-18% for sugars and organic acids, and bioaccessibility indexes (BI) exhibited slightly higher values (Table 5.1). Formic acid revealed the highest BI (23.88 ± 2.51%) and fructose the lowest BI (15.92 ± 3.93%). After SGD, formic acid was the most bioaccessible compound. On the other hand, mannitol was less affected by SGD digestion until intestine (RI = 46.40 ± 0.99%) and was the most bioaccessible sugar

(BI = 19.86 ± 2.37%). At the end of digestion, 52.42 ± 1.63 mg/g LOPP DW of mannitol (Figure 5.2) were available to exert its functional benefits.

Higher recovery index values of soluble sugars and organic acids were attained in colon available fraction (OUT). Fructose (89.32 ± 11.42%) and mannitol (71.43 ± 8.72%) exhibited higher RI values, followed by glucose (61.52 ± 7.56%) and formic acid (43.11 ± 2.32%). Despite the higher RI of fructose, mannitol (214.08 ± 26.13 mg/g LOPP DW) and glucose (75.55 ± 9.28 mg/g LOPP DW) were the predominant sugars on the colon (Figure 5.2). Mannitol has been used in medicine to promote digestive and urinary excretion of toxins (orally in combination with activated charcoal by gastroclysis), as an enema to prepare the bowel for gastrointestinal procedures and also to reduce hepatic encephalopathy. Intestinal mannitol could increase the molarity of intestinal lumen, decrease hyperammonemia (hepatic inability to remove nitrogenous products generated by protein metabolism of intestinal microbiota) and reduce oxidative stress (excessive production of reactive oxygen species) (Montes-Cortés *et al.*, 2018). These potential effects could also be expected with LOPP's ingestion due to the higher amount of mannitol available in the colon.

Regarding the high amount of formic acid retained in the colon, formic acid could be used by gut microbiota to produce colonic acetate through the Wood–Ljungdahl pathway (Ríos-Covián *et al.*, 2016), which could have potential health benefits in several organs (Koh *et al.*, 2016).

**Table 5.1. Recovery index (RI%) and Bioaccessibility index (BI%) of bioactive compounds simulated gastrointestinal digestion (SGD) from LOPP.**

| | Recovery Index (%) | | | | | Bioaccessibility Index (%) |
|---|---|---|---|---|---|---|
| | Oral | Gastric | Intestinal | IN (Serum available) | OUT (Colon available) | |
| ***Sugars & Organic acids*** | | | | | | |
| *Glucose* | 51.28 ± 0.88 $^{b}$ | 41.92 ± 0.60 $^{c}$ | 41.37 ± 0.80$^{c}$ | 15.19 ± 0.53 $^{d}$ | 61.52 ± 7.56 $^{a}$ | 19.95 ± 1.66 $^{g}$ |
| *Fructose* | 60.54 ± 1.53 $^{b}$ | 47.04 ± 3.65 $^{b}$ | 47.43 ± 2.81 $^{b}$ | 16.58 ± 1.63 $^{c}$ | 89.32 ± 11.42 $^{a}$ | 15.92 ± 3.93 $^{g}$ |
| *Mannitol* | 59.07 ± 1.39 $^{b}$ | 43.21 ± 1.41 $^{c}$ | 46.40 ± 0.99 $^{c}$ | 17.49 ± 0.55 $^{d}$ | 71.43 ± 8.72 $^{a}$ | 19.86 ± 2.37 $^{g}$ |
| *Formic acid* | 59.69 ± 2.66 $^{a}$ | 35.84 ± 1.31 $^{b}$ | 38.74 ± 6.57 $^{b}$ | 13.51 ± 1.37 $^{c}$ | 43.11 ± 2.32 $^{b}$ | 23.88 ± 2.51 $^{fg}$ |
| ***Minerals*** | | | | | | |
| *Phosphorus* | 19.43 ± 2.53 $^{a}$ | 17.24 ± 0.61 $^{ab}$ | 16.15 ± 0.51 $^{ab}$ | 5.23 ± 0.05 $^{c}$ | 11.79 ± 1.68 $^{b}$ | 36.22 ± 3.25 $^{e}$ |
| *Magnesium* | 18.94 ± 1.13 $^{a}$ | 18.39 ± 0.57 $^{a}$ | 15.59 ± 0.81 $^{a}$ | 5.10 ± 0.16 $^{c}$ | 11.90 ± 1.19 $^{b}$ | 35.36 ± 2.40 $^{ef}$ |
| *Calcium* | 13.29 ± 1.00 $^{a}$ | 12.89 ± 0.87 $^{a}$ | 13.00 ± 1.52 $^{a}$ | nd | 8.83 ± 1.88 $^{a}$ | nq |
| *Potassium* | 19.57 ± 1.04 $^{a}$ | 19.59 ± 0.88 $^{a}$ | 16.95 ± 1.52 $^{a}$ | 12.71 ± 0.19 $^{b}$ | 12.00 ± 1.24 $^{b}$ | 57.42 ± 3.18 $^{d}$ |
| ***Phenolic compounds*** | | | | | | |
| *TPC* | 6.03 ± 1.14 $^{b}$ | 13.35 ± 2.76 $^{a}$ | 3.92 ± 0.44 $^{bc}$ | 5.63 ± 0.76 $^{bc}$ | 2.77 ± 0.67 $^{c}$ | 65.52 ± 6.82 $^{cd}$ |
| *Hydroxytyrosol glucoside* | 8.46 ± 5.61 $^{b}$ | 5.57 ± 3.05 $^{c}$ | 6.07 ± 2.65 $^{bc}$ | 11.61 ± 0.17 $^{a}$ | 3.76 ± 2.53 $^{c}$ | 75.47 ± 2.84 $^{ab}$ |
| *Hydroxytyrosol* | 7.52 ± 1.48 $^{b}$ | 10.27 ± 2.21 $^{a}$ | 0.73 ± 0.12 $^{c}$ | 1.47 ± 0.11 $^{c}$ | 0.32 ± 0.05 $^{c}$ | 82.10 ± 2.59 $^{a}$ |
| *Tyrosol glucoside* | 14.65 ± 3.21 $^{b}$ | 12.37 ± 2.76 $^{b}$ | 5.45 ± 0.89 $^{c}$ | 35.87 ± 4.26 $^{a}$ | 13.88 ± 1.16 $^{b}$ | 71.93 ± 3.58 $^{bc}$ |
| *Tyrosol* | 17.62 ± 4.72 $^{b}$ | 14.32 ± 3.63 $^{b}$ | 5.42 ± 0.90 $^{c}$ | 48.00 ± 1.60 $^{a}$ | 13.78 ± 2.02 $^{b}$ | 77.79 ± 2.03 $^{ab}$ |
| *Caffeic acid* | 5.32 ± 0.72 $^{b}$ | 13.52 ± 2.11 $^{a}$ | 0.84 ± 0.22 $^{c}$ | nd | 0.89 ± 0.11 $^{c}$ | 0.00 ± 0.00 $^{h}$ |
| *Verbascoside* | 17.14 ± 2.75 $^{a}$ | 12.82 ± 2.36 $^{a}$ | nd | nd | nd | nq |
| *p-coumaric acid* | 24.21 ± 4.48 $^{b}$ | 34.62 ± 7.44 $^{a}$ | 9.03 ± 1.69 $^{cd}$ | 4.84 ± 1.08 $^{d}$ | 15.40 ± 0.79 $^{bc}$ | 24.51 ± 3.81 $^{fg}$ |
| *Caffeoyl-6'-secologanoside* | 13.76 ± 2.89 $^{b}$ | 22.22 ± 2.82 $^{a}$ | 2.83 ± 0.07 $^{cd}$ | nd | 4.45 ± 0.69 $^{c}$ | nq |

nd – not detected; nq – not quantified. Results are the means of three determinations ± standard deviation. Values with different letters in the same line to RI (%) and the same column to BI (%) are significantly different, as determined by one-way ANOVA test ($p < 0.05$), respectively.

#### 5.3.1.2. Minerals

The concentration of minerals (phosphorus, magnesium, calcium and potassium) throughout the SGD is presented in Figure 5.2. Minerals have several potential health benefits. Potassium was the predominant mineral in LOPP (5.431 ± 0.11 g/100 g DW). Other minerals, like phosphorus, magnesium, calcium and sodium, were also detected. Potassium is well-known to positively affect the maintenance of normal blood pressure, and consequently, associated with cardiovascular disease prevention (EFSA, 2016). EFSA considers that a potassium intake of 3.5 g/day has beneficial effects on adults' blood pressure (EFSA, 2016). Therefore, the high amount of potassium makes of LOPP a potential agent for cardiovascular diseases. Besides that, minerals have been correlated positively with antioxidant proprieties (Olson and Kobayashi, 1992). Still, to ensure the potential health benefits of minerals from LOPP, it is essential to assess its bioaccessibility after SGD.

Minerals were considerably affected by the SGD in all phases and, as a result, the intestinal RI ranged between 13-17% (calcium < magnesium < phosphorus < potassium) (Table 5.1). The amount of minerals that reached the gut was significantly lower than the amount present in the initial LOPP ($p < 0.05$), as observed in Figure 5.2. A similar negative effect of in vitro digestion on minerals was reported by Costa *et al.*, (2019) in grape pomace extract.

The bioaccessibility of all minerals was also negatively affected by SGD. Calcium was not bioaccessible, phosphorus and magnesium exhibited similar low BI values (≈35-36%). Only potassium was detected in higher amounts in the serum available fraction (IN) than in the colon available fraction (OUT), presenting a BI of 57.42 ± 3.18%. The bioaccessibility of minerals is influenced by many factors, enhancing or inhibiting their absorption on the human diet. Generally, phenolics and dietary fibres are the main inhibitors of the absorption, while organic acids act as enhancers. Therefore, the low recovery and bioaccessibility of minerals in LOPP could be linked to its high phenolics amount. Generally, phenolics reduce the absorption of minerals by chelation mechanisms leading to the sequestration of minerals. For example, calcium and magnesium bioaccessibility has been negatively correlated with the phenolic content in whole grain tea-biscuits (Vitali *et al.*, 2008).

Potassium has been significantly affected by SGD. Only 6.91 ± 0.09 mg of potassium was delivered by 1 g of LOPP. This amount of potassium represents only ≈ 0.2% of the dietary reference value established (3.5 g of potassium/day) by the EFSA to have beneficial effects on blood pressure (EFSA, 2016, 2010). However, when integrated int an equilibrated diet, LOPP could help reach the potassium intake of 3.5 g (90 mmol)/day which has beneficial effects on blood pressure and reduces the risk of stroke in adults, without adverse effects on heart function or undesirable gastrointestinal symptoms (EFSA, 2016, 2010). However, future *in vivo* studies needed to be done to validate potassium uptake, since it is regulated by several mechanisms (Youn, 2013).

#### 5.3.1.3. Phenolic compounds

Total phenolic compound (TPC) content of LOPP was determined using spectrophotometric Folin-Ciocalteau method (Figure 5.3). Before and after *in vitro* digestion, LOPP's individual phenolic compounds were identified by using HPLC-DAD and LC-ESI-UHR-QqTOF-MS detection (Table 5.2). The main individual phenolics were quantified by HPLC-DAD (Figure 5.3).

TPC amount of LOPP decreased significantly after the SGD. An evident decrease of TPC occurred in the mouth phase (RI 6%) followed by a slight increase in the stomach (RI 13%) and a decrease in the intestine (RI 4%). This pronounced negative effect of SGD on olive phenolics has also been reported to olive tables (Fernández-Poyatos *et al.*, 2019).

The TPC increment on stomach could be explained by the increase of HYD, *p*-coumaric and caffeic acid. In this phase of the digestion, there was also the detection of other derivative compounds by LC-ESI-UHR-QqTOF-MS. One example of this was the ion at TR = 7.5 with m/z 315.1085 which may have the formula $C_{14}H_{20}O_8$ (error = -0.2 mDa, mSigma = 7.3), corresponding to an isomer of HYD glucoside. Furthermore, during SGD, pH has a vital role as a protector of phenolics against degradation in the stomach (acidic conditions) and as a promotor of degradation in the small intestine (mild alkaline conditions) (Gayoso *et al.*, 2016).

During the intestinal absorption phase, an increase of TPC was observed principally in the serum available fraction (IN), which could be explained by the sample's additional contact time with the intestinal fluids and intestinal digestive enzymes (plus 2 h after the small intestine phase). This additional contact time facilitates the release of phenolics linked to the matrix by the action of enzymes present in pancreatin (extract from porcine pancreas composed by proteolytic, lipolytic, amylolytic, and nucleic acid splitting enzymes). A similar effect was also observed for apple phenolics (Bouayed *et al.*, 2011).

Regarding LOPP individual phenolics, the results obtained by HPLC were in line with TPC results. High correlation coefficients ($r^2 \geq 0.9$) between TPC and individual phenolics throughout SGD were obtained. HYD and caffeic acids exhibited better correlation with TPC ($r^2 = 0.99$), followed by HYD glucoside and caffeoyl-6'-secologanoside ($r^2 = 0.98$). Tyrosol ($r^2 = 0.90$) and tyrosol glucoside ($r^2 = 0.93$) revealed a lower correlation with TPC.

The high correlation of some individual phenolics with TPC was evident when the recovery indexes of individual phenolics was analysed (Table 5.1). For example, HYD content decreased in the mouth (RI = 7.52%) followed by an increase in the stomach (RI = 10.27%), and a considerable decrease in the small intestine phase (RI = 0.73%) and a slight increase after the intestinal absorption phase in the serum bioaccessible fraction (IN) (RI =1.47%). As shown above, TPC and HYD had a similar behaviour throughout SGD simulation. On the other hand, tyrosol glucoside, tyrosol and *p*-coumaric acid were not so positively correlated with TPC, mainly due to its high amount in the serum-bioaccessible fraction (IN) which led to higher RI values (36-48%).

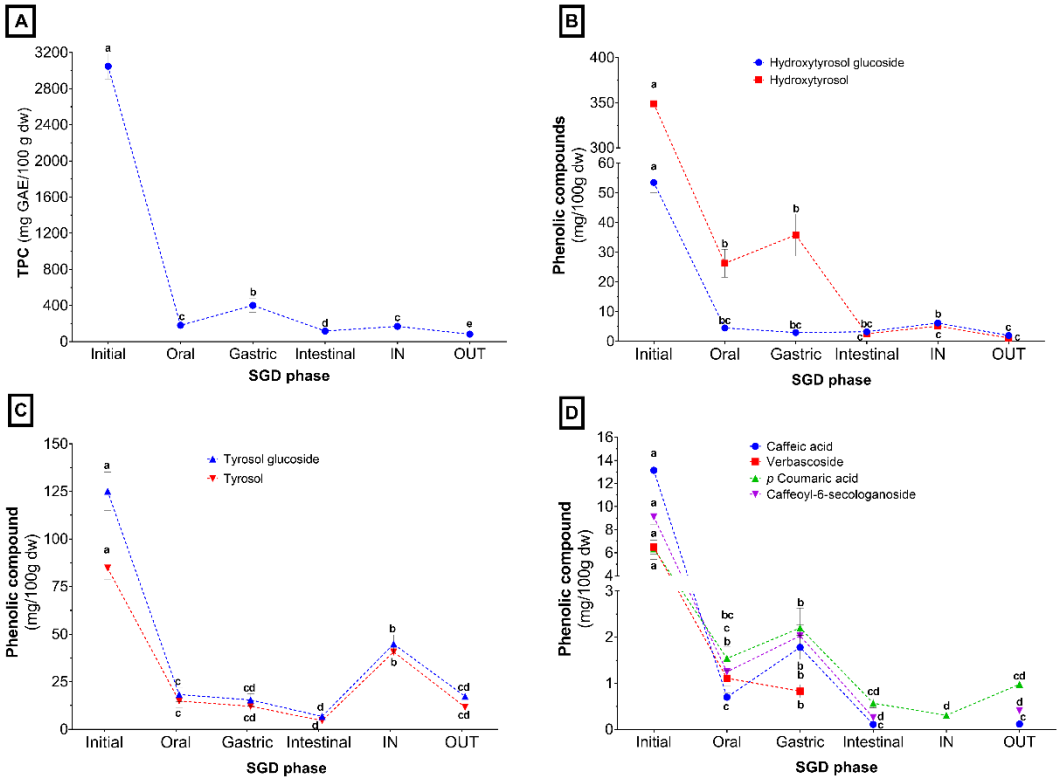



**Figure 5.3. Stability and bioaccessibility of phenolic compounds obtained after each step of simulated (SGD) gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP. (A)Total phenolic compounds (mg GAE/g DW) and (B) (C) (D) the concentration of the main individual phenolic compounds (mg/100 g DW).** Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

Note: The initial amount before digestion ($BC_{TF}$) and the amounts detected in the digested sample for each digestion step ($BC_{DF}$) expressed in this figure were used to calculate the Recovery Index (RI %) of total phenolic compounds and each phenolic enunciated in Table 1. On the other hand, to calculate the Bioaccessibility Index (BI %) of total phenolic compounds and each individual phenolic compound, the $BC_S$ amount detected in the digested sample after the duodenal dialysis step (IN) and $BC_{DFE}$ content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

An essential class of phenolic compounds belonging to elenolic-acid derivatives was also identified by LC-ESI-UHR-QqTOF-MS (Table 5.2). Elenolic acid is formed, together with the HYD, tyrosol, and glucose, by hydrolysis of oleuropein (Thielmann *et al.*, 2017). Among these, hydrated and hydroxylated products of dialdehydic form of decarboxymethyl-elenolic acid and aldehydic form of decarboxymethyl-elenolic acid were found in all LOPP digestion phases. All elenolic-acid derivatives have been previously reported in olive-oil wastes (Lozano-Sánchez *et al.*, 2013). Elenolic acid derivatives are the most potent antimicrobial compounds in olive and may exert potential health benefits in the gut (Thielmann *et al.*, 2017). Another compound with potential antioxidant and antimicrobial action identified by LC-ESI-UHR-QqTOF-MS was quinic acid. Quinic acid has demonstrated to be an antioxidant agent and, together with other acids, to act as an inhibitor of virulence traits of oral pathogens (Marrubini *et al.*, 2015).

Besides the negative effect of SGD on HYD and derivatives, the phenolic compounds mentioned above exhibited the highest bioaccessibility indexes (Table 5.1), being HYD the most bioaccessible compound (BI 82.10 ± 2.59%) followed by tyrosol (77.79 ± 2.03%). Some studies have studied the

effect of the gastrointestinal tract on olive phenolics, but most of these studies have been carried out with olive oil (Seiquer *et al.*, 2015) or table olives (Fernández-Poyatos *et al.*, 2019). Seiquer *et al.* (2015) reported that, in olive oil, the most bioaccessible and stable compounds after in vitro digestion were also tyrosol and HYD. Other studies explored the bioaccessibility, and potential bioavailability of phenolics from olive leaf extracts (González *et al.*, 2019), but OP extracts bioaccessibility has been less examined. Rubió *et al.* (2014) assessed the bioaccessibility of phenolic compounds using the gastrointestinal *in vitro* model and cell models from an OP extract, a thyme extract and a conjugation of both. In this study, the HYD and secoiridoids' bioaccessibility from OP extract and both extracts' conjugations was 13.3% and 23.0%, respectively. Results also indicated that HYD's bioaccessibility was enhanced by thyme, and no significant differences were observed in HYD transport after Caco-2 cells exposure.

Considering the positive health claim approved by EFSA, the recommended consumption for HYD and derivatives is 5 mg/day (EFSA, 2016). Based on the present study results, the levels of LOPP ingested directly as nutraceutical allowed to estimate that a daily dose of 5.2 g was enough to deliver 5 mg to human blood. However, as a food ingredient, the food matrix may play a crucial role in LOPP phenolics' bioaccessibility, affecting its absorption positively or negatively. Consequently, it is needed to study the effect of SGD using cellular or in vivo models and after LOPP incorporation in different food matrixes to clarify LOPP phenolics' bioaccessibility.

**Table 5.2. Retention time, maximum wavelength absorbance and MSn fragmentations profiles of phenolic compounds identified in LOPP obtained before (initial) and after each step of in vitro gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) and the bound phenolics of dietary fibre achieved by AOAC and after the tract.**

| Compound | Molecular formula | RT (min) | $\lambda_{max}$ | m/z calcd | m/z exptl | Err [mDa] | mSigma | Major fragments ESI negative MS/MS ions | *In vitro* gastrointestinal digestion step |
|---|---|---|---|---|---|---|---|---|---|
| **Quinic acid** [*2] | $C_7H_{12}O_6$ | 1.6 | 269 | 191.0561 | 191.0565 | -0.4 | 12.2 | 191.0563; 127.0398; 93.0398; 87.0089; 85.097 | Initial, Oral, Gastric, Intestinal, IN, OUT |
| **Protocatechuic acid**[*3] | $C_7H_6O_4$ | 6.8 | 203 | 153.0197 | 153.0193 | -0.4 | 7 | 153.0197; 109.0298 | SDF Tract |
| **Hydroxytyrosol glucoside**[*2] | $C_{14}H_{20}O_8$ | 7.1 | 279 | 315.1088 | 315.1085 | -0.2 | 4 | 315.1092; 153.0556; 123.0451 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC |
| **Hydrated product of the dialdehydic form of decarboxymethyl-elenolic acid**[*2] | $C_9H_{14}O_5$ | 7.3 | 197/281 | 201.077 | 201.0768 | -0.2 | 4 | 201.0405; 153.0553; 123.0449; 95.0502 | Initial, Oral, Intestinal, IN, OUT, SDF AOAC |
| **Hydroxytyrosol** [*1] | $C_8H_{10}O_3$ | 7.4 | 280 | 153.0556 | 153.0557 | 0.1 | 5.7 | 153.0452; 123.0457 | Initial, Oral, Intestinal, IN, OUT, SDF AOAC, SDF Tract |
| **Hydroxytyrosol glucoside isomer**[*2] | $C_{14}H_{20}O_8$ | 7.5 | 279 | 315.1087 | 315.1085 | -0.2 | 7.3 | 315.1089; 151.0398; 123.0449 | Gastric |
| **Loganin**[*2] | $C_{17}H_{26}O_{10}$ | 7.6 | 281 | 389.1455 | 389.1453 | -0.2 | 8.9 | 151.0763; 113.0244; 101.0244 | Initial, Oral |
| **Oleoside derivative isomer**[*2] | $C_{17}H_{28}O_{11}$ | 7.7 | 228 | 407.1557 | 407.1559 | -0.5 | 11.7 | 151.0760; 119.0346 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC |
| **Decarboxylated form of hydroxy-elenolic acid**[*2] | $C_{10}H_{14}O_5$ | 7.9 | 197/280 | 213.0765 | 213.0768 | 0.1 | 5.6 | 213.0920; 137.0601; 121.0665; 111.0085 | Initial, Oral, Gastric, Intestinal, OUT |
| **Tyrosol glucoside**[*3] | $C_{14}H_{20}O_7$ | 8.1 | 227/276 | 299.1139 | 299.1136 | -0.3 | 3.2 | 119.0505; 137.0244; 135.0299 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC, SDF Tract |
| **4-Hydroxybenzoic acid**[*4] | $C_7H_6O_3$ | 8.2 | 204 | 137.0241 | 137.0244 | 0.4 | 4 | 137.0241; 138.0280 | SDF Tract |
| **Oleoside**[*2] | $C_{16}H_{22}O_{11}$ | 8.4 | 277 | 389.1093 | 389.1089 | -0.4 | 4.3 | 389.1088; 183.0664; 165.0557 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC, SDF Tract |
| **p-Coumaroyl-D-glucose**[*3] | $C_{15}H_{18}O_8$ | 8.7 | 195/280 | 325.0925 | 325.0929 | 0.4 | 0.9 | 326.0976; 163.0402; 119.0501 | Initial, Oral, Gastric, Intestinal, IN, OUT |
| **Caffeic acid** [*1] | $C_9H_8O_4$ | 9.3 | 323 | 179.0350 | 179.0350 | -0.1 | 7.9 | 179.0350; 135.0448 | Initial, Oral, Gastric, Intestinal, OUT, SDF Tract |
| **10-Hydroxyloganin** [*2] | $C_{17}H_{26}O_{11}$ | 9.5 | 197 /278 | 405.1402 | 405.1406 | -0.4 | 5.7 | 405.1402; 165.0557; 139.0035; 123.045 | Initial, Oral, Gastric |
| **Tyrosol** [*1] | $C_8H_{10}O_2$ | 9.8 | 277 | 137.0608 | 137.0608 | 0.5 | 4.5 | 111.0084; 95.0510 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC |
| ***p* - Coumaric acid** [*1] | $C_9H_8O_3$ | 10.6 | 309 | 163.0403 | 163.0401 | -0.2 | 10.5 | 163.0397; 119.0499 | Initial, Oral, Gastric, Intestinal, IN, OUT,SDF Tract |
| **Verbascoside** [*3] | $C_{29}H_{36}O_{15}$ | 10.7 | 245/330 | 623.1988 | 623.1981 | -0.7 | 2.4 | 623.1983; 161.0244; 461.1665 | Initial, Oral, Gastric |
| **Caffeoyl-6-secologanoside**[*3] | $C_{25}H_{28}O_{14}$ | 10.8 | 221/326 | 551.1407 | 551.1406 | 0 | 18.1 | 551.1416; 161.0245; 507.1504 | Initial, Oral, Gastric, Intestinal, OUT |
| **Hydroxylated form of elenolic acid**[*2] | $C_{11}H_{14}O_7$ | 10.9 | 280/320 | 257.0666 | 257.0667 | 0.1 | 2.7 | 257.1271; 181.0505; 137.0603; 109.0655; 95.0498 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC; SDF Tract |
| **Elenolic acid** [*2] | $C_{11}H_{14}O_6$ | 12.5 | 197/288 | 241.0720 | 241.0718 | -0.2 | 0.5 | 241.0737; 139.0035; 127.0398; 111.0086; 95.0551 | Initial, Oral, Gastric, Intestinal, IN, OUT, SDF AOAC |
| **Comsegoloside**[*3] | $C_{25}H_{28}O_{13}$ | 12.6 | 223/311 | 535.1462 | 535.1457 | -0.5 | 6.4 | 535.1465; 145.0296; 491.1558 | Initial, Oral, Gastric, Intestinal, OUT |
| **Luteolin** [*1] | $C_{15}H_{10}O_6$ | 15.9 | 268/349 | 285.0406 | 285.0405 | -0.1 | 0.8 | 285.0414; 151.0037 | Initial, Oral, Gastric, Intestinal, OUT, SDF AOAC, SDF Tract |
| **Quercetin** [*1] | $C_{15}H_{10}O_7$ | 15.0 | | 301.0357 | 301.0354 | -0.3 | 3.4 | 301.0359; 151.0035; 178.9988 | Initial |
| **Apigenin** [*1] | $C_{15}H_{10}O_5$ | 18.0 | 268/338 | 269.0460 | 269.0455 | -0.5 | 4.8 | 269.0461; 151.0035 | Initial |

[*1] Hydroxytyrosol, tyrosol, luteolin, quercetin, apigenin, caffeic acid and coumaric acid were identified by comparison with the standards. The other compounds were tentatively identified by LC-ESI-UHR-QqTOF-MS based on accurate mass, isotope rate calculations designated mSigma and literature data [*2] Jerman Klen & Mozetič Vodopivec, 2012; [*3] Lozano-Sánchez *et al.*, 2013.

### 5.3.1.4. Dietary fibre

The amount of dietary fibre (DF) present in LOPP and its composition (neutral sugars, uronic acids and phenolics) throughout SGD are showed in Table 5.3. In the LOPP fraction, soluble dietary fibre (SDF) represents 9% of its composition. SDF has an essential role in health. As a relevant LOPP component, SDF could confer intestinal health benefits like the capacity to slow intestinal transit, delay gastric emptying, and slow glucose/sterol absorption (Bertolino *et al.*, 2015). Nonetheless, the beneficial effects of DF depend not only of its solubility in water but also on (i) fibre composition, (ii) the bioactive compounds associated with it, mainly phenolic compounds which are directly related to the antioxidant properties (Saura-Calixto, 2011) and (iii) the changes produced during gastrointestinal digestion.

The effect of the SGD on dietary fibre had a meaningful impact on SDF and all its fibre components. The simulated mastication and stomach digestion lead, probably, to a higher vegetable cell rupture, and compounds release (Grundy *et al.*, 2016), which reflected in lower SDF amount after the SGD. Uronic acids and neutral sugars decreased significantly after the SGD, except for glucose that increased significantly. In the SGD, glucose was the predominant neutral sugar (37.73 ± 3.44 mg/g fibre DW*)* followed by galactose and arabinose. The action of the tract enzymes of SGD probably led to the release of polysaccharides rich in glucose by the cleavage of glycosidic bonds (Ríos-Covián *et al.*, 2016).

**Table 5.3. Dietary composition (g/100 g DW) and profile (mg/g fibre DW) of LOPP by using modified AOAC dietary fibre analysis method and simulated gastrointestinal system.**

| | **SDF** g/100 g DW | **Monosaccharide composition** (mg/g fibre DW) | | | | ***UA*** *2 | ***BPC*** *3 |
|---|---|---|---|---|---|---|---|
| | | *Glucose* | *Galactose* | *Arabinose* | *NS* | | |
| **AOAC method** *1 | 9.20 ± 1.18 | 25.04 ± 1.77 | 36.00 ± 6.00 | 28.75 ± 3.97 | 89.79 | 16.47 ± 2.46 | 14.56 ± 1.74 |
| **SGD** | 4.01 ± 0.53 | 37.73 ± *3.44* | 22.59 ± 1.65 | 12.20 ± 0.79 | 72.52 | 12.93 ± 1.49 | 8.38± 0.79 |

Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as ANOVA ($p < 0.05$) determined.
NS – Neutral sugars; UA – Uronic acids; RP – Resistant protein; KL – Klason lignin; BPC: Bound phenolic compounds; TDF – Total dietary fibre; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. *1 – Data from previous work Tânia Ribeiro *et al.* (2020b); *2 – mg GUAE /g fibre dry weight; *3 - mg GAE/ g fibre DW.

The amount of bound phenolic compounds was also affected by the SGD, being the decrease in TPC amount linked to fibre (Table 5.3), in comparison to the AOAC method. Nonetheless, the amount of total phenolic compounds related to fibre was considerable when expressed per 100 g of LOPP (33.60 ± 3.15 mg GAE/100 g DW). The identification, by LC-ESI-UHR-QqTOF-MS (Table 5.2) and quantification by HPLC (Figure 5.4), of bound phenolics in SDF obtained by AOAC and after SGD allowed to explain the differences in TPC. Regarding HYD and its derivatives, only tyrosol glucoside was identified in SDF after SGD. On the other hand, 4-hydroxybenzoic acid was only detected in SDF after SGD. The higher release of compounds after SGD hypothesised could explain the no detection of HYD and its higher liberation from fibre and consecutive decarboxylation (α-oxidation dihydroxylation) into protocatechuic acid followed by dihydroxylation into 4-hydroxybenzoic acid

(López de las Hazas *et al.*, 2016). SDF also exhibited a higher concentration of p-coumaric after SGD, which might be related to SDF higher glucose composition. The linkage between *p*-coumaric and glucose-rich polysaccharides has been verified in several *p*-coumaric extraction studies (Jiang *et al.*, 2016).

Concerning the AOX of bound phenolics in SDF (Figure 5.4), as for the TPC, the values were higher when using the AOAC method than after the SGD. Only the antioxidant potential by DPPH was not negatively affected by the gastrointestinal tract.

The significant amount of phenolics and antioxidant potential of SDF, although not bioaccessible, could exert antioxidant and antimicrobial activity to improve the gut health (Arranz *et al.*, 2010; Papillo *et al.*, 2014).

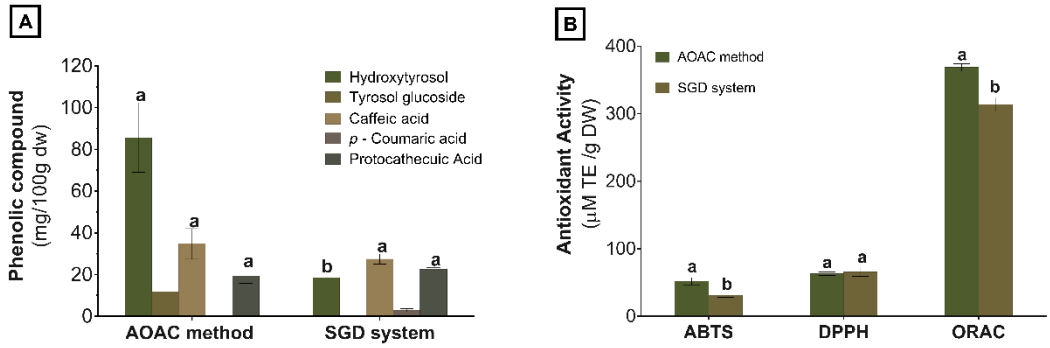



**Figure 5.4. The concentration of (A) main phenolics (mg/100 g sample dry weight) present in fibre fraction of LOPP and respective (B) antioxidant activity using ABTS, DPPH and ORAC methods of soluble fibre from by AOAC method and SGD system.** Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as determined by the t-Student test ($p < 0.05$).

### 5.3.2. Effect of *in vitro* gastrointestinal digestion on bioactivities

#### 5.3.2.1. Antioxidant activity: ABTS, DPPH e ORAC

The results obtained in the *in vitro* ABTS, DPPH and ORAC assays showed evidence that LOPP AOX was negatively affected by SGD (Figure 5.5). In all tests, non-digested LOPP presented higher antioxidant capacity than the digested LOPP available, to be assimilated, in the small intestine ($p < 0.05$).

Correlation of antioxidant results from ABTS and DPPH ($r^2 = 0.97$) and from ABTS and ORAC ($r^2 = 0.97$) were higher than with DPPH and ORAC ($r^2 = 0.96$)]. On the other hand, ABTS values were slightly higher than DPPH, and ORAC values were much higher than the values obtained by DPPH and ABTS. Similar differences were also observed in previous works reporting antioxidant assays for olives (Gouvinhas *et al.*, 2017) and OP (Uribe *et al.*, 2014). These differences probably arise from different mechanisms to measure the antioxidant capacity of each methodology. According to the chemical reaction used, methods can be mainly grouped into two classes: electron transfer and hydrogen atom transfer-based methods. Electron transfer methods measure the ability of a potent

antioxidant to transfer 1 electron to reduce radicals, and hydrogen atom transfer-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation. DPPH/ABTS assays are examples of electron transfer methods, and ORAC is a hydrogen atom transfer-based method. DPPH and ABTS capture are based on oxide-reduction reactions, so it is normal the similarity of their results. However, ABTS allows the measure of AOX of hydrophilic and lipophilic compounds, but DPPH can only be dissolved in organic media (especially in alcoholic media), not in aqueous media, which is an important limitation when interpreting the role of hydrophilic antioxidants (Arnao, 2000). The limitation of the antioxidant evaluation of the hydrophilic compounds using DPPH could explain its lower results. On the other hand, ORAC assay is based on the reaction of water and lipid-soluble substances with peroxyl free radical from ROS generator AAPH ((2,2′-azobis(2-methylpropionamidine) dihydrochloride)) (Tabart *et al.*, 2010), which can explain the higher correlation of ORAC/ABTS. To interpret the AOX results, it is imperative to understand the action mechanism and limitations of each AOX methodology.

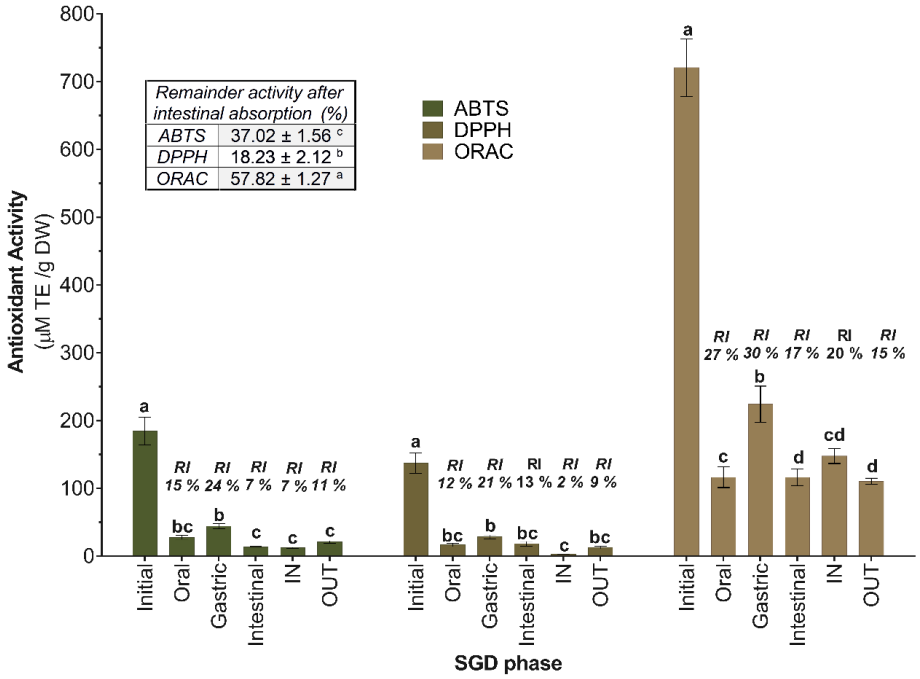



**Figure 5.5. Effect of *in vitro* gastrointestinal digestion on LOPP bioactivities. Antioxidant properties of LOPP measured by ABTS, DPPH and ORAC assays after each step of *in vitro* gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT) of LOPP.** Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

A higher loss of antioxidant capacity occurred in the small intestinal phase, which was more evident in ABTS (RI 7%), followed by DPPH (RI 13 %) and lastly on ORAC (26%). However, after the simulated intestinal absorption, ORAC values (57.82 ± 1.27%) remained at a higher proportion in the serum-available fraction (IN) than in the colon-available fraction (OUT). However, in ABTS (37.02 ± 1.56%) and DPPH (18.23 ± 2.12%), only a small proportion of the total AOX at the end of the digestion (IN + OUT) was detected in the serum-available fraction. The compounds evaluated by

ABTS or ORAC were more bioaccessible, which could be linked to the higher hydrophilic nature of the antioxidants present in LOPP serum available fraction (Martínez *et al.*, 2012). Despite the loss of a significant amount of antioxidant potential after SGD, the bioaccessible LOPP fraction represents a good source of antioxidant compounds (ABTS: 12.25 ± 0.76 mM TE/g DW; DPPH: 2.75 ± 0.15 mM TE/g DW; ORAC: 147.60 ± 10.99 mM TE/g DW).

Several works reported that phenolic compounds largely contribute to the antioxidant properties presenting good correlations between these compounds and AOX (Gullon *et al.*, 2015). ORAC exhibited a better correlation with TPC ($r^2$ = 0.98) followed by ABTS ($r^2$ = 0.97) and DPPH ($r^2$ = 0.97). The good correlations of AOX methodologies with TPC demonstrate that phenolic compounds are the main contributors to LOPP's antioxidant properties. In addition, it was demonstrated that HYD ($r^2$ ≥ 0.97), caffeic acid ($r^2$ ≥ 0.96), caffeoyl-6'-secologanoside ($r^2$ ≥ 0.96), HYD glucoside ($r^2$ ≥ 0.93) and *p*-coumaric acid ($r^2$ ≥ 0.91) were highly correlated with antioxidant capacity. However, tyrosol glucoside ($r^2$ ≥ 0.82) and tyrosol ($r^2$ ≥ 0.73) exhibited good correlations with antioxidant methodologies, but not as strong.

The different correlations coefficients between individual phenolics and the antioxidant assays used were related to phenolic chemical structure. For example, tyrosol's antioxidant action is only as hydroxyl radical scavenger or at most α-tocopherol regenerator (Bonechi *et al.*, 2019). None of the mechanism of the action of tyrosol was individually evaluated by antioxidant assays used. So, the lower correlation of tyrosol and tyrosol glucoside with TPC ($r^2$ ≥ 0.90) compared to the other LOPP phenolics ($r^2$ ≥ 0.97) might be related to the limited antioxidant effect of these compounds (only carry one hydroxyl group in *para*-position) (Bonechi *et al.*, 2019). Folin-Ciocalteu's procedure could also be interpreted as an alternative way to measure the sample's total reducing capacity as the reagent reacts with any reducing substance (Milella *et al.*, 2014).

Not only phenolic compounds influence the AOX trough gastrointestinal tract, but also, minerals could have an important role in AOX of digested LOPP (Olson and Kobayashi, 1992). Minerals were strongly correlated with $ABTS^{\cdot+}$, DPPH and ORAC ($r^2$ ≥ 0.97). This correlation between AOX and metals is related to metals' ability to react with phenolic compounds to form complex compounds that can increase or decrease antioxidant properties (Samsonowicz *et al.*, 2019).

Despite the negative effect of the SGD in LOPP AOX (until the small intestine phase) and bioaccessibility, an increase of the AOX was revealed during the intestinal absorption phase by ABTS and ORAC methods, mainly on the non-bioaccessible fraction (OUT). As emphasised before, the 2 h extra of contact of LOPP with intestinal fluids and intestinal digestive enzymes during the dialysis process, may have facilitated the release of antioxidant components in the colon fraction. Besides that, the possibility of interaction between phenolics and minerals could explain the high AOX by ABTS and DPPH on the OUT fraction. This higher AOX of LOPP in the colon after the whole digestion may significantly decrease local oxidative stress and improve microbiota composition, consequently improving gut permeability and boosting anti-inflammatory/immunity mechanisms (Papillo *et al.*, 2014).

The variation in LOPP AOX (by different assays) throughout the SGD allowed an understanding that phenolic compounds bioaccessibility has an essential role in AOX. However, the possible formation of metal-phenolic complexes causes changes in the molecular size and electronic charge distribution in the ligand molecule and consequently alters its bioaccessibility and antioxidant functions (Rasouli *et al.*, 2017). Thus, these chelating roles observed in LOPP besides having a negative effect on the bioaccessibility might be of potential interest as dietary antioxidants (Rasouli *et al.*, 2017). Future studies to establish a better correlation between minerals and phenolics regarding their AOX and bioaccessibility will be needed to design a more effective functional ingredient from LOPP.

#### 5.3.2.2. Antihypertensive and antidiabetic activity

LOPP exhibited a higher ACE inhibitory activity (91.98 ± 3.24%) and a higher α-glucosidase inhibitory activity (87.77± 1.04%), using a tested dose (50 mg/mL) (Figure 5.6). As an effect of the SGD, ACE (RI 31%) and α-glucosidase (RI 50%) inhibitory activity decreased significantly in small intestine phase ($p < 0.05$). In the case of ACE inhibitory activity, a considerable increase occurred during the dialysis process. The highest release of bioactive compounds during the dialysis process allowed the recovery of 57% of LOPP initial ACE inhibitory activity in the serum available fraction and the retention of 71% of that initial potential in the OUT fraction. Even though, after SGD, more or less of 50% ACE inhibitory activity was measured in the serum-available fraction (IN). Regarding α-glucosidase inhibitory activity, at least 50 % of LOPP's initial potential was verified in the small intestine, where α-glucosidase hydrolyses the disaccharides into simple sugars, facilitating intestinal sugar absorption (Collado-González *et al.*, 2017). Using the tested dose (50 mg/mL) of LOPP, at least 43 % of the activity of α-glucosidase could be inhibited. In conclusion, at least 50 % of both inhibitory activities of LOPP were maintained after SGD.

Several studies reported that plants phenolics have potential ACE (Al Shukor *et al.*, 2013), and α-glucosidase inhibition capacity (Rasouli *et al.*, 2017). TPC was significantly correlated with α-glucosidase ($r^2 = 0.69$). Indeed, the high degradation of LOPP phenolic compounds verified after gastrointestinal tract seemed negatively influenced the α-glucosidase inhibitory activity. Between the LOPP phenolics, *p*-coumaric acid was highly correlated with α-glucosidase inhibitory activity ($r^2 = 0.70$), but substantial correlation was also found between HYD ($r^2 = 0.62$), caffeic acid ($r^2 = 0.64$) and caffeoyl-6'-secologanoside ($r^2 = 0.74$) with the α-glucosidase inhibitory activity. However, tyrosol and its glucoside were weakly correlated with α-glucosidase inhibitory activity ($r^2 < 0.30$). Similar interactions have been reported for olive oils (Figueiredo-González *et al.*, 2019, 2018; Hadrich *et al.*, 2015; Nadour *et al.*, 2015).

ACE inhibitory activity was also relatively influenced by the loss and low bioaccessibility of phenolic compounds (TPC) throughout the SGD ($r^2 = 0.65$). In previous studies, ACE inhibitory activity of different phenolics was variable and dependent of its chemical structure (Al Shukor *et al.*, 2013). For example, phenolic acids as HYD ($r^2 = 0.62$), tyrosol ($r^2 = 0.61$) and its derivatives, HYD glucoside ($r^2 = 0.62$) and tyrosol glucoside ($r^2 = 0.68$), were probably key compounds to explain ACE inhibitory activity of the bioaccessible fraction (IN). Similar results were observed in previous works in olive oil

(Alcaide-Hidalgo *et al.*, 2020). On the other hand, the presence of caffeic acid (Al Shukor *et al.*, 2013) in the non-bioaccessible fraction (OUT) explains, in part, its high ACE inhibitory activity ($r^2$ = 0.73).

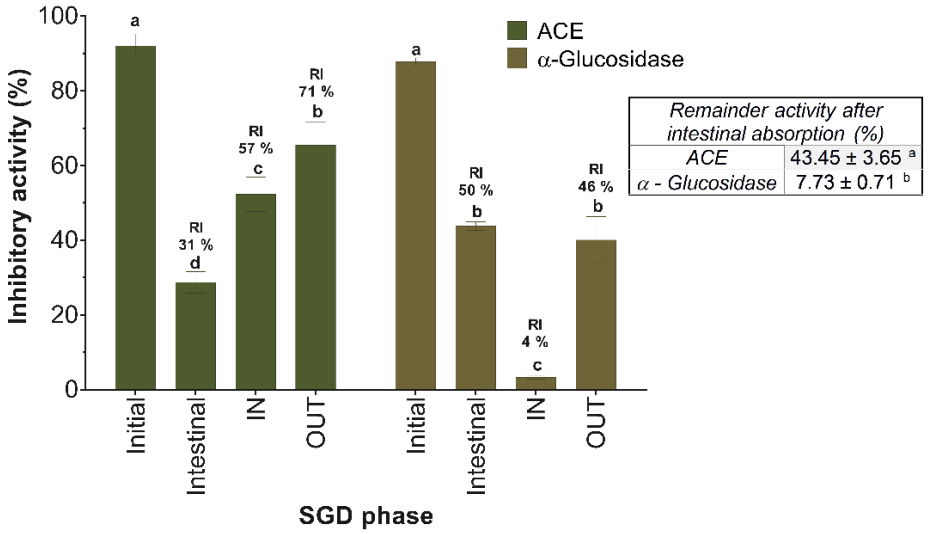



**Figure 5.6. Effect of *in vitro* gastrointestinal digestion on LOPP bioactivities.ACE and α - glucosidase inhibitory activity of LOPP before and after simulated gastrointestinal digestion (intestinal, after dialysis IN and OUT) of LOPP.** Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

Nevertheless, not only phenolic compounds inhibit α-glucosidase and ACE activity, mannitol (Chukwuma *et al.*, 2019; Hagiwara *et al.*, 2005) and glucose (Endringer *et al.*, 2014) from LOPP could also have a substantial role as reported in previous works. A high correlation between α-glucosidase inhibitory activity and mannitol or glucose was attained ($r^2 \geq 0.86$). However, regarding ACE inhibitory activity, a lower correlation with mannitol and glucose was observed ($r^2 \geq 0.58$). Therefore, other compounds besides phenolics and sugars could be present in LOPP and influence ACE inhibitory activity. Among ACE inhibitor compounds not evaluated in the present study were triterpenoids and small peptides with attested olive oil activity (Alcaide-Hidalgo *et al.*, 2020).

Minerals have been reported to possess a significant α-glucosidase inhibitory activity (Avcı *et al.*, 2019). In LOPP, the α-glucosidase inhibitory activity was strongly correlated with minerals content ($r^2 \geq 0.70$). The considerable loss of phenolics and minerals explained the loss of 50% of the initial α-glucosidase inhibitory activity until small intestine.

In the future, the addition of ascorbic acid to LOPP as a protective agent of phenolics (Peters *et al.*, 2010) and enhancer of minerals absorption (Singh & Prasad, 2018) throughout gastrointestinal tract might be explored to improve, not only LOPP AOX but also, its α-glucosidase and ACE inhibitory activity. Other solutions as the conjugation with other phenolic extracts (Rubió *et al.*, 2014) or the development of micro and nano delivery systems (Aliakbarian *et al.*, 2018) could be considered in the future to improve the bioaccessibility of LOPP bioactive compounds.

## 5.4. Conclusion

The bioaccessibility/stability of all liquid-enriched olive pomace powder (LOPP) bioactive compounds (soluble sugars, organic acids, minerals and phenolic compounds) and not only phenolics have been analysed throughout the different phases of gastrointestinal digestion. This study demonstrates that the gastrointestinal tract has a substantial effect in all LOPP bioactive compounds and that all these changes affected LOPP potential health benefits. Phenolics and minerals were the most affected LOPP compounds by the gastrointestinal tract. Its low stability was reflected in the significantly lower values of LOPP antioxidant activity throughout the gastrointestinal tract. Even though notable bioaccessibility indexes (> 50%) were obtained to potassium and hydroxytyrosol derivatives, which could exert its potential cardiovascular health benefits. Furthermore, substantial antihypertensive activity and α-glucosidase inhibitory activity were maintained (50% of the initial inhibitory activities).

Additionally, a significant amount of soluble sugars and formic acid, and a considerable content of phenolics were retained in the non-bioaccessible fraction (OUT), which could be interesting to study in the future to understand its possible benefits in the gut health.

In the future, new strategies to improve the bioaccessibility of LOPP bioactive compounds and to assess the bioaccessibility and potential health benefits from LOPP may be studied. *In vitro* experiments represent a consistent approach to evaluate the health effect of new functional ingredients, but future studies in cell lines and *in vivo* studies are needed before drawing final conclusions. More research into in vitro–in vivo correlations is required to achieve more realistic *in vitro* models and, therefore, screen the bioaccessibility and digestibility of foods.

Finally, the valorisation of bioactive compounds in olive pomace to increase the sustainability and circular bioeconomy is a key option for the olive oil industry, taking into account the high nutritional value identified in this study.

# Chapter 6.

# Study of olive pomace antioxidant dietary fibre powder throughout gastrointestinal tract as multisource of phenolics, fatty acids and dietary fibre

## Abstract

Pulp-enriched powder (POPP) was obtained from olive pomace solid fraction, a derived from the new value chain established for olive by-products. As a multifunctional powder, POPP retains several bioactive compounds (fatty acids, dietary fibre and phenolics) under potential synergic interaction, even more, reactive throughout the digestion. So, in this study, the potential multifunctionality of POPP was evaluated after the gastrointestinal tract. A significant loss of phenolics occurred during oral digestion (62.48%). However, the potential role of dietary fibre as phenolics' carrier and its possible liberation in the stomach allowed recovering a significant amount of phenolics (77.11%) and a bioaccessibility index of at least 50% (mainly for tyrosol and its glucoside). POPP also provides a high content of dietary fibre mainly insoluble fibre (69.68 g/100 g dry weight) linked to a substantial amount of bound phenolics (7.63 mg of gallic acid equivalents/g fibre dry weight), with a positive effect on the fatty acids bioaccessibility [decreased the saturated (5-6%) and facilitated the unsaturated fatty acids bioaccessibility (4-11%)]. PCA analysis revealed the negative effect of simulated gastrointestinal digestion upon POPP as mainly linked to phenolics' loss. Despite all negative effects of the simulated digestion on POPP bioactive composition, phenolics and unsaturated fatty acids showed to be bioaccessible in a significant amount, and the amount of bound phenolics associated to fibre retained in the colon have the potential to exert gut health benefits.

**Keywords:** olive pomace; antioxidant dietary fibre; multifunctional powder; *in vitro* digestion.

## 6.1. Introduction

Olive oil industry produces a large volume of by-products annually. Nowadays, the most implemented two-phase system, where no water is added, allowed to reduce the wastewater production and disposal verified in the past three-phase system (Lafka *et al.*, 2011). However, it is obtained a semisolid mixture of water, olive pulp, skin and stones, called olive pomace (OP), challenging to treat due to its organic and moisture content ( $\geq$ 65%). Only in the Andalucía, from the 5.8 million tonnes of olives annually processed, more than 4 million tons of OP are generated, representing approximately a 65% of the initial weight (AGAPA, 2015).

Severe environmental problems and waste management costs have been associated with OP (Moreno-Maroto *et al.*, 2019). However, this by-product and its solid fraction have been highlighted by its composition rich in dietary fibre, phenolic compounds and substantial antioxidant capacity (AOX) (Pérez-Jiménez *et al.*, 2015; Ribeiro *et al.*, 2020c). These characteristics seem to be aligned to the concept of antioxidant dietary fibre (ADF) proposed by Saura-Calixto (1998), i.e. ADF should contains over 50% (dry weight, DW) of dietary fibre and high AOX. ADF combines the health and technological benefits of DF and phenolics together (Beres *et al.*, 2016; Silva *et al.*, 2018). Hence, the search for natural ADF sources to the food industry has been emerging and could be an excellent opportunity of moving olive oil industry towards a sustainable circular economy model (Campos *et al.*, 2020a; Quirós-Sauceda *et al.*, 2014).

A new promising ADF source was obtained from OP solid fraction using a fractionation approach. The pulp-enriched powder (POPP) obtained in our previous study revealed a rich composition in DF (68.14 ± 0.54 g/100 g DW), mainly insoluble dietary fibre (IDF) (52.17 ± 0.01 g/100 g DW), but also a significant amount of free phenolic compounds (extractable) and bound phenolic compounds linked to the lipids, proteins and dietary fibre (Ribeiro *et al.*, 2020b). POPP also exhibited a high level (about 19 g/ 100 g DW) of unsaturated fatty acids (UFAs), principally in the monounsaturated form (>16%), but also in the polyunsaturated form (> 1%) Oleic acid was the UFA detected in higher amount followed by linoleic acid (Ribeiro *et al.*, 2020b). Both these UFAs are known for their beneficial effects on reducing cholesterol and triglycerides (Lopez-Huertas, 2010). Regarding POPP bound phenolics, it was estimated that more than half of its total bound phenolics (≈ 54%) were linked to dietary fibre (Ribeiro *et al.*, 2020b). In literature, the bound phenolic compounds have been described as significant contributors of the health-related properties attributed to dietary fibre, due to its capacity to pass through the gastrointestinal tract almost intact reaching the colon linked to dietary fibre (González-Sarrías *et al.*, 2017; Liu *et al.*, 2019). In the colon, they can be liberated exerting potential health antioxidant benefits which have been neglected (Silva *et al.*, 2018).

In our previous work, POPP's complete chemical characterisation was achieved showing that POPP is an attractive add-value powdered product with the advantage of retaining several functional compounds, namely UFAs, dietary fibre, and phenolics (Ribeiro *et al.*, 2020b). The retention of these functional compounds together could interact with each other synergically ascribing multifunctional properties to food (García-Lomillo *et al.*, 2014; Saura-Calixto, 1998). However, the digestive tract has been described as a releaser of bioactive compounds, causing negative and positive effects in

bioactive compounds bioaccessibility. Indeed, the phenolics and fatty acids (FAs) that are released by chemical hydrolysis may differ from those liberated during gastrointestinal tract, i.e. mastication, acidic pH and digestive enzymes can trigger the release of food matrix compounds more efficiently than aqueous-organic solvents (Gouw *et al.*, 2017; Jakobek, 2015). However, studies showed also that only a low amount of phenolics reach the intestine, and even a minor portion can pass the gut barrier. Even in the case of dietary fibre, its amount and composition measured by chemical methods could diverge from those reaching the gut (Gouw *et al.*, 2017). Besides that, phenolics, dietary fibre and lipids showed to interact positively and negatively with each other throughout the gastrointestinal tract (Jakobek, 2015).

Dietary fibre can act as carriers of phenolics throughout the gastrointestinal tract, protecting the phenolics from oxidative degradation, but also affecting its bioaccessibility by entrapping bound and free phenolic compounds and restricting the diffusion of digestive enzymes (Bohn, 2014; Jakobek, 2015; Jakobek and Matić, 2019). Several studies showed dietary fibre's role as uptake enhancers of some phenolics (Schramm *et al.*, 2003; Serra *et al.*, 2010). Nevertheless, even non-released phenolics could have a potentially decisive role in gut health, as mentioned above (González-Sarrías *et al.*, 2017). Similarly, lipids-phenolics interactions have been linked with positive effects on lipid oxidation inhibition, lipase activity and fat absorption. On the other hand, lipids could improve phenolics' stability during the gastrointestinal tract by their “capture” protecting them (Jakobek, 2015). For example, in a rat model study, tyrosol and hydroxytyrosol (HYD) absorption from a lipid-rich matrix (olive oil) were higher (≈ 25%) than that from an aqueous solution (Bohn, 2014). However, other works suggested a small impact of lipids on phenolics absorption (Schramm *et al.*, 2003).

As described above, the content and interaction of dietary fibre, lipids and phenolics throughout the gastrointestinal tract are considered essential factors for the healthiness impact of functional ingredients like POPP. Therefore, the present study intends to assess the impact of *in vitro* simulation of gastrointestinal digestion (SGD) on the POPP composition (ADF, soluble sugars, FAs, phenolics) and AOX. The information obtained in this work may help to clarify the potential health benefits of multifunctional ingredients as POPP.

## 6.2. Materials and methods

### 6.2.1. Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS diammonium salt (2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), 2,2’-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, methanol and potassium persulfate were purchased from Sigma-Aldrich (Sintra, Portugal). Folin-Ciocalteu's reagent and sodium carbonate were purchased from Merck (Algés, Portugal). Trolox, gallic acid, p-coumaric and caffeic acid standards were obtained from Sigma-Aldrich (Sintra, Portugal), whereas HYD, tyrosol and luteolin were purchased from Extrasynthese (Lyon, France).

### 6.2.2. Preparation of pulp-enriched olive pomace powder

OP was collected from an olive mill from Oliveira do Hospital, Portugal. Homogenous samples were packed in polyethene flasks and kept on a freezer at - 80 $^0$C until use to avoid the phenolics damage.

Pulp-enriched olive pomace powder (POPP) samples were obtained according to previous work, briefly described as follows: OP was centrifuged (10,000×g for 10 min), the solid fraction was oven-dried (90 °C, water activity < 0.4, 90 minutes), milled using a coffee grinder and sieved (mesh 40). All the pieces of stones were removed to obtain a potentially food-grade ingredient free of physical hazards such as small stones.

### 6.2.3. *In vitro* digestion

SGD of POPP was performed according to the method described by Costa *et al.*, (2019) with dialyses process, to simulate the intestinal and blood absorption (Ribeiro *et al.*, 2020a). The complete procedure was described in section 5.2.3.

#### 6.2.3.1. Recovery and bioaccessibility index

The results of each extract determination (sample, after mouth, gastric and intestinal digestion) were reported in 100 g of DW of POPP.

Recovery index (RI%) and bioaccessibility index (BI%) were performed to study the effect of digestion on a multifunctional ingredient as POPP evaluating its principal nutritional/ bioactive compounds throughout SGD. The values of all nutritional, bioactive compounds and AOX of POPP before digestion were assumed as 100% (Gullon *et al.*, 2015; Lucas-Gonzalez *et al.*, 2016).

According to the methodology of Lucas-Gonzalez *et al.*, (2016) the recovery index allows determining the amount of a given main components' group of the tested food present in the digested sample after oral, gastric and intestinal digestion, according to:

$$Recovery\ index\ (\%) = \left(\frac{BC_{DF}}{BC_{TF}}\right) \times\ 100 \qquad Equation\ (1)$$

Where: $BC_{DF}$ is the bioactive content (mg) in the digested sample, and $BC_{TF}$ is the bioactive content (mg) quantified in the test matrix.

The bioaccessibility index is defined as the percentage of the bioactive compound that is solubilised after intestinal dialysis step. Thus, this index defines the proportion of the bioactive compound that could become available for absorption into the systematic circulation:

$$Bioaccessibility\ index\ (\%) = \left(\frac{BC_S}{BC_{DF}}\right) \times\ 100 \qquad Equation\ (2)$$

where: $BC_S$ is the bioactive content (mg) in the digested sample after the dialysis step (IN) and $BC_{DF}$ is the total bioactive content (mg) in the digested sample after the dialysis step (IN + OUT).

At this point, it is essential to define the term “bioaccessibility” to avoid the confusion with “bioavailability” or “permeability”. Bioaccessibility is described as the amount of a compound that is released from its matrix in the digestive tract, becoming available for bloodstream absorption. Bioavailability expresses the fraction of ingested bioactive compound or nutrient that reaches the systemic circulation and finally utilised. Before becoming bioavailable, bioactives must be released from the food matrix and modified in the gastrointestinal tract. Thus, bioavailability includes the term bioaccessibility (Torres-Palazzolo *et al.*, 2018). In this work, bioaccessibility was evaluated using the *in vitro* SGD, and permeability will be assessed in future using Caco-2 cell cultures.

#### 6.2.3.2. Dietary fibre composition

The digested sample was filtered using a sintered glass crucible (no. 2). The IDF was retained in the crucible, and the supernatant was saved for soluble dietary fibre (SDF) analysis. The procedure used to determine the total amount of IDF, SDF and its composition was described in the previous chapter.

#### 6.2.3.3. Bioactive compounds determination and quantification: Sugars and organic acids, fatty acids and phenolic compounds

All bioactive compounds were determined in lyophilised samples. The analysis procedure for each POPP component throughout SGD (sugars, organic acids, FAs, total phenolic compounds (TPC) and individual phenolics was enlightened in the previous chapter section 5.2.4.

#### 6.2.3.4. Nutritional fatty acids quality indices

Nutritional fatty acids (FAs) quality indices of POPP after and before each step of SGD were analysed from FAs composition data. The indices of thrombogenicity (*TI*) and atherogenicity (*AI*) were calculated using Eqs. (3) and (4), respectively. Other nutritional quality indices, namely PUFA/SFA and Saturation Index (*SI*) (Eq. (5)) were also determined (de Alba *et al.*, 2019).

$$TI = \frac{[C14{:}\,0 + C16{:}\,0 + C18{:}\,0]}{\left[0.5 \times (\sum MUFA + \sum n6) + 3 \times \sum n3 + \frac{\sum n3}{\sum n6}\right]} \qquad Equation\ (3)$$

$$AI = \frac{[C12{:}\,0 + 4 \times C14{:}\,0 + C16{:}\,0]}{[\sum MUFA + \sum PUFA]} \qquad Equation\ (4)$$

$$SI = \frac{[C14{:}\,0 + C16{:}\,0 + C18{:}\,0]}{[\sum MUFA + \sum PUFA]} \qquad Equation\ (5)$$

#### 6.2.3.5. Antioxidant capacity: ABTS, DPPH e ORAC

The antioxidant capacity (AOX) of POPP during SGD was achieved according to DPPH (Alexandre *et al.*, 2019), ABTS and ORAC (Costa *et al.*, 2019) methods, using a multidetection plate reader (Synergy H1, Vermont, USA). The radical stock solutions were freshly prepared. Lyophilised samples were dissolved in methanol to obtain a concentration comprised between 20 and 200 mg/mL. All analyses were performed in triplicate and expressed in mM of Trolox-equivalents (TE)/g DW.

### 6.2.4. Statistical analysis

All experiments were carried out in triplicates, and data were reported as mean ± standard deviation. Software R was used to carry out statistical analyses. The Shapiro - Wilk test was used to test the normality of data distribution and then analysed by one-way analysis of variance (ANOVA). Tukey's post hoc test was applied for comparison of means; differences were considered significant at $p < 0.05$. Correlation analysis (Pearson correlation analysis) and principal component analysis (PCA) were performed to evaluate the potential associations between the bioactive compounds of POPP and its AOX through throughout SGD. Correlations between different parameters were considered significant at $r > 0.95$ ($p < 0.05$).

## 6.3. Results and Discussion

### 6.3.1. Dietary fibre

The amount of dietary fibre, its composition (neutral sugars, uronic acids, TPC and individual phenolics) and AOX measured after SGD are presented in Figure 6.1, together with the previous results of dietary fibre profile of POPP obtained using the AOAC (Association of Official Analytical Chemists) method (Ribeiro *et al.*, 2020b). The comparison between the results achieved using the AOAC methodology and SGD allowed to understand the possible effects of digestion in POPP dietary fibre content and composition, but also to estimate their potential beneficial effects on human health.

After SGD, POPP maintained its higher content of IDF *versus* SDF. Indeed, the amount quantified after SGD (69.68 ± 0.79 g/ 100 g DW) was higher than that estimated by AOAC (52.17 ± 0.01 g/ 100 g DW) and the SDF content decreased after SDG (4.49 ± 0.24 g/ 100 g DW) in comparison to AOAC results (9.89 ± 0.54 g/ 100 g DW) as can be seen in Figure 6.1. Regarding IDF composition (Figure 6.1), some significant differences were detected ($p < 0.05$) after SGD. The content of uronic acids and lignin from SGD were significantly ($p < 0.05$) higher than those obtained by the AOAC method, and lower for neutral sugars (mainly to xylose). The higher amount of lignin could be explained by the complex macromolecular structure of lignin and its consequent resistance to digestive enzymes (Gouw *et al.*, 2017). The lower content of neutral sugars (mainly xylose, but also glucose) was probably related to the release of a higher level of compounds by chemical and mechanical reactions occurred during SGD (Grundy *et al.*, 2016).

The higher amount of lignin reported could be an advantage in terms of gut health benefits. Lignified fibres have been described as potent *in vitro* source of antioxidants and adsorbers of hydrophobic carcinogens in the whole intestine (Mudgil, 2017; Sato *et al.*, 2011). Lignin, and more generally IDF, have been claimed as carriers of phenolics throughout the gastrointestinal tract. The principal bound phenolic compound associated with IDF and SDF after AOAC and SGD were identified by LC-ESI-UHR-QqTOF-MS (Table 6.1) and quantified by HPLC (Figure 6.1).

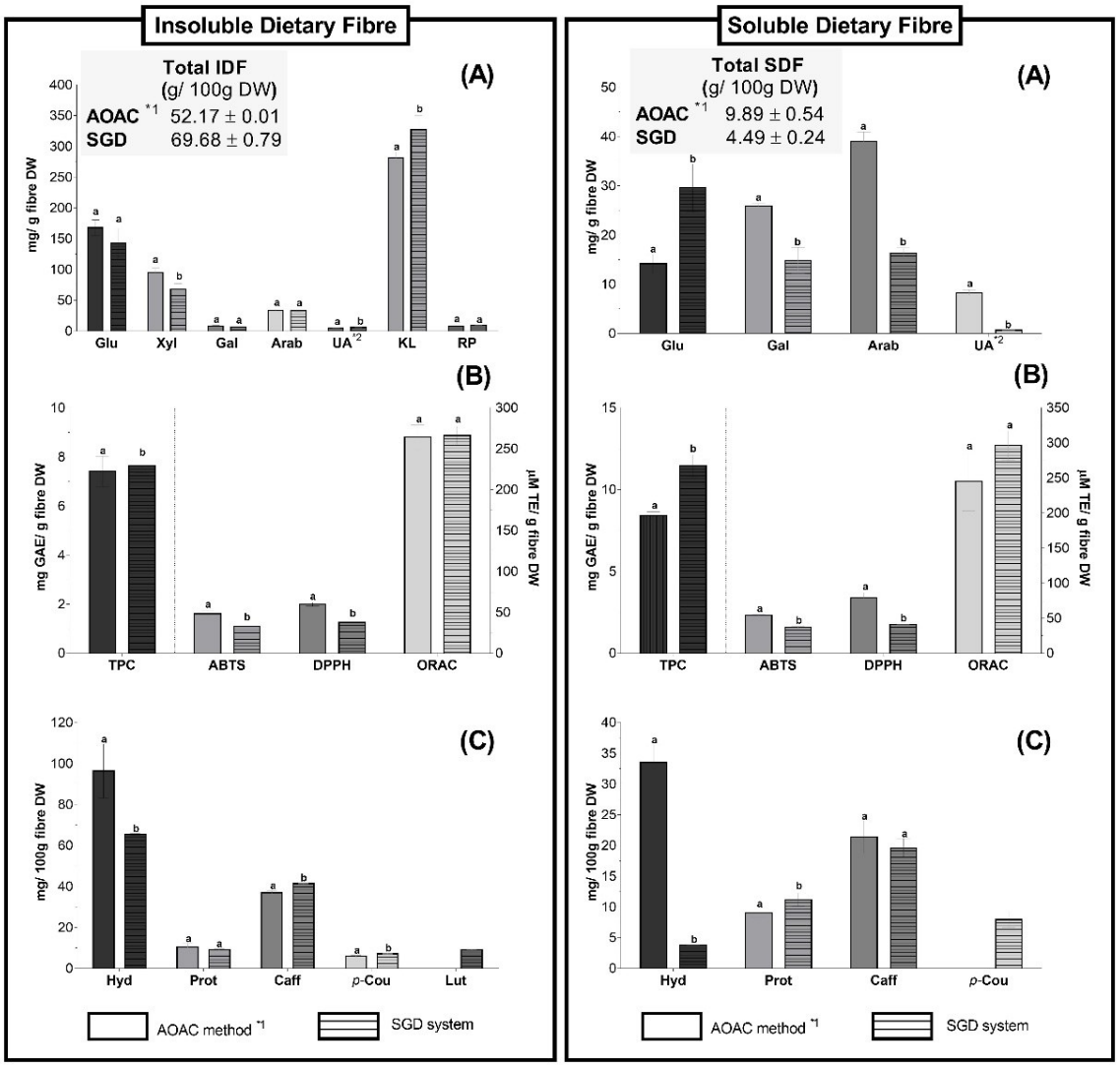



**Figure 6.1. POPP dietary fibre composition and antioxidant activity using modified AOAC dietary fibre analysis method and simulated gastrointestinal system. (A) Determination (g/ 100 g DW) and profile (mg/g fibre DW) of dietary fibre; (B) Total phenolic compounds (TPC) expressed as mg GAE/ g fibre DW and antioxidant capacity using ABTS, DPPH and ORAC methods of phenolics associated to fibre fraction (µM TE/ g DW); (C) Concentration of main individual phenolics (mg/100 g DW) associated to fibre fraction**

DW – dry weight; Glu – Glucose; Xyl – Xylose; Gal – Galactose; Arab – Arabinose; UA – Uronic acids; KL – Klason lignin; RP – Resistant protein; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre; GAE - gallic acid equivalents; TE – Trolox equivalents; Hyd - Hydroxytyrosol; Prot - Protocathecuic acid; Caff - Caffeic acid; p-Cou - p-Coumaric; Lut - Luteolin. *1 Data from previous paper Ribeiro *et al.* (2020b); *2 mg galacturonic acid equivalents (GUAE)/g fibre DW. Results are the means of three determinations ± standard deviation. Different letters in the same column are significantly different, as ANOVA ($p < 0.05$) determined.

**Table 6.1. Retention time, maximum wavelength absorbance and MSn fragmentations profiles of bound phenolics of dietary fibre achieved by AOAC and after in vitro simulated gastrointestinal digestion (SGD).**

| Compound | Molecular formula | RT (min) | $\lambda_{max}$ | m/z calcd | m/z exptl | Err [mDa] | mSigma | Major fragments ESI negative MS/MS ions | AOAC | | After SGD | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | IDF | SDF | IDF | SDF |
| **Dihidroxytyrosol** [*2] | $C_8H_{10}O_4$ | 1.4 | 279 | 169.0506 | 169.0504 | 0 | 7.9 | 151.0397; 123.0448 | × | × | ✓ | × |
| **Hydrozylated form of elenolic acid**[*2] | $C_{11}H_{14}O_7$ | 6.9 | 280/320 | 257.0667 | 257.0666 | 0 | 12.4 | 125.0968; 151.0758; 257.0681 | × | × | × | ✓ |
| **Vanillin** [*1] | $C_8H_8O_3$ | 7.3 | 230 | 151.0401 | 151.0399 | 0.1 | 5.6 | 123.0450; 109.0291; 151.0401 | ✓ | ✓ | ✓ | × |
| **Hydroxytyrosol** [*1] | $C_8H_{10}O_3$ | 7.4 | 280 | 153.0556 | 153.0557 | 0.1 | 5.7 | 153.0452; 123.0457 | ✓ | ✓ | ✓ | × |
| **Tyrosol glucoside**[*3] | $C_{14}H_{20}O_7$ | 8.1 | 227/276 | 299.1139 | 299.1136 | -0.3 | 3.2 | 119.0505; 137.0244; 135.0299 | ✓ | ✓ | ✓ | ✓ |
| **4-Hydroxybenzoic acid** [*2] | $C_7H_6O_3$ | 8.2 | 193 | 137.0244 | 137.0241 | 0.3 | 2.6 | 136.0166; 137.0236; 108.0215 | × | × | ✓ | × |
| **Oleoside**[*2] | $C_{16}H_{22}O_{11}$ | 8.4 | 277 | 389.1093 | 389.1089 | -0.4 | 4.3 | 389.1088; 183.0664; 165.0557 | ✓ | ✓ | ✓ | ✓ |
| **Caffeic acid** [*1] | $C_9H_8O_4$ | 9.3 | 323 | 179.0350 | 179.0350 | -0.1 | 7.9 | 179.0350; 135.0448 | ✓ | ✓ | ✓ | ✓ |
| **Tyrosol** [*1] | $C_8H_{10}O_2$ | 9.8 | 277 | 137.0608 | 137.0608 | 0.5 | 4.5 | 111.0084; 95.0510 | ✓ | ✓ | × | × |
| **Coumaric acid** [*1] | $C_9H_8O_3$ | 10.6 | 309 | 163.0403 | 163.0401 | -0.2 | 10.5 | 163.0397; 119.0499 | ✓ | ✓ | ✓ | ✓ |
| **Luteolin – 7 – *O* – glucoside** [*1] | $C_{21}H_{20}O_{11}$ | 12.0 | 237/266/352 | 447.0928 | 447.0933 | 0.5 | 0.5 | 447.0924; 285.0399 | ✓ | ✓ | ✓ | ✓ |
| **Luteolin** [*1] | $C_{15}H_{10}O_6$ | 15.9 | 268/349 | 285.0406 | 285.0405 | -0.1 | 0.8 | 285.0414; 151.0037 | ✓ | ✓ | ✓ | ✓ |

[*1] Hydroxytyrosol, tyrosol, luteolin-7-O-glucoside, luteolin, vanillin, caffeic acid and coumaric acid were identified by comparison with the standards. The other compounds were tentatively identified by LC-ESI-UHR-QqTOF-MS based on accurate mass, isotope rate calculations designated mSigma and literature.[*2] Lozano-Sánchez *et al.*, (2013), [*3]Jerman Klen & Mozetič Vodopivec (2012).

IDF revealed a significantly higher content of total bound phenolic compounds after SGD ($p < 0.05$), but also a higher amount of the individual phenolics caffeic, *p*-coumaric acid and luteolin which appeared to be related to IDF higher amount of lignin detected after SGD (Figure 6.1). Hydroxycinnamic acid derivatives like *p*-coumaric and caffeic acid are commonly found linked to cell-wall cellulose and lignin through ester bonds (Acosta-Estrada *et al.*, 2014; Calvache *et al.*, 2016).

Between the bound phenolics associated with IDF, HYD seemed to be the only bound phenolic negatively modified by SGD. HYD linked to IDF exhibited a significantly lower amount after SGD compared to AOAC data ($p < 0.05$), which could explain the lower AOX reported by ABTS and DPPH results (Figure 6.1). The amount of HYD and electron transfer methods showed to be strongly correlated [HYD/ABTS ($r^2$ =0.80) and HYD/DPPH ($r^2$ =0.70)]. On the other hand, the ORAC values of IDF were not negatively influenced by SGD. Overall, the AOX results of IDF from POPP revealed that a substantial bound phenolic compounds amount could hypothetically reach the colon intact and further be liberated to exert their potential gut health benefits.

The profile of SDF was negatively changed by SGD (Figure 6.1), principally the neutral sugars. All neutral sugars were significantly lower ($p < 0.05$) after SGD than those determined by the AOAC method; only glucose was significantly higher ($p < 0.05$). Total phenolic compounds (TPC) and the

individual phenolics protocatechuic and *p*-coumaric acid amounts were also significantly higher after SGD. The higher concentrations of glucose and phenolics as *p*-coumaric acid after SGD system might be related as reported in previous studies of *p*-coumaric acid extraction (Jiang *et al.*, 2016). Equally, the higher concentration of protocatechuic acid could be associated with the lower detection of HYD, i.e. the higher release of HYD from fibre after the SGD system could explain a possible consecutive decarboxylation (α-oxidation dihydroxylation) into protocatechuic acid (López de las Hazas *et al.*, 2016). Concerning the AOX of bound phenolic compounds present in SDF, the values were higher using the AOAC method than after the SGD system. The ORAC was the only AOX methodology that not detected negative changes in AOX of bound phenolics linked to SDF after SGD. The higher TPC detected for bound phenolic compounds after SGD could be related to higher ORAC values estimated after SGD. ORAC was strongly correlated with TPC ($r^2$ =0.86) than ABTS and DPPH ($r^2$ =0.76).

### 6.3.2. Bioactive compounds trough *in vitro* simulation of the gastrointestinal tract

#### 6.3.2.1. Soluble sugars and organic acids

The soluble sugars and organic acids RI and concentration throughout SGD are presented in Table 6.2 and Table 6.3, respectively.

After SGD, the soluble sugars amount changed significantly ($p < 0.05$). Fructose was the sugar most affected (Table 6.2). In the mouth, a higher release of fructose was verified (RI = 106.32 ± 8.96%), that is maintained until the intestine (RI = 104.14 ± 3.46%) and increased during the simulated intestinal absorption (dialysis) as shown in Table 6.2. RI higher than 100% was registered. A liberation of compounds from POPP by the action of pH and digestive enzymes; and chemical modifications in these compounds, such as sugar isomerisation, could have taken place, thus justifying this RI value.

Glucose and mannitol exhibited similar behaviour through SGD (Table 6.2). In the mouth, the RI of these soluble sugars decreased (glucose: RI = 69.02 ± 4.40%; mannitol: RI = 66.83 ± 4.37%) and increased significantly after simulated intestinal absorption (dialysis) ($p < 0.05$). As mentioned above, a higher release of carbohydrates throughout SGD was observed in the dietary fibre profile, which possibly influenced positively the recovery of the soluble sugar detected. This higher soluble sugar release was especially evident during the simulated intestinal absorption, perhaps a consequence of the long incubation time (more 2 h) which means that reactions (proteolytic, lipolytic, amylolytic, etc.) between intestinal digestive enzymes used and dietary fibre, as well its contact with water, are much more lasting and thorough (Singh *et al.*, 2014; Zhou *et al.*, 2018) Other reactions of isomerisation reactions of glucose into fructose and mannose, and subsequent hydrogenation into mannitol under alkaline conditions (pH of the intestine), could eventually explain the higher amount of fructose and mannitol (Makkee *et al.*, 1985).

The formic acid concentration in POPP also increased considerably after SGD (Table 6.3), possibly because of the oxidation of FAs that could occur in each step of the SGD, for example, studies

reported that acidic pH of stomach enhanced lipid oxidation (Kanner and Lapidot, 2001; Van Hecke *et al.*, 2017). A similar increase of formic acid was detected through the olive oil storage and throughout *in vitro* SGD of the liquid-enriched fraction powder from OP of our previous work (Ribeiro *et al.*, 2020a).

Regarding bioaccessibility (Table 6.2), the BI of soluble sugars and organic acids after SGD were all higher than 60 % [Formic acid (83.22 ± 3.24%) > Glucose (75.94 ± 3.99%) > Mannitol (70.83 ± 4.71%) > Fructose (63.25 ± 6.58%)]. All the soluble sugars and formic acid were detected at higher amounts in the absorbable fraction (IN) than in the non-absorbable fraction (OUT) (Table 6.3). Nevertheless, the higher amount of mannitol (2.47± 0.35 g/100g DW) in the IN fraction in comparison to glucose (2.21 ± 0.26 g/100g DW) and fructose (0.62 ± 0.11 g/100g DW) could be related to potential glycaemic control activity of mannitol (Chukwuma *et al.*, 2019).

#### 6.3.2.2. Fatty acids

The effect of SGD on the total FA profile of POPP is expressed in Table 6.3. Significant variations in FAs profile occurred throughout SGD ($p < 0.05$). Fat digestion occurs mainly in the intestine where about 80% of the lipolysis reaction occurs; however, oral and gastric digestion could have a preeminent action on the facilitation of lipid intestinal digestion (Ye *et al.*, 2019). This action was noticed by the changes in the FAs profile in the mouth and stomach phases. The negative effect of mastication in the mouth and acidic pH of the stomach, reported before to other fat-rich foods (Kanner and Lapidot, 2001; Van Hecke *et al.*, 2017), appears to have a strong degradation effect on lipid fraction (RI between 20 - 40%). Another factor that could explain the extensive loss of FAs was the oxygen presence in all SGD steps (Tullberg *et al.*, 2019). The use of $N_2$ gas at the start of each digestion step of fat-rich foods should be applied to reduce fat oxidation.

The SGD negative effect was noticed in the same degree for all FAs (Table 6.2). Generally, SFAs were more stable than PUFAs, but the presence of phenolics on POPP could explain the better stability of PUFAs detected (Jakobek, 2015).

The MUFA oleic acid was identified as the main FA of POPP, being also the most predominant in all digestion phases (Table 6.2) and the most bioaccessible (BI =11.89 ± 0.90%) (Table 6.2). As verified by other *in vitro* studies, the most abundantly released FAs were also the most abundant (Ye *et al.*, 2019). After oleic acid, PUFAs showed higher BI (Table 6.2). Linoleic (C18:2 *c9c12*) and α-linolenic (α C18:3 *c*9*c*12*c*15) exhibit BI values of 7.06 ± 0.56 and 4.00 ± 0.53, respectively. The degree of saturation seems to have a relevant impact on the FAs digestion since it was noticed a higher BI to UFAs than SFAs (Table 6.2). POPP's rich dietary fibre composition could justify the lower SFAs bioaccessibility (palmitic and stearic) due to the oil holding capacity of dietary fibre to retain SFAs, influencing their bioaccessibility. This possible slow down effect of dietary fibre on SFAs bioaccessibility was reported previously in a study with fortified wheat bread (Kurek *et al.*, 2018). The retention of FAs by dietary fibre was also supported to the detection of the higher amount of FAs (higher proportion of SFAs than UFAs) in the pellet (PF) than in the soluble fraction (SF) (Table 6.3).

**Table 6.2. Recovery index (RI%) and bioaccessibility index (BI%) of POPP bioactive compounds/ antioxidant capacity throughout simulated gastrointestinal digestion (SGD).**

| | Recovery index (%) | | | | | Bioaccessibility index (%) |
|---|---|---|---|---|---|---|
| | Oral | Gastric | Intestinal | IN (absorbable) | OUT (non-absorbable) | |
| ***Sugars & Organic acids*** | | | | | | |
| *Glucose* | 69.02 ± 4.40 $^{a}$ | 70.07 ± 0.47 $^{a}$ | 75.08 ± 2.58 $^{a}$ | 62.6 ± 7.36 $^{a}$ | 19.55 ± 1.99 $^{b}$ | 75.94 ± 3.99 $^{ab}$ |
| *Fructose* | 106.32 ± 8.96 $^{ab}$ | 95.37 ± 1.00 $^{ab}$ | 104.14 ± 3.46 $^{ab}$ | 121.90 ± 22.31 $^{a}$ | 62.94 ± 6.94 $^{b}$ | 63.25 ± 6.58 $^{b}$ |
| *Mannitol* | 66.83 ± 4.37 $^{a}$ | 56.05 ± 0.61 $^{a}$ | 65.01 ± 2.31 $^{a}$ | 61.17 ± 8.58 $^{a}$ | 24.77 ± 2.19 $^{b}$ | 70.83 ± 4.71 $^{ab}$ |
| *Formic acid* | 94.01 ± 2.99 $^{a}$ | 78.64 ± 4.40 $^{a}$ | 101.20 ± 16.58 $^{a}$ | 83.12 ± 17.10 $^{a}$ | 16.11 ± 0.58 $^{b}$ | 83.22 ± 3.24 $^{a}$ |
| ***SFA*** | | | | | | |
| *C14:0* | nd | nd | nd | nd | nd | 0.00 ± 0.00 $^{d}$ |
| *C16:0* | 20.80 ± 0.41 $^{b}$ | 22.75 ± 0.22 $^{ab}$ | 23.81 ± 2.33 $^{ab}$ | 1.38 ± 0.26 $^{c}$ | 27.39 ± 1.20 $^{a}$ | 5.06 ± 0.76 $^{bc}$ |
| *C18:0* | 21.92± 0.44 $^{b}$ | 24.00 ± 0.46 $^{b}$ | 26.68 ± 2.50 $^{ab}$ | 1.82 ± 0.19 $^{c}$ | 30.73 ± 1.09 $^{a}$ | 5.76 ± 0.49 $^{bc}$ |
| *C20:0* | 22.27 ± 0.74 $^{b}$ | 23.98 ± 0.54 $^{b}$ | 24.77 ± 2.51 $^{ab}$ | 0.00 ± 0.00 $^{c}$ | 31.56 ± 4.04 $^{a}$ | 0.00 ± 0.00 $^{d}$ |
| ***MUFA*** | | | | | | |
| *C16:1 c9* | 20.99 ± 0.35 $^{a}$ | 23.17 ± 0.46 $^{a}$ | 22.85 ± 2.21 $^{a}$ | 1.30 ± 0.24 $^{b}$ | 27.11 ± 2.08 $^{a}$ | 4.83 ± 0.53 $^{c}$ |
| *C16:1 t9* | 22.86 ± 0.41 $^{a}$ | 24.67 ± 0.78 $^{a}$ | 23.90 ± 3.08 $^{a}$ | 0.00 ± 0.00 $^{b}$ | 28.64 ± 2.24 $^{a}$ | 0.00 ± 0.00 $^{d}$ |
| *C17:1 c10* | nd | nd | nd | nd | nd | 0.00 ± 0.00 $^{d}$ |
| *C18:1 c9* | 20.83 ± 0.39 $^{a}$ | 23.29 ± 0.39 $^{a}$ | 23.12 ± 2.22 $^{a}$ | 1.29 ± 0.16 $^{c}$ | 9.93 ± 0.49 $^{b}$ | 11.89 ± 0.90 $^{a}$ |
| *C18:1 c11* | 20.57 ± 0.60 $^{b}$ | 23.52 ± 0.51 $^{b}$ | 23.39 ± 2.34 $^{b}$ | 0.99 ± 0.19 $^{b}$ | 385.34 ± 48.14 $^{a}$ | 0.27 ± 0.04 $^{d}$ |
| *C20:1 c9* | 23.81 ± 0.49 $^{a}$ | 25.21 ± 0.47 $^{a}$ | 21.76 ± 2.16 $^{a}$ | 0.00 ± 0.00 $^{b}$ | 27.38 ± 2.44 $^{a}$ | 0.00 ± 0.00 $^{d}$ |
| ***PUFA*** | | | | | | |
| *C18:2 c9c12* | 20.99 ± 0.36 $^{a}$ | 23.50 ± 0.36 $^{a}$ | 23.31 ± 2.22 $^{a}$ | 2.00 ± 0.31 $^{b}$ | 27.61± 2.27 $^{a}$ | 7.06 ± 0.56 $^{b}$ |
| *$\alpha$ C18:3 c9c12c15* | 30.98 ± 0.54 $^{a}$ | 34.96 ± 0.44 $^{a}$ | 33.99 ± 2.86 $^{a}$ | 1.56 ± 0.35 $^{b}$ | 39.98 ± 3.21 $^{a}$ | 4.00 ± 0.53 $^{c}$ |
| ***Phenolic compounds*** | | | | | | |
| *TPC* | 37.52 ± 3.85 $^{c}$ | 77.11 ± 1.40 $^{a}$ | 46.08 ± 1.80 $^{b}$ | 14.48 ± 2.71 $^{d}$ | 14.12 ± 2.21 $^{d}$ | 51.39 ± 3.34 $^{d}$ |
| *Hydroxytyrosol glucoside* | 27.32 ± 4.13 $^{c}$ | 60.81 ± 2.77 $^{a}$ | 49.29 ± 2.86 $^{b}$ | 33.63 ± 3.49 $^{c}$ | 26.74 ± 1.75 $^{c}$ | 54.93 ± 1.74 $^{cd}$ |
| *Hydroxytyrosol* | 17.88 ± 2.90 $^{b}$ | 58.33 ± 4.83 $^{a}$ | 54.27 ± 9.93 $^{a}$ | 4.14 ± 0.80 $^{c}$ | 4.74 ± 0.85 $^{c}$ | 45.80 ± 3.54 $^{de}$ |
| *Tyrosol glucoside* | 24.52 ± 4.55 $^{b}$ | 22.68 ± 4.51 $^{b}$ | 26.73 ± 4.22 $^{b}$ | 48.81 ± 1.27 $^{a}$ | 18.97 ± 3.44 $^{b}$ | 69.36 ± 3.22 $^{b}$ |
| *Tyrosol* | 49.90 ± 1.32 $^{a}$ | 53.74 ± 8.92 $^{a}$ | 42.07 ± 2.30 $^{ab}$ | 45.39 ± 6.36 $^{a}$ | 28.02 ± 3.92 $^{b}$ | 63.06 ± 7.92 $^{bc}$ |
| *Caffeic acid* | 42.92 ± 7.26 $^{a}$ | 39.78 ± 3.18 $^{ab}$ | 31.98 ± 2.27 $^{bc}$ | 24.27 ± 2.97 $^{c}$ | 3.42 ± 0.56 $^{d}$ | 87.68 ± 0.73 $^{a}$ |
| *p-coumaric acid* | 29.02 ± 2.56 $^{bc}$ | 31.41 ± 3.91 $^{b}$ | 55.43 ± 6.35 $^{a}$ | 12.55 ± 2.22 $^{d}$ | 22.45 ± 1.61 $^{cd}$ | 35.20 ± 3.40 $^{e}$ |
| *Luteolin* | 14.09 ± 0.50 $^{a}$ | 16.85 ± 2.45 $^{a}$ | 16.37 ± 1.39 $^{a}$ | 0.00 ± 0.00 $^{c}$ | 10.80 ± 0.87 $^{b}$ | 0.00 ± 0.00 $^{f}$ |
| **Antioxidant capacity** | | | | | | |
| *ABTS* | 52.90 ± 9.42 $^{b}$ | 92.84 ± 7.55 $^{a}$ | 37.12 ± 5.09 $^{c}$ | 35.61 ± 6.95 $^{c}$ | 47.59 ± 5.37 $^{bc}$ | 43.00 ± 8.99 $^{a}$ |
| *DPPH* | 21.36 ± 8.82 $^{c}$ | 41.46 ± 1.58 $^{b}$ | 43.82 ± 2.27 $^{b}$ | 65.40 ± 3.03 $^{a}$ | 5.98 ± 0.42 $^{d}$ | 91.63 ± 0.17 $^{b}$ |
| *ORAC* | 70.22 ± 8.61 $^{b}$ | 104.14 ± 10.40 $^{a}$ | 65.61 ± 3.16 $^{b}$ | 10.16 ± 1.31 $^{e}$ | 40.73 ± 3.12 $^{c}$ | 24.93 ± 4.20 $^{d}$ |

Nd- non-detected; SFA - Saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. C14:0 – Myristic acid; C16:0 – Palmitic acid; C18:0 – Stearic acid; C20:0 – Arachidic acid; C16:1 *c*9 – Palmitoleic acid; C16:1 *t9* - trans-palmitoleic acid; C17:1 *c10* - cis-10-heptadecenoic acid; C18:1 *c9* – Oleic acid; C18:1 *c11* - cis-Vaccenic acid; C20:1 *c9* - cis-Eicosanoid acid; C18:2 *c9c12* – Linoleic acid; $\alpha$ C18:3 *c9c12c15* - $\alpha$-linolenic acid. Results are the means of three determinations ± standard deviation. Values with different letters in the same line to RI (%) and the same column to BI (%) are significantly different, as determined by one-way ANOVA test ($p < 0.05$), respectively.

**Table 6.3. Soluble sugars/organic acids, fatty acid and phenolics concentration obtained after the simulated gastrointestinal digestion (SGD) of POPP.**

| *Bioactive component* | Initial | Oral | | | Gastric | | | Intestinal | | | IN | OUT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SF | PF | Total | SF | PF | Total | SF | PF | Total | Total | SF | PF | Total |
| **Sugars & Organic acids (g/ 100g DW)** | | | | | | | | | | | | | | |
| *Glucose* | 3.53 ± 0.28 $^{a}$ | | | 2.44 ± 0.16 $^{b}$ | | | 2.47 ± 0.02 $^{b}$ | | | 2.65 ± 0.09 $^{b}$ | 2.21 ± 0.2 $^{b}$ | | | 0.69± 0.07 $^{c}$ |
| *Fructose* | 0.41 ± 0.06 $^{ab}$ | | | 0.54 ± 0.05 $^{a}$ | | | 0.49 ± 0.01 $^{ab}$ | | | 0.53 ±0.02 $^{a}$ | 0.62 ± 0.11 $^{a}$ | | | 0.35 ± 0.04 $^{c}$ |
| *Mannitol* | 4.03 ± 0.49 $^{a}$ | | | 2.69 ± 0.18 $^{b}$ | | | 2.26 ± 0.02 $^{b}$ | | | 2.62 ± 0.09 $^{b}$ | 2.47 ± 0.35 $^{b}$ | | | 1.00 ± 0.09 $^{c}$ |
| *Formic acid* | 0.47 ± 0.02 $^{a}$ | | | 0.42 ± 0.01 $^{a}$ | | | 0.35 ± 0.02 $^{a}$ | | | 0.46 ± 0.07 $^{a}$ | 0.37 ± 0.08 $^{a}$ | | | 0.07 ± 0.00 $^{c}$ |
| **Saturated fatty acids (mg/ g DW)** | | | | | | | | | | | | | | |
| *C14:0* | nd | 0.07 ± 0.01 $^{ab}$ | 0.01 ± 0.00 $^{de}$ | 0.08 ± 0.00 $^{a}$ | 0.04 ± 0.01 $^{cd}$ | 0.01 ± 0.00 $^{de}$ | 0.05 ± 0.00 $^{bc}$ | 0.02± 0.01 $^{de}$ | 0.01 ± 0.00 $^{de}$ | 0.03 ± 0.00 $^{cde}$ | 0.00 ± 0.00 $^{e}$ | 0.03 ± 0.00 $^{cde}$ | 0.01± 0.00 $^{de}$ | 0.03 ± 0.00 $^{cd}$ |
| *C16:0* | 33.69 ± 0.39 $^{a}$ | 0.39 ± 0.08 $^{g}$ | 6.62 ± 0.09 $^{cd}$ | 7.01 ± 0.14 $^{c}$ | 0.23 ± 0.05 $^{g}$ | 7.74 ± 0.03 $^{c}$ | 7.67 ± 0.09 $^{bc}$ | 2.89 ± 0.14 $^{f}$ | 5.13 ± 0.65 $^{de}$ | 8.02 ± 0.79 $^{bc}$ | 0.46 ± 0.09 $^{g}$ | 5.26 ± 0.54 $^{de}$ | 3.97 ± 0.18 $^{ef}$ | 9.23 ± 0.40 $^{b}$ |
| *C18:0* | 4.98 ± 0.07 $^{a}$ | 0.07± 0.01 $^{h}$ | 1.03 ± 0.02 $^{de}$ | 1.10 ± 0.02 $^{cde}$ | 0.03 ± 0.00 $^{h}$ | 1.15 ± 0.01 $^{cd}$ | 1.18 ± 0.01 $^{cd}$ | 0.49 ± 0.02 $^{g}$ | 0.84 ± 0.10 $^{ef}$ | 1.33 ± 0.12 $^{bc}$ | 0.09 ± 0.01 $^{h}$ | 0.90 ± 0.07 $^{e}$ | 0.64 ± 0.03 $^{fg}$ | 1.54 ± 0.05 $^{b}$ |
| *C20:0* | 1.10 ± 0.03 $^{a}$ | 0.02 ± 0.01 $^{f}$ | 0.23 ± 0.00 $^{cd}$ | 0.24 ± 0.01 $^{cd}$ | 0.01 ± 0.00 $^{f}$ | 0.26 ± 0.00 $^{cd}$ | 0.26 ± 0.01 $^{cd}$ | 0.10 ± 0.01 $^{ef}$ | 0.18 ± 0.02 $^{de}$ | 0.27 ± 0.03 $^{c}$ | nd | 0.26 ± 0.04 $^{cd}$ | 0.13 ± 0.00 $^{e}$ | 0.39 ± 0.02 $^{b}$ |
| ***Total*** | 38.67 ± 0.46 $^{a}$ | | | 8.19 ± 0.15 $^{c}$ | | | 8.89 ± 0.08 $^{bc}$ | | | 9.38 ± 0.91 $^{bc}$ | 0.55 ± 0.10 $^{d}$ | | | 10.80 ± 0.46 $^{b}$ |
| **Monounsaturated fatty acids (mg/ g DW)** | | | | | | | | | | | | | | |
| *C16:1 c9* | 3.75 ± 0.04 $^{a}$ | 0.06 ± 0.01 $^{cde}$ | 0.74 ± 0.01 $^{h}$ | 0.80 ± 0.01 $^{cd}$ | 0.03 ± 0.00 $^{bcd}$ | 0.84 ± 0.00 $^{h}$ | 0.88 ± 0.02 $^{bc}$ | 0.32 ± 0.01 $^{g}$ | 0.55 ± 0.07 $^{ef}$ | 0.87 ± 0.08 $^{bc}$ | 0.05 ± 0.01 $^{h}$ | 0.66 ± 0.08 $^{de}$ | 0.37 ± 0.02 $^{fg}$ | 1.03 ± 0.08 $^{b}$ |
| *C16:1 t9* | 0.21 ± 0.01 $^{a}$ | 0.01 ± 0.00 $^{gh}$ | 0.04 ± 0.00 $^{cd}$ | 0.05 ± 0.00 $^{bc}$ | 0.00 ± 0.00 $^{gh}$ | 0.05 ± 0.00 | 0.05 ± 0.00 $^{bc}$ | 0.02 ± 0.00 $^{fg}$ | 0.03 ± 0.00 $^{de}$ | 0.05 ± 0.01 $^{bc}$ | 0.00 ± 0.00 $^{h}$ | 0.04 ± 0.00 $^{cd}$ | 0.02 ± 0.00 $^{ef}$ | 0.06 ± 0.00 $^{b}$ |
| *C17:1 c10* | nd | 0.01 ± 0.00 $^{g}$ | 0.13 ± 0.00 $^{bcd}$ | 0.13 ± 0.00 $^{bcd}$ | 0.00 ± 0.00 $^{g}$ | 0.14 ± 0.00 $^{abc}$ | 0.15 ± 0.00 $^{abc}$ | 0.05 ± 0.00 $^{f}$ | 0.10 ± 0.01 $^{de}$ | 0.15 ± 0.02 $^{ab}$ | 0.00 ± 0.00 $^{g}$ | 0.11 ± 0.01 $^{cd}$ | 0.06 ± 0.00 $^{ef}$ | 0.18 ± 0.01 $^{a}$ |
| *C18:1 c9* | 160.58 ± 1.91 $^{a}$ | 1.83 ± 0.40 $^{g}$ | 31.62 ± 0.31 $^{bc}$ | 33.45 ± 0.62 $^{b}$ | 0.92 ± 0.09 $^{g}$ | 36.12 ± 0.14 $^{b}$ | 37.40 ± 0.63 $^{b}$ | 13.67 ± 0.61 $^{ef}$ | 23.47 ± 2.95 $^{cd}$ | 37.14 ± 3.56 $^{b}$ | 2.07 ± 0.25 $^{fg}$ | nd | 15.95 ± 0.78 $^{de}$ | 15.95 ± 0.78 $^{de}$ |
| *C18:1 c11* | 7.82 ± 0.06 $^{b}$ | 0.09 ± 0.02 $^{c}$ | 1.52 ± 0.03 $^{bc}$ | 1.60 ± 0.05 $^{bc}$ | 0.06 ± 0.03 $^{c}$ | 1.77 ± 0.01 $^{bc}$ | 1.83 ± 0.04 $^{bc}$ | 0.66 ± 0.03 $^{c}$ | 1.16 ± 0.15 $^{c}$ | 1.82 ± 0.18 $^{bc}$ | 0.08 ± 0.01 $^{c}$ | 29.26 ± 3.76 $^{a}$ | 0.79 ± 0.04 $^{c}$ | 30.06 ± 3.76 $^{a}$ |
| *C20:1 c9* | 0.75 ± 0.01 $^{a}$ | 0.03 ± 0.00 $^{ghi}$ | 0.14 ± 0.00 $^{cde}$ | 0.18 ± 0.00 $^{bc}$ | 0.02 ± 0.00 $^{hi}$ | 0.16 ± 0.00 $^{bcd}$ | 0.19 ± 0.00 $^{b}$ | 0.06 ± 0.00 $^{gh}$ | 0.11 ± 0.01 $^{ef}$ | 0.16 ± 0.02 $^{bcd}$ | 0.00 ± 0.00 $^{i}$ | 0.13 ± 0.02 $^{de}$ | 0.07 ± 0.00 $^{fg}$ | 0.21 ± 0.02 $^{b}$ |
| ***Total*** | 173.24 ± 2.01 $^{a}$ | | | 36.21 ± 0.67 $^{b}$ | | | 40.50 ± 0.70 $^{b}$ | | | 40.18 ± 3.87 $^{b}$ | 2.20 ± 0.27 $^{c}$ | | | 47.48 ± 3.82 $^{b}$ |
| **Polyunsaturated fatty acids (mg/ g DW)** | | | | | | | | | | | | | | |
| *C18:2 c9c12* | 14.73 ± 0.17 $^{a}$ | 0.16 ± 0.03 $^{h}$ | 2.93 ± 0.03 $^{cde}$ | 3.09 ± 0.05 $^{cd}$ | 0.08 ± 0.01 $^{h}$ | 3.35 ± 0.01 $^{bcd}$ | 3.46 ± 0.05 $^{bc}$ | 1.27 ± 0.06 $^{g}$ | 2.16 ± 0.27 $^{ef}$ | 3.43 ± 0.33 $^{bcd}$ | 0.29 ± 0.05 $^{h}$ | 2.62 ± 0.34 $^{de}$ | 1.45 ± 0.08 $^{fg}$ | 4.07 ± 0.33 $^{b}$ |
| $\alpha$ *C18:3 c9c12c15* | 1.17 ± 0.03 $^{a}$ | 0.02 ± 0.00 $^{h}$ | 0.34 ± 0.00 $^{cde}$ | 0.36 ± 0.01 $^{cd}$ | 0.01 ± 0.00 $^{h}$ | 0.40 ± 0.00 $^{bc}$ | 0.41 ± 0.01 $^{bc}$ | 0.15 ± 0.01 $^{g}$ | 0.25 ± 0.03 $^{ef}$ | 0.40 ± 0.03 $^{bc}$ | 0.02 ± 0.00 $^{h}$ | 0.30 ± 0.03 $^{de}$ | 0.17 ± 0.01 $^{fg}$ | 0.47 ± 0.04 $^{b}$ |
| ***Total*** | 16.47 ± 0.19 $^{a}$ | | | 3.45 ± 0.06 $^{b}$ | | | 3.87 ± 0.06 $^{b}$ | | | 3.83 ± 0.36 $^{b}$ | 0.31 ± 0.05 $^{c}$ | | | 4.53 ± 0.37 $^{b}$ |
| **Polyphenolic compounds (mg/ 100 g DW)** | | | | | | | | | | | | | | |
| *Hydroxytyrosol glucoside* | 9.44 ± 1.28 $^{a}$ | 2.15 ± 0.42 $^{e}$ | 0.41 ± 0.05 $^{f}$ | 2.61 ± 0.40 $^{e}$ | 5.48 ± 0.26 $^{bc}$ | 0.26 ± 0.01 $^{f}$ | 5.74 ± 0.26 $^{b}$ | 4.30 ± 0.24 $^{cd}$ | 0.33 ± 0.04 $^{f}$ | 4.65 ± 0.27 $^{bc}$ | 3.17 ± 0.33 $^{de}$ | 2.37 ± 0.15 $^{e}$ | 0.14 ± 0.03 $^{f}$ | 2.49 ± 0.16 $^{e}$ |
| *Hydroxytyrosol* | 14.73 ± 0.94 $^{a}$ | 2.64 ± 0.39 $^{d}$ | 0.10 ± 0.01 $^{e}$ | 2.63 ± 0.43 $^{d}$ | 7.90 ± 0.81 $^{b}$ | 0.46 ± 0.09 $^{e}$ | 8.59 ± 0.71 $^{b}$ | 5.24 ± 0.26 $^{c}$ | 0.07 ± 0.01 $^{e}$ | 5.39 ± 0.41 $^{c}$ | 0.61 ± 0.12 $^{e}$ | 0.70 ± 0.13 $^{e}$ | 0.02 ± 0.00 $^{e}$ | 0.70 ± 0.13$^{e}$ |
| *Tyrosol glucoside* | 19.42 ± 2.50 $^{a}$ | 4.37 ± 0.91 $^{c}$ | 0.40 ± 0.02 $^{d}$ | 4.76 ± 0.88 $^{c}$ | 4.40 ± 0.88 $^{c}$ | 0.00 ± 0.00 | 4.40 ± 0.88 $^{c}$ | 4.64 ± 0.89 $^{c}$ | 0.55 ± 0.09 | 5.19 ± 0.82 $^{c}$ | 8.75 ± 1.82 $^{b}$ | 3.60 ± 0.67 $^{c}$ | 0.21 ± 0.03 $^{d}$ | 3.81 ± 0.69 $^{c}$ |
| *Tyrosol* | 20.11 ± 1.59 $^{a}$ | 8.16 ± 1.22 $^{c}$ | 0.77 ± 0.16 $^{f}$ | 8.84 ± 1.30 $^{c}$ | 12.37 ± 2.25 $^{b}$ | 1.15 ± 0.00 $^{def}$ | 13.39 ± 2.19 $^{b}$ | 7.69 ± 0.51 $^{c}$ | 0.77 ± 0.21 $^{f}$ | 8.46 ± 0.46 $^{c}$ | 9.32 ± 1.70 $^{c}$ | 4.42 ± 0.85 $^{de}$ | 0.94 ± 0.00 $^{ef}$ | 4.73 ± 0.61 $^{d}$ |
| *Caffeic acid* | 3.03 ± 0.35 $^{a}$ | 1.23 ± 0.23 $^{bc}$ | 0.07 ± 0.01 $^{g}$ | 1.30 ± 0.22 $^{b}$ | 0.79 ± 0.06 $^{d}$ | 0.41 ± 0.06 $^{ef}$ | 1.21 ± 0.10 $^{bc}$ | 0.90 ± 0.07 $^{cd}$ | 0.07 ± 0.01 $^{g}$ | 0.97 ± 0.07 $^{bcd}$ | 0.74 ± 0.09 $^{de}$ | 0.10 ± 0.02 $^{fg}$ | 0.00 ± 0.00 $^{g}$ | 0.10 ± 0.02 $^{fg}$ |
| *p-coumaric acid* | 4.04 ± 0.55 $^{a}$ | 1.01 ± 0.09 $^{cde}$ | 0.15 ± 0.02 $^{g}$ | 1.17 ± 0.10 $^{cd}$ | 0.79 ± 0.13 $^{def}$ | 0.48 ± 0.05 $^{fg}$ | 1.27 ± 0.16 $^{c}$ | 2.12 ± 0.26 $^{b}$ | 0.11 ± 0.09 $^{g}$ | 2.22 ± 0.26 $^{b}$ | 0.51 ± 0.09 $^{efg}$ | 0.88 ± 0.06 $^{cdef}$ | 0.02 ± 0.00 $^{g}$ | 0.89 ± 0.06 $^{cdef}$ |
| *Luteolin* | 15.34 ± 1.00 $^{a}$ | 0.17 ± 0.02 $^{d}$ | 1.97 ± 0.11 $^{bc}$ | 2.07 ± 0.16 $^{bc}$ | 0.19 ± 0.04 $^{d}$ | 2.52 ± 0.27 $^{b}$ | 2.60 ± 0.26 $^{b}$ | 0.32 ± 0.05 $^{d}$ | 2.19 ± 0.25 $^{bc}$ | 2.51 ± 0.21 $^{b}$ | 0.00 ± 000 | 0.00 ± 0.00 | 1.66 ± 0.25 $^{c}$ | 1.66 ± 0.25 $^{c}$ |

Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$). PF – pellet fraction; SF - soluble fraction; DW – dry weight. *C14:0 – Myristic acid;* C16:0 – Palmitic acid; C18:0 – Stearic acid; C20:0 – Arachidic acid; C16:1 *c*9 – Palmitoleic acid; C16:1 *t9* - trans-palmitoleic acid; C17:1 *c10* - cis-10-heptadecenoic acid; C18:1 *c9* – Oleic acid; C18:1 *c11* - cis-Vaccenic acid; C20:1 *c9* - cis-Eicosanoid acid; C18:2 *c9c12* – Linoleic acid; $\alpha$ C18:3 *c9c12c15* - $\alpha$-linolenic acid.

Nutritional quality indices (PUFA/SFA, SI, AI and TI) of POPP throughout SGD are shown in Table 6.4. To the authors' knowledge, this is the first time that effect of SGD on these indices have been calculated to potential functional ingredients. Alba *et al.* (2019) explained that AI and TI measure the influence of diet on coronary heart disease. AI relates the risk of atherosclerosis and is based on UFAS (C12:0, C14:0 and C16:0) that can increase or UFAS that can decrease (∑MUFA, ∑PUFA) the level of blood cholesterol. TI values relate to the tendency to form clots in the blood vessels, defined as the relationship between the pro-thrombogenic (SFAs) and the anti-thrombogenicity acids (MUFAs, n-6 PUFAs and n-3 PUFAs). In the present study, the low AI and TI values of POPP were maintained throughout SGD (Table 6.4). Low values for AI and TI are recommended, indicating POPP positive health benefits. POPP revealed similar AI (0.18 ± 0.00), and TI (0.39 ± 0.00) values to the values reported to olive oil (AI =0.14 and 0.32) (Alba *et al.*, 2019). Other good indicators of the nutritional value of dietary fat are PUFA/SFA ratio and SI index (Table 6.4). PUFA/SFA ratio in the human diet should be above 0.45. The SI indicates the relationship between the sum of SFAs (pro-thrombogenic) and UFAs (anti-thrombogenic). There are no numerical values assigned to SI, but food with lower values of these SFAs compared to UFAs would be considered healthier food (Alba *et al.*, 2019). SGD modified PUFA/SFA and SI positively and significantly ($p < 0.05$). PUFA/SFA ratio changed from the initial 0.43 ± 0.00 to 0.57 ± 0.01 on the absorbable fraction (IN) and SI kept a low value throughout SGD (0.20 - 0.22).

The higher UFAs proportion and the low values of AI, TI, SI and PUFA/SFA above 0.45 detected in absorbable fraction (IN) after SGD makes POPP a highly advisable food or nutraceutical ingredient from a nutritional standpoint associated to health benefits as reduction of the risk of cardiovascular disease, hypertension or general inflammation even more after SGD (Lopez-Huertas, 2010).

**Table 6.4. Nutritional value of POPP fatty acid profile throughout the SGD.**

| | Initial | Oral | Gastric | Intestinal | IN | OUT |
|---|---|---|---|---|---|---|
| *PUFA/SFA* | 0.43 ± 0.00 $^{b}$ | 0.42 ± 0.00 $^{b}$ | 0.43 ± 0.00 $^{b}$ | 0.41 ± 0.00 $^{b}$ | 0.57 ± 0.01 $^{a}$ | 0.42 ± 0.03 $^{b}$ |
| *TI* | 0.39 ± 0.00 $^{a}$ | 0.39 ± 0.00 $^{a}$ | 0.38± 0.00 $^{a}$ | 0.41 ± 0.00 $^{a}$ | 0.40 ± 0.02 $^{a}$ | 0.40 ± 0.02 $^{a}$ |
| *AI* | 0.18 ± 0.00 $^{a}$ | 0.18 ± 0.00 $^{a}$ | 0.17± 0.00 $^{a}$ | 0.18 ± 0.02 $^{a}$ | 0.18 ± 0.01 $^{a}$ | 0.18 ± 0.01 $^{a}$ |
| *SI* | 0.20 ± 0.00 $^{a}$ | 0.21 ± 0.00 $^{a}$ | 0.20 ± 0.01 $^{a}$ | 0.21 ± 0.00 $^{a}$ | 0.22 ± 0.01 $^{a}$ | 0.21 ± 0.01 $^{a}$ |

PUFA/SFA: polyunsaturated fatty acids/saturated fatty acids. SI: saturation index. AI: index of atherogenicity. TI: index of thrombogenicity. Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

### 6.3.2.3. Phenolic compounds

Table 6.2 showed BI and RI of TPC obtained for POPP after SGD phases. All digestion phases revealed a significant decrease in TPC amount ($p < 0.05$) of POPP (Table 6.2). This negative effect of SGD on olive phenolics was also observed in other studies with table olives (Fernández-Poyatos *et al.*, 2019). Fernández-Poyatos *et al.* (2019) reported a loss of approximately 75% of the phenolic

content after SGD. The mouth step lowered TPC recovery (RI = 37.52%), but the gastric digestion increased TPC recovery (RI = 77.11%) from POPP. The higher amount of TPC in the stomach phase could be related to the breakdown of bound phenolic compounds from macromolecules, evidencing the phenolics' carriers role of dietary fibre, lipids and protein (Jakobek, 2015). The stomach's acidic pH also explains this higher release and stability of phenolics (Jakobek and Matić, 2019; Seiquer *et al.*, 2015). On the other hand, the alkaline conditions of intestine influenced TPC recovery (RI = 46.08%) significantly and even more, during intestinal absorption phase (RI ≈ 14% for IN and OUT fraction) (Seiquer *et al.*, 2015). The great and lower stability of olive phenolics to gastric and intestinal digestion respectively were reported in other studies with table olives (Fernández-Poyatos *et al.*, 2019), olive leaf (González *et al.*, 2019) and olive oil (Seiquer *et al.*, 2015). These studies suggested that a proportion of TPC could be transformed during digestion into different structural forms with different chemical properties and bioaccessibility, especially after the intestinal phase (Seiquer *et al.*, 2015). These results supported the evidence that the gastrointestinal tract can simultaneously act as a releaser and damaging agent of phenolics (Gouw *et al.*, 2017).

A significant loss of phenolics occurred throughout SGD of POPP, but at least 50% of the TPC amount that reaches the intestine were bioaccessible (BI = 51.39 ± 3.34%) to be absorbed, metabolised and exert their potential beneficial effects as shown in Table 6.2 (Lucas-Gonzalez *et al.*, 2016). Similar results of olive phenolics bioaccessibility (> 50%) were reported for olive leaf (González *et al.*, 2019) and olive oil (Seiquer *et al.*, 2015). The detection of higher TPC content on the soluble (SF) than in pellet fraction (PF) also demonstrated that POPP phenolics were released from a solid matrix and available to be metabolised. A similar TPC proportion on SF and PF fractions was described to other by-products' powders (Lucas-Gonzalez *et al.*, 2016).

Individual phenolics of POPP before and after SGD were identified using LC-ESI-UHR-QqTOF-MS analyses (Table 6.5). The main individual phenolics were quantified by HPLC-DAD (Table 6.3). Regarding individual phenolics, HYD, tyrosol and its derivatives were the predominant phenolics. Correlations between individual phenolics and TPC (Supplementary Material) validated the majority role of HYD glucoside ($r^2$ = 0.90), HYD ($r^2$ = 0.95) and tyrosol ($r^2$ =0.87) among all phenolics detected on POPP throughout SGD.

SGD negatively affected all phenolics, principally in the mouth step (RI varies between 14 – 50%) (Table 6.2). Similar results were reported in the literature that estimates that 90% of phenolics are digested before reaching the intestine (Costa *et al.*, 2019). An interdependent relation could also be expected between phenolics and lipids. Not only the lipids could protect the phenolics, but also phenolics could create a positive antioxidant environment or react with harmful products of the lipid peroxidation to neutralise them (Gorelik *et al.*, 2013).

Tyrosol was the most abundant and the most stable POPP phenolic throughout SGD (Table 6.3). After the significant loss on the mouth (RI = 49.90%), the RI value variation for each digestion was minimal (50-40%) as presented in Table 6.2. The good stability of tyrosol has been reported in previous works. Tyrosol has less tendency to react with other medium macromolecular components and remains stable during the digestion with no dramatic changes in its structure and properties

(Dinnella *et al.*, 2007). On the other hand, HYD underwent more changes throughout the SGD (Table 6.2). Despite the significant loss in the mouth (RI=17.88%), the HYD amount increased under the stomach's acidic conditions (RI=58.33%). However, during the intestinal absorption step, HYD concentration declined substantially, which may be attributed to its high instability under alkaline conditions (González *et al.*, 2019). Nevertheless, its glucosidic form (HYD glucoside) increased during the intestinal absorption phase. Tyrosol glucoside also increased in this step. LC-ESI-UHR-QqTOF-MS confirmed the presence of HYD glucoside (m/z 315.1085) and tyrosol glucoside (m/z 299.1139). The higher release of these glucosidic compounds during intestinal absorption could be related to the action of α-amylase present in the pancreatin extract used in SGD. This pancreatin is an extract from the porcine pancreas composed of different enzymes, classified as proteolytic, lipolytic, amylolytic, and nucleic acid splitting enzymes. α-Amylase (EC 3.2.1.1), the main amylolytic enzyme in pancreatin, is an endohydrolase specific for α-(1→4) glycosidic bonds (Singh *et al.*, 2014).

Regarding absorbable fraction (IN), tyrosol and its glucoside exhibited the highest RI values (45.39% and 48.81%, respectively) and elevated BI (63.06% and 69.36%, respectively) as can be seen in Table 6.2. Tyrosol and its glucoside presented several potential health benefits. Tyrosol glucoside is suggested to act as an anti-ageing, anti-inflammatory and anticancer compound, mostly due to its role as an adaptogen, i.e., a biologically-active compound that is supposed to increase resistance in humans to different stress-related disorders and other diseases. Tyrosol was shown to inhibit the oxidation of low-density lipoprotein (LDL) and prevent the risk of reactive oxygen metabolite-mediated diseases inhibiting leukocyte 5-lipooxygenase (Dinnella *et al.*, 2007). Other POPP phenolic with higher BI (87.68%) that also possess a potential inhibition action on leukocyte 5-lipooxygenase was caffeic acid. Caffeic acid is known to exhibit antimutagenic, carcinogenic and antioxidant activities *in vitro* (Sato *et al.*, 2011).

Luteolin and *p*-coumaric acid were detected in significant amounts in the non-absorbable fraction (OUT) due to its low BI values (0.00 and 35.20%, respectively). More than 30% of the initial amount of luteolin and *p*-coumaric acid were available in the colon, where they potentially could be metabolised by the microflora, increasing their presumed biological activity (Table 6.2). Luteolin has been pointed out as a potent intestinal anti-inflammatory agent by different mechanisms using *in vitro* gut inflammation models (Mizuno and Nishitani, 2013). Tyrosol, tyrosol glucoside and HYD glucoside were also present in significant amounts in OUT fraction (more than 70% of the initial amount of these compounds present on POPP), where they could also encourage the growth of healthy bacteria (Liu *et al.*, 2019), act as an anti-inflammatory agent on the gut (González-Sarrías *et al.*, 2017) and protecting the Caco-2 intestinal mucosal cells against the cytostatic and cytotoxic effect of oxidised LDL (Bonechi *et al.*, 2019).

**Table 6.5. Retention time, maximum wavelength absorbance and MSn fragmentations profiles of phenolic compounds identified in POPP through in vitro simulated gastrointestinal digestion.**

| Compound | Molecular formula | RT (min) | $\lambda_{max}$ | m/z calcd | m/z exptl | Err [mDa] | mSigma | Major fragments ESI negative MS/MS ions |
|---|---|---|---|---|---|---|---|---|
| **Protocatechuic acid** [*3] | $C_7H_6O_4$ | 6.8 | 203 | 153.0197 | 153.0193 | -0.4 | 7 | 153.0197; 109.0298 |
| **Hydroxytyrosol glucoside**[*2] | $C_{14}H_{20}O_8$ | 7.1 | 279 | 315.1088 | 315.1085 | -0.2 | 4 | 315.1092; 153.0556; 123.0451 |
| **Hydroxylated product of the dialdehydic form of decarboxymethyl-elenolic acid** [*1] | $C_9H_{12}O_5$ | 7.2 | 197/279 | 199.0615 | 199.0612 | -0.3 | 9.6 | 198.5356; 111.0814; 123.0453; 97.0296 |
| **Vanillin** [*1] | $C_8H_8O_3$ | 7.3 | 230 | 151.0401 | 151.0399 | 0.1 | 5.6 | 123.0450; 109.0291; 151.0401 |
| **Hydrated product of the dialdehydic form of decarboxymethyl-elenolic acid**[*2] | $C_9H_{14}O_5$ | 7.3 | 197/281 | 201.077 | 201.0768 | -0.2 | 4 | 201.0405; 153.0553; 123.0449; 95.0502 |
| **Hydroxytyrosol** [*1] | $C_8H_{10}O_3$ | 7.4 | 280 | 153.0556 | 153.0557 | 0.1 | 5.7 | 153.0452; 123.0457 |
| **Loganin**[*2] | $C_{17}H_{26}O_{10}$ | 7.6 | 281 | 389.1455 | 389.1453 | -0.2 | 8.9 | 151.0763; 113.0244; 101.0244 |
| **Oleoside derivative isomer**[*2] | $C_{17}H_{28}O_{11}$ | 7.7 | 228 | 407.1557 | 407.1559 | -0.5 | 11.7 | 151.0760; 119.0346 |
| **Decarboxylated form of hydroxy-elenolic acid**[*2] | $C_{10}H_{14}O_5$ | 7.9 | 197/280 | 213.0765 | 213.0768 | 0.1 | 5.6 | 213.0920; 137.0601; 121.0665; 111.0085 |
| **Tyrosol glucoside**[*3] | $C_{14}H_{20}O_7$ | 8.1 | 227/276 | 299.1139 | 299.1136 | -0.3 | 3.2 | 119.0505; 137.0244; 135.0299 |
| **Oleoside**[*2] | $C_{16}H_{22}O_{11}$ | 8.4 | 277 | 389.1093 | 389.1089 | -0.4 | 4.3 | 389.1088; 183.0664; 165.0557 |
| **Caffeic acid** [*1] | $C_9H_8O_4$ | 9.3 | 323 | 179.0350 | 179.0350 | -0.1 | 7.9 | 179.0350; 135.0448 |
| **10-Hydroxyloganin** [*2] | $C_{17}H_{26}O_{11}$ | 9.5 | 197 /278 | 405.1402 | 405.1406 | -0.4 | 5.7 | 405.1402; 165.0557; 139.0035; 123.045 |
| **Tyrosol** [*1] | $C_8H_{10}O_2$ | 9.8 | 277 | 137.0608 | 137.0608 | 0.5 | 4.5 | 111.0084; 95.0510 |
| **Aldehydic form of decarboxymethyl-elenolic acid** [*2] | $C_{10}H_{16}O_5$ | 10.1 | 198/283 | 215.0927 | 215.0925 | -0.2 | 5 | 215.0923; 171.1025; 153.0918; 141.0917; 125.0696 |
| **Coumaric acid** [*1] | $C_9H_8O_3$ | 10.6 | 309 | 163.0403 | 163.0401 | -0.2 | 10.5 | 163.0397; 119.0499 |
| **Caffeoyl-6-secologanoside**[*3] | $C_{25}H_{28}O_{14}$ | 10.8 | 221/326 | 551.1407 | 551.1406 | 0 | 18.1 | 551.1416; 161.0245; 507.1504 |
| **hydroxylated form of elenolic acid**[*2] | $C_{11}H_{14}O_7$ | 10.9 | 280/320 | 257.0666 | 257.0667 | 0.1 | 2.7 | 257.1271; 181.0505; 137.0603; 109.0655; 95.0498 |
| **Luteolin – 7 – *O* – glucoside** [*1] | $C_{21}H_{20}O_{11}$ | 12.0 | 237/266/352 | 447.0928 | 447.0933 | 0.5 | 0.5 | 447.0924; 285.0399 |
| **Elenolic acid** [*2] | $C_{11}H_{14}O_6$ | 12.5 | 197/288 | 241.0720 | 241.0718 | -0.2 | 0.5 | 241.0737; 139.0035; 127.0398; 111.0086; 95.0551 |
| **Comsegoloside**[*3] | $C_{25}H_{28}O_{13}$ | 12.6 | 223/311 | 535.1462 | 535.1457 | -0.5 | 6.4 | 535.1465; 145.0296; 491.1558 |
| **Luteolin** [*1] | $C_{15}H_{10}O_6$ | 15.9 | 268/349 | 285.0406 | 285.0405 | -0.1 | 0.8 | 285.0414; 151.0037 |
| **Quercetin** [*1] | $C_{15}H_{10}O_7$ | 15.0 | | 301.0357 | 301.0354 | -0.3 | 3.4 | 301.0359; 151.0035; 178.9988 |
| **Apigenin** [*1] | $C_{15}H_{10}O_5$ | 18.0 | 268/338 | 269.0460 | 269.0455 | -0.5 | 4.8 | 269.0461; 151.0035 |

[*1] Hydroxytyrosol, tyrosol, luteolin-7-O-glucoside, luteolin, quercetin, apigenin, vanillin, caffeic acid and coumaric acid were identified by comparison with the standards. The other compounds were tentatively identified by LC-ESI-UHR-QqTOF-MS based on accurate mass, isotope rate calculations designated mSigma and literature.[*2] Lozano-Sánchez *et al.*, (2013), [*3] Jerman Klen & Mozetič Vodopivec (2012).

### 6.3.2.4. Antioxidant capacity: ABTS, DPPH e ORAC

The AOX of POPP was negatively influenced by SGD (Figure 6.2). At the end of the SGD, the AOX measured by ABTS, DPPH and ORAC were lower than the initial AOX of POPP. DPPH /ABTS assays are examples of electron transfer methods, and ORAC is a hydrogen atom transfer (HAT) method. DPPH was more efficient to measure AOX of less polar compounds, due to its solubilisation only in organic media (Arnao, 2000). On the other hand, the ORAC peroxyl free radical from ROS generator AAPH ((2,2′-azobis(2-methylpropionamidine) dihydrochloride)) only react with water and lipid-soluble substances (Tabart *et al.*, 2010).

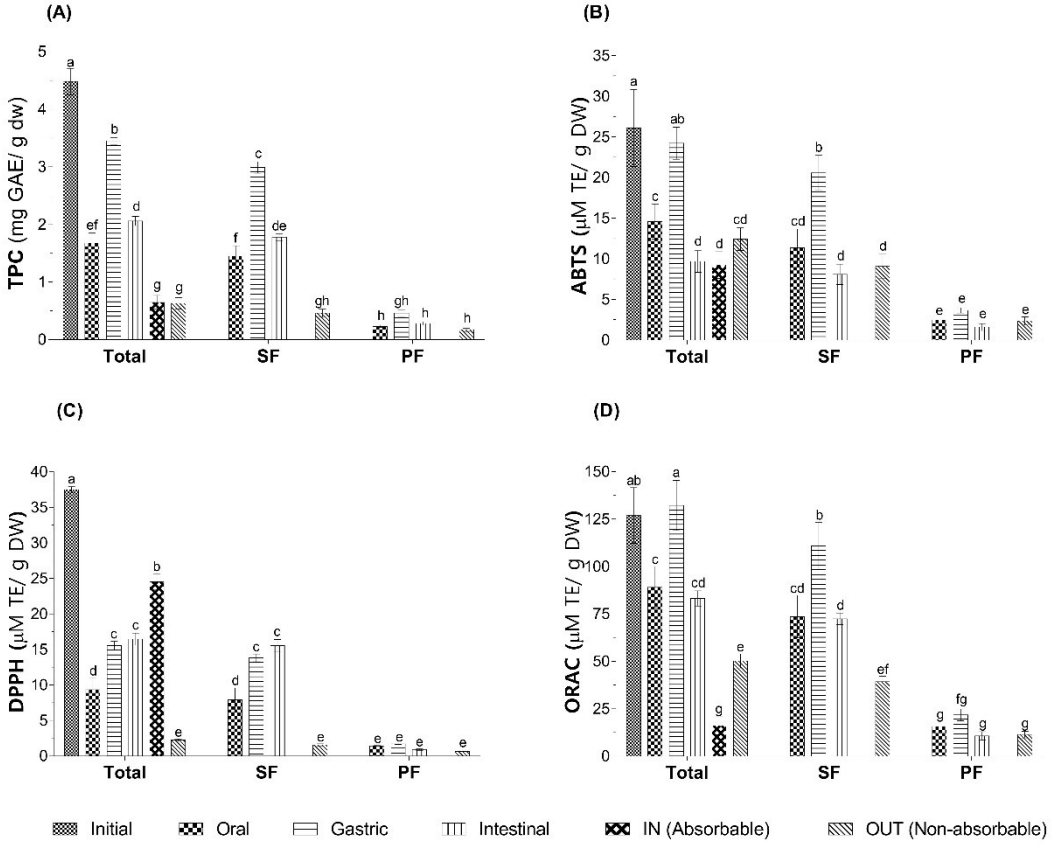



**Figure 6.2. Effect of in vitro gastrointestinal digestion on POPP total phenolic compounds (TPC) and antioxidant properties after each step of in vitro gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT). (A) TPC (mg GAE/g DW). (B) Antioxidant capacity measured by ABTS (µM TE/g DW). (C) Antioxidant capacity measured by DPPH (µM TE/g DW). (D) Antioxidant capacity measured by ORAC (µM TE/g DW).**
PF – pellet fraction; SF - soluble fraction; DW – dry weight; GAE - gallic acid equivalents; TE – Trolox equivalents Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

The correlation between the different AOX methods could be an excellent approach to help the evaluation of the results (Table 6.6). Usually, ABTS and DPPH exhibit a strong correlation; however, POPP's less polar nature (generous fat content) could provoke different behaviour between these methods ($r^2$ =0.53). Similar low correlation ($r^2$ =0.69) between ABTS and DPPH were also reported to 33 fruits after SGD (Chen *et al.*, 2014). ABTS was more correlated with ORAC ($r^2$ =0.78). DPPH

allowed validating the role of the less – polar composition of POPP and ABTS/ORAC evaluated more the potential of POPP soluble compounds by two different AOX mechanisms.

Phenolics were known to their AOX (Gullon *et al.*, 2015; Lucas-Gonzalez *et al.*, 2016), so Pearson's correlation coefficients between the TPC/ individual phenolics amount and their AOX were analysed (Table 6.6). The phenolics' contribution to AOX was supported by the good correlations between TPC and AOX. ORAC exhibited a better correlation with TPC ($r^2$ = 0.86) than ABTS ($r^2$ = 0.76) and DPPH ($r^2$ = 0.75). These good correlations of all AOX methods validated the importance of POPP phenolics as antioxidant compounds. These results are in agreement with several previous studies that reported high correlations between phenolics and AOX throughout SGD including other by-products powders and extracts as pomegranate peel flour (Gullon *et al.*, 2015) and *Cinnamomum camphora* seed kernel extracts (Zhang *et al.*, 2020). FAs appeared to have a lower impact on the AOX, influencing in the same degree the DPPH ($r^2$ = 0.59) and ABTS ($r^2$ = 0.59 - 0.60) and even less ORAC ($r^2$ = 0.45-0.46).

**Table 6.6. Pearson correlation coefficient calculated among measured antioxidant activities and quantified chemical compounds.**

| | TPC | ABTS | DPPH | ORAC |
|---|---|---|---|---|
| *TPC* | - | 0.76 | 0.76 | 0.86 |
| *ABTS* | 0.76 | - | 0.53 | 0.78 |
| *DPPH* | 0.76 | 0.53 | - | 0.46 |
| *ORAC* | 0.86 | 0.78 | 0.47 | - |
| *Hydroxytyrosol glucoside* | 0.90 | 0.75 | 0.85 | 0.76 |
| *Hydroxytyrosol* | 0.95 | 0.70 | 0.80 | 0.75 |
| *Tyrosol glucoside* | 0.50 | 0.39 | 0.83 | 0.27 |
| *Tyrosol* | 0.87 | 0.74 | 0.75 | 0.65 |
| *Caffeic acid* | 0.76 | 0.55 | 0.88 | 0.56 |
| *p-Coumaric acid* | 0.64 | 0.41 | 0.83 | 0.46 |
| *Luteolin* | 0.48 | 0.25 | 0.66 | 0.15 |
| *Glucose* | 0.64 | 0.43 | 0.77 | 0.45 |
| *Mannitol* | 0.61 | 0.42 | 0.84 | 0.36 |
| *Fructose* | -0.04 | -0.04 | 0.16 | -0.03 |
| *Formic acid* | 0.27 | 0.09 | 0.49 | 0.14 |
| *Total SFA* | 0.51 | 0.59 | 0.59 | 0.45 |
| *Total MUFA* | 0.52 | 0.60 | 0.59 | 0.46 |
| *Total PUFA* | 0.52 | 0.60 | 0.59 | 0.46 |

During SGD, the mouth phase changed more profoundly the AOX of POPP (Table 6.2). Only 19.29%, 52.90% and 70.22% of initial DPPH, ABTS and ORAC value of POPP were recovered after oral digestion, respectively. In the stomach phase, AOX increased again as a result of phenolics liberation in the gastric phase (higher TPC); principally in the AOX measured by ABTS (RI = 92.84%) and ORAC (RI=104.14%). This AOX increase in the stomach supported the importance of bound phenolic compounds linked to macromolecules to the high AOX potential of POPP (Jakobek, 2015; Silva *et al.*, 2018). The phenolics influence on AOX was also noticed in the intestine, where the lower amount of phenolics decreased AOX (Figure 6.2). Besides the higher and lower amount of phenolics in stomach and intestine, respectively reported in Table 2, the pH changes that occur during the SGD

could be another factor that probably affected phenolics' reactivity (creation of enantiomers with different biological reactivity). Thus, phenolics have been reported as more reactive, i.e. antioxidants, at the stomach step's acidic pH than at the intestinal pH close to neutrality (Gullon *et al.*, 2015).

The RI values of ABTS and ORAC reported in stomach decreased significantly to about half in the intestine (37.12% and 65.61%, respectively) ($p < 0.05$). Only DPPH values did not exhibit significant differences between the gastric and intestinal steps. This stability of DPPH from the stomach to intestine digestion could be associated with the increase of the amount of *p*-coumaric acid and stability of HYD, tyrosol, tyrosol glucoside and caffeic acid (Table 6.3). DPPH revealed reliable and better correlations ($r^2$ between 0.75 and 0.88) with these phenolics than ABTS and ORAC and luteolin only exhibited a good correlation with DPPH ($r^2 = 0.66$). Nevertheless, between all phenolics analysed, HYD ($r^2 = 0.75$) and its glucoside ($r^2 = 0.76$) showed to be the compounds with better correlation with ORAC and their significant loss during the intestinal absorption (Table 6.3) could explain the significant decrease of AOX measured by ORAC to the absorbable fraction (IN) of POPP (Figure 6.2). The good correlation between DPPH and tyrosol and its derivative could also validate the higher DPPH value of absorbable fraction (IN).

Regardless of the AOX loss throughout SGD, a significant AOX was retained in bioaccessible fraction (IN) (Table 6.2). DPPH exhibited a higher amount of AOX kept in fraction IN (91.63%) followed by ABTS (43.00%) and ORAC (24.93%). The higher retention of AOX in absorbable fraction by DPPH may be related to the high recovery and bioaccessibility of phenolics as HYD glucoside, tyrosol, tyrosol glucoside and caffeic acid, but also mannitol which is also considered an antioxidant agent (Ribeiro *et al.*, 2020a). The good correlation between DPPH and tyrosol, its derivative and mannitol ($r^2 \geq 0.75$) validated this higher DPPH value. Borges *et al.* (2017) and Seiquer *et al.* (2015) described similar higher retention of AOX assessed by DPPH (> 90%) to the bioaccessible fraction (IN) of extra virgin olive oils.

As stated in previous works, the main POPP phenolics, i.e. tyrosol, HYD and its derivatives, are potent antioxidants that retain their biological activities after ingestion (Karković Marković *et al.*, 2019). Besides that, tyrosol and its derivatives are characterised by a lower AOX than HYD (González *et al.*, 2019). However, the potential conversion of tyrosol into HYD reported *in vivo* in humans allowed to expect an AOX higher than the reported (Boronat *et al.*, 2019). The HYD AOX is very strong due to its potential as free radical-scavenger, metal-chelator and activator of different cellular signalling pathways to increase the defences against oxidative stress (Karković Marković *et al.*, 2019).

The lower AOX retention in the absorbable fraction (IN) than in non-absorbable fraction (OUT) measured by ABTS and ORAC (Table 6.2) could be related to the affinity of these methods with more polar compounds. Nevertheless, this low bioaccessibility allowed obtaining an OUT fraction with significant AOX with potential health benefits to the gut (González-Sarrías *et al.*, 2017; Liu *et al.*, 2019).

### 6.3.3. Interactions between all bioactives throughout *in vitro* gastrointestinal digestion

A PCA was performed, reducing the multidimensional structure of the data and providing a two-dimensional map to explain the AOX variance of POPP observed throughout SGD. The scree plot of PCA analysis, the graph of the loadings plot and scores plot of different SGD phases impacting POPP are presented in Figure 6.3. The scree plot indicates that the first two principal components account for 73.1% of the total variance (PC1 = 60.1% and PC2 = 13.0%). Most of the variables were essential contributors to PC1 and PC2. A high cos2 indicates a good representation of the variable on the principal component. All variables exhibited a significant high cos2 value (higher than 0.6), except for ORAC that showed an intermediate cos2 value.

The most significant contributors to the PC1 were the amount of the individual phenolic (HYD glucoside, HYD, tyrosol, caffeic acid, p-coumaric acid, luteolin), TPC, DPPH, mannitol and glucose. As presented in the loadings plot shown in Figure 6.3**,** most parameters are positioned close to each other, indicating high positive correlations between them.

PCA shows that SGD has a significant effect on the bioactives composition and AOX of POPP. Before the digestion, POPP was positioned mainly on IV quadrant, and all steps of digested POPP were placed mostly on the III and II quadrants. Scores plot allows easy and quick insight into the effect of SGD upon POPP based on the most critical factor (PC1), which contributes with 60.0% of the total variance and contains information about TPC, AOX (mainly DPPH), main individual phenolics and sugars.

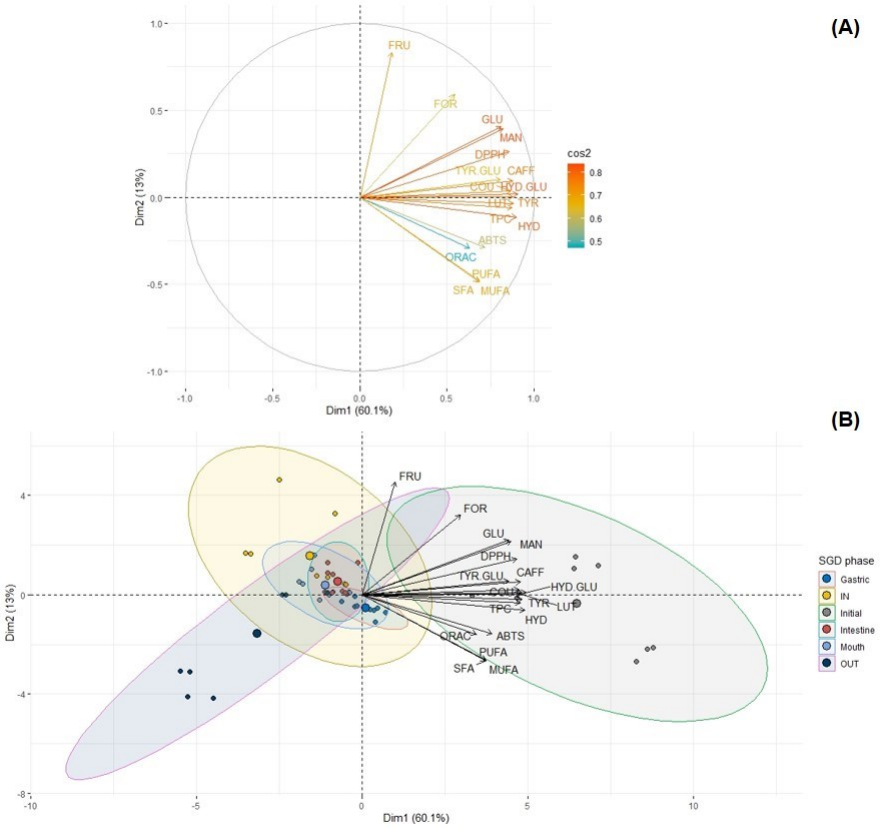



**Figure 6.3. PCA of POPP digestion. (A) Scree plot of the principal component analysis for POPP SGD phases. (B) Scores plot of different phases of the digestion of POPP.**

## 6.4. Conclusion

Based on the results obtained, it can be concluded that phenolics' behaviour during digestion is strongly correlated with POPP's potential biological effect. Despite the higher loss of phenolics at the beginning of digestion, the bound phenolics liberation in the stomach allowed to recover a significant amount of these antioxidant compounds. Dietary fibre and fatty acids appeared to act as phenolic carriers through *in vitro* tract with positive effects on the antioxidant potential of absorbable fraction and non-absorbable fraction. Besides, POPP showed to be an interesting dietary fibre source, which also provides a considerable amount of free and bound phenolics that reach the colon where they could exert potential health benefits (antioxidant, antimicrobial and anti-inflammatory activity). Dietary fibre also demonstrated a potential positive interaction with lipids, decreasing the bioaccessibility of saturated fatty acids and facilitating the absorption of the unsaturated fatty acids. PCA analysis allowed to validate the negative impact of digestion, principally in phenolics and antioxidant capacity. Notwithstanding the negative effect of digestion on POPP bioactive composition and antioxidant capacity, not only dietary fibre, phenolics and unsaturated fatty acids benefits were bioaccessible in a significant amount but also phenolics were retained in the colon where they could exert potential gut health benefits. Further studies need to be developed *in vitro as* transepithelial diffusion across intestinal (Caco-2) cell layers and *in vivo* or clinical trials, to validate the findings and implications resulting from the *in vitro* experiments described in the present work.

# Chapter 7.

# Prebiotic effects of olive pomace powders in the gut: *In vitro* evaluation of the inhibition of adhesion of pathogens, prebiotic and antioxidant effects

## Abstract

Olive pomace is a biowaste rich in phenolics and insoluble dietary fibre with high potential to develop new value chains towards a sustainable and circular bioeconomy. Regarding gut health, olive pomace phenolics and insoluble dietary fibre (after possible fermentation) could act as antioxidants, antimicrobial and prebiotic agents. The gut's potential beneficial effects were analysed for two powders from olive pomace: liquid-enriched powder (LOPP) - mostly source of phenolics - and pulp-enriched powder (POPP) - the main source of insoluble dietary fibre. LOPP and POPP were subjected to an *in vitro* simulated gastrointestinal digestion followed by *in vitro* faecal fermentation. The undigested fraction retained in the colon was analysed regarding its potential antioxidant, antimicrobial and prebiotic effects. LOPP and POPP did not impact the gut microbiota diversity negatively, showing a similar ratio of Firmicutes/Bacteroidetes compared to a positive control (fructooligosaccharides - FOS). LOPP exhibit a positive (similar to FOS) effect on the *Prevotella* spp./*Bacteroides* spp. ratio. Both powders promoted more the production of short-chain fatty acids (mainly acetate > butyrate > propionate) than FOS and also showed significant total phenolic content and oxygen radical absorbance capacity during faecal fermentation until 48 h. Besides that, these powders showed mucin-adhesion inhibition ability against pathogens, principally POPP against *Bacillus cereus* (22.03 ± 2.45%) and *Listeria monocytogenes* (20.01 ± 1.93%). This study demonstrates that olive pomace powders have prebiotic effects on microbiota, including the stimulation of short-chain fatty acids production, potential antioxidant and antimicrobial activity, which could improve the human gut health.

**Keywords:** olive pomace; biowaste, dietary fibre; prebiotic effect; antioxidant activity; gut health.

## 7.1. Introduction

Sustainable and functional ingredients have been developed from several food by-products. This trend towards a circular economy model has been rising as an opportunity to manage today's society's environmental and nutritional problems, also allowing the generation of economic gains for the food industry (Torres-León *et al.*, 2018). Presently, there is a stimulus to reduce the disposal of olive oil production by-products, specifically the olive pomace (OP), which represents the major environmental problem and cost of this industry (Conterno *et al.*, 2019).

OP has been extensively investigated to obtain new functional and bioactive ingredients due to its richness in olive oil phenolics (Nunes *et al.*, 2016). Recently, OP dietary fibre (DF) has also been explored (Galanakis, 2011; Ying *et al.*, 2017). However, until now, these functional ingredients have been developed involving the use of organic solvents or requiring higher operational costs and valorising only a small fraction of the OP. So, to fulfil this lack, we have developed a liquid-enriched olive pomace powder (LOPP) and pulp-enriched olive pomace powder (POPP). These OP powders have been described as important DF sources, olive oil phenolics and soluble sugars (Ribeiro *et al.*, 2020a). If LOPP is mainly a source of phenolics and sugars, POPP contains a significant amount of insoluble dietary fibre (IDF), mainly constituted by lignin and cellulose hemicellulose and bound phenolics associated with DF.

The main OP powder phenolics (hydroxytyrosol (HYD) and derivatives) were known to have several health benefits linked to cardiovascular disease prevention (Robles-Almazan *et al.*, 2018) (e.g. protectors from oxidative damage of low-density lipoprotein (LDL) particles (EFSA, 2011)). However, OP phenolics have also been described as antimicrobial agents against both Gram-positive/Gram-negative bacteria and stimulants of the growth of beneficial bacteria in the gut (Karković Marković *et al.*, 2019; Mosele *et al.*, 2014). Not only DF and its associated bound phenolics, often referred to as non-digestible polysaccharides, may remain unaltered until reaching the colon (Arranz *et al.*, 2010; Papillo *et al.*, 2014; Sato *et al.*, 2011), but also not all polyphenols are absorbed in the small intestine and could reach the colon (Mosele *et al.*, 2014). In the gut, DF and phenolics could be fermentable and undergo biotransformation by the gut microbiota (Lattimer and Haub, 2010; Williams *et al.*, 2017) increasing the beneficial bacteria content, the short-chain fatty acids (SCFAs) production and exerting other potential health benefits associated to phenolics action (Arranz *et al.*, 2010; Papillo *et al.*, 2014; Sato *et al.*, 2011).

The literature supported the prebiotic potential of OP powders. According to the most recent definition of ‘prebiotic’ proposed by ISAPP consensus panel as ‘a substrate that is selectively utilised by host microorganisms conferring a health benefit’, other substances such as polyphenols and not only the usual carbohydrate-based compounds, might fit the updated definition, assuming adequate evidence of health benefit for the target host (Gibson *et al.*, 2017). Phenolic compounds were pointed as potential prebiotic candidates, with enhancer benefits on microbiota composition, gut permeability and anti-inflammatory/immunity mechanisms (Papillo *et al.*, 2014; Williams *et al.*, 2017). However, far more studies in the target host are required to validate phenolic ‘prebiotic effects’ (Gibson *et al.*,

2017). In conclusion, prebiotics are compounds that have the potential to improve human health and reduce the risk of diseases mediated by microbiota dysbiosis (Gibson *et al.*, 2017).

The specific stimulation of the growth and/ or activity of intestinal bacteria associated with health and well-being was considered a 'prebiotic effect'. In this field, the sequencing techniques, especially 16S rRNA-based approaches, have been revolutionising the study of changes in human faecal microbial communities (Wang *et al.*, 2019). On the other hand, the release of SCFAs (acetate, propionate and butyrate) due to microbial fermentation of phenolics and DF, if measurable, as distinct from a control, could also constitute a 'prebiotic effect'. These SCFAs are metabolised by the colonic epithelium (butyrate), liver (propionate) and muscle (acetate), exerting different functions with an essential role in health and disease (Laparra and Sanz, 2010). At the same time, an increase in the antioxidant phenolic compounds generated as microbial metabolites could also be found with the potential to decrease the local oxidative stress. So, besides the 'prebiotic effects', phenolics could also exert potential antioxidant and/ or antimicrobial effects (against pathogens) on the gut.

The gastrointestinal tract impact on OP powders, regarding the recovery and bioaccessibility of bioactive compounds, has been assessed (Ribeiro *et al.*, 2020a). However, the potential beneficial effect on the gut of DF and phenolics (free and bound form) retained in significant amounts in the colon fraction has not been disclosed yet. Few studies have analysed the potential prebiotic and antioxidant impact on the gut microbiota of olive oil (Liehr *et al.*, 2017; Mosele *et al.*, 2014; Santos *et al.*, 2012) and olive leaf extracts (Aponte *et al.*, 2018; Haddadin, 2010), but until now the influence of OP on the composition of human gut microbiota was only assessed by Conterno *et al.*, (2019). In this study, the 16S rRNA gene analysis allowed to validate that OP incorporated into biscuits did not negatively impact the faecal microbiota's diversity. The effect of the OP oil as a dietary supplement on the rumen microbial community profile in Comisana ewes was also investigated decreasing the population of lipase-producing microorganisms (*Anaerovibrio* spp.), lipolysis rate and the concentration of polyunsaturated fatty acids involved in biohydrogenation (Mannelli *et al.*, 2018).

According to our knowledge, there are no studies where the OP food ingredients prebiotic potential was assessed by *in vitro* fermentation with faecal inocula. Therefore, for the first time, the present study intends to analyse the potential prebiotic effect of OP powders and their potential antioxidant and antimicrobial effects on gut health. Not only the OP powders capacity to modulate the gut microbiota composition and its metabolic activity were studied by a copy of the 16S rRNA gene of the bacterial population and quantifying the amount of SCFAs produced after the in vitro colonic fermentation, but also their ability to create an antioxidant environment in the gut by liberation of phenolic metabolites (LC-ESI-UHR-QqTOF-MS) and to inhibit the adhesion of pathogens (antiadhesion ability) were assessed. In short, a complete *in vitro* screen of the OP powders potential as prebiotics and gut-health benefits ingredients is aimed in this study.

## 7.2. Materials and methods

### 7.2.1. Preparation of olive pomace powders

OP was collected from an olive mill from Oliveira do Hospital, Portugal, mainly composed of olive cultivar Galega Vulgar (80% of the olive heritage). Samples from different olive oil production hours were taken and transported to the laboratory, where they were mixed. The homogenous sample was packed in polyethene flasks and kept in a freezer at - 80 ºC until use.

OP was fractionated by centrifugation (10,000 *g* for 10 min). The liquid fraction was freeze-dried (Telstar Lyo Quest HT 40) with 2% of mannitol (as a cryoprotectant and to prevent aggregation), and the powder obtained was denominated liquid-enriched olive pomace powder (LOPP). The solid fraction was oven-dried (90 °C, water activity < 0.4, 90 min), milled using a coffee grinder and sieved (mesh 40). All the pieces of stones were removed to obtain a potentially food-grade ingredient free of the small stones, which is a potential physical hazard. This fraction was denominated pulp-enriched olive pomace powder (POPP).

### 7.2.2. Simulated *in vitro* gastrointestinal digestion and large intestine fermentation model

POPP and LOPP *in vitro* simulations of gastrointestinal digestion (SGD) were performed according to the method that includes the dialysis process in order to simulate intestinal and blood absorption described by (Ribeiro *et al.*, 2020a). Mouth digestion was conducted with 0.6 mL of 100 U/mL α–amylase solution (Sigma) and incubation took place for 1 min, at 37 °C and 200 rpm. For gastric digestion, the pH was adjusted to 2.0 with concentrated HCl (1 mol $L^{-1}$), and the mixture was incubated with 25 mg/mL of pepsin (from porcine stomach mucosa, pepsin A 250 U/mg, Sigma), at a rate of 0.05 mL/mL of sample, in a shaking bath, for 60 min at 37 °C. Intestinal digestion was performed by adjusting pH to 6.0 with $NaHCO_3$ (1 mol $L^{-1}$), before the addition of 2 g/L of pancreatin (from porcine pancreas 8 x USP, Sigma) and 12 g/L of bile salts (Sigma), at a ratio of 0.25 mL/mL of sample, and further incubation of the mixture for an additional 120 min at 37 °C. In the last phase of intestinal digestion, a segment (10 cm) of dialysis tubing (3.5 kDa molecular weight cut-off) filled with $NaHCO_3$ (1 mol $L^{-1}$) was placed inside of screw-topped bottles filled with digested samples and incubated for 2 h in a shaking water bath, at 37 °C and 50 rpm.

At the end of the incubation process, the solution left outside the dialysis tubing (OUT) represented the non-absorbable sample (colon-available). The OUT sample was homogenised and partitioned in two portions. One portion was centrifuged for 12 min at 8000 *g* at 4 °C, yielding the soluble fractions and the pellet fractions. Soluble fractions were lyophilised and used to evaluate the antiadhesion ability. The other OUT portion was lyophilised and then exposed to faecal fermentation using fresh faecal inoculum (maintained under anaerobic conditions, for a maximum of 2 h before being used) obtained from five healthy donors (A-E, two men and three women, ages between 23 to 63 years old) according to the methodology developed by Gullon *et al.* (2015) and Gullón *et al.* (2014). The faecal inocula (FI) were prepared in an anaerobic workstation by diluting (100 g $L^{-1}$) the faecal matter in Reduced Physiological Salt solution (RPS) (constituted by 0.5 g $L^{-1}$ cysteine-HCl (Merck,

Darmstadt, Germany) and 8.5 g $L^{-1}$ NaCl (LabChem, Zelienople, USA) with a final pH value of 6.8 (10% $CO_2$, 5% $H_2$ and 85% $N_2$). The flasks containing 50 mL of Nutrient Base Medium were capped and autoclaved. The Nutrient Base Medium medium was comprised of 5.0 g $L^{-1}$ trypticase soy broth without dextrose (Fluka Analytical, St. Louis, Missouri, EUA), 5.0 g $L^{-1}$ bactopeptone (Becton Dickinson Biosciences, New Jersey, USA), 0.5 g $L^{-1}$ cysteine-HCl (Merck, Darmstadt, Germany), 1.0% (v/v) of salt solution A [100.0 g $L^{-1}$ $NH_4Cl$ (Merck, Darmstadt, Germany), 10.0 g $L^{-1}$ $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 10.0 g $L^{-1}$ $CaCl_2 \cdot 2H_2O$ (Carlo Erba, Chaussée du Vexin, France)], 1.0% (v/v) of trace mineral solution (ATCC, Virginia, USA), 0.2% (v/v) of salt solution B [200.0 g $L^{-1}$ $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany)] and 0.2% (v/v) of a 0.5 g $L^{-1}$ resazurin solution (Sigma-Aldrich Chemistry, St. Louis, USA). The medium final pH value was adjusted to 6.8 and was then bubbled with $N_2$ until it presented a translucent/yellowish colour.

Following sterilisation, and before adding the faecal inocula, the freeze-dried digested olive pomace powders biomass was added to the respective vessels at a final concentration of 20 g.$L^{-1}$. In other flasks, FOS (Nutripar, Matosinhos, Portugal) in the same concentration as a positive control were added. FOS are well-known prebiotics, which is often used to be positive controls in fermentation experiments *in vitro*. Flasks without substrate only NBS (60 mL) were used as blanks. Then, the atmosphere of each flask was refluxed with a gas mixture (10% $CO_2$, 5% $H_2$ and 85% $N_2$) sterilised using a 0.22 µm filter (Millipore, Burlington, USA) followed by inoculation at 2% (v/v) with FI and incubated for 48 h at 37 ºC under anaerobic atmosphere (10% $CO_2$, 5% $H_2$ and 85% $N_2$). Samples were collected after 0, 12, 24 and 48 h of incubation (4 mL) and the pH values were measured using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). The positive control and blank were respectively designated as FOS and Blank (only faecal inocula), while the powders obtained from the olive pomace digested were dubbed as POPP for pulp-enriched powder and LOPP for liquid-enriched powder. Afterwards, the samples were stored at -30 ºC until analysis. All the steps considered in this section were carried out inside an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

Aliquots (4 mL) of each sample collected were centrifuged for 6 min. The resulting pellet was used to extract the genomic DNA. On the other hand, the supernatants were used to evaluate SCFA production, the amount of total phenolic acids (TPC), identification of phenolic acids and its metabolites and even the potential antioxidant activity (AOX).

### 7.2.3. Bacterial population analysis

#### 7.2.3.1. DNA extraction and Real-Time Quantitative Polymerase Chain Reaction

DNA was extracted using an NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) according to the manufacturers' instructions, with some adaptations (Supplementary Material 7.2). Afterwards, copy numbers of the 16S rRNA gene from the *Firmicutes*, *Lactobacillus* spp., *Enterococcus* spp., *Clostridium leptum*, *Bacteroidetes*, *Bacteroides* spp., *Prevotella* spp., and *Bifidobacterium* spp. groups were determined using primers obtained from STABvida (Lisbon, Portugal), according to a real-time polymerase chain reaction (RT-PCR) using a CFX96 Touch™ Real-Time PCR Detection

System (Bio-Rad Laboratories, Inc., Hercules, USA), under conditions adapted from Marques *et al.* (2016).

Briefly, pellets were washed with TE (pH 8.0; Tris EDTA buffer), vortexed and centrifuged at 4000 g for 10 min, a process that was repeated until the supernatant was colourless. Then, 180 µL of a freshly prepared lysozyme solution (10 mg $mL^{-1}$ lysozyme in a NaCl-EDTA solution; 30 mmol $L^{-1}$ NaCl and 10 mM EDTA) was added and incubated for a period of 1 h, at 37 ºC, with periodic shaking. Afterwards, 350 µL of NT1- buffer were added to samples which were then vortexed and incubated at 95 ºC. After 10 min, samples were centrifuged (11000 *g*, 10 min, 4 ºC), supernatants (200 µL) were mixed with 25 µL of proteinase K, and incubate at 70 ºC during 10 min. The remaining steps were performed accordingly to the manufacturer's instructions. After extraction, DNA's purity and concentration were assessed using a Thermo Scientific™ µDrop™ Plate coupled with a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientifc, Waltham, USA).

The real-time polymerase chain reaction (RT-PCR) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) was developed under the conditions described in Table 7.1.

**Table 7.1. Real-time PCR conditions.**

| PCR stage | Temperature | Time | Number of Cycles |
|---|---|---|---|
| **Initial denaturation/ enzyme activation** | 95 ºC | 10 min | 45 |
| **Denaturation** | 95 ºC | 10 s | |
| **Annealing** | Specific temperature for each primer | 1 min | |
| **Extension *** | 72 ºC | 15 s | |
| **Melting curve*** | 60-97 ºC, with an increment of 0.5 ºC for 0.05 min | 0.05 s | |

*Stages in which fluorescence is measured.

The PCR reaction mixture comprised of 5 µL of 2x iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA), 2 µL of ultrapure water, 1 µL of sample DNA (equilibrated to 20 ng $µL^{-1}$) and 1 µL of forward and reverse primers (100 nmol $L^{-1}$) targeting the 16S rRNA gene. The primers used were obtained from STABvida (Lisbon, Portugal) and are listed in Table 7.2. Standard curves were constructed using tenfold dilutions (from 2 log to 6 logs of the number of copies of 16S rRNA gene $µL^{-1}$) of bacterial genomic DNA standards (DSMZ, Braunschweig, Germany) (Table 7.2) and were drawn using the number of copies of the 16S rRNA gene of each bacterial strain in relation to the quantification cycles (Cq). Melting curve analysis was performed for each PCR to evaluate the specificity of the amplification, considering a temperature interval from 60 to 97 ° C. All assays were performed in quadruplicate.

**Table 7.2. Primer sequences targeting bacterial groups, genomic DNA standards and PCR product size.**

| Target group | Primer sequence (5'-3') | Genomic DNA standard | PCR Product size | Annealing temperatures (ºC) |
|---|---|---|---|---|
| *Lactobacillus* | F GAG GCA GCA GTA GGG AAT CTT C<br>R GGC CAG TTA CTA CCT CTA TCC TTC TTC | *Lactobacillus gasseri* ATCC 33323 | 126 | 55 |
| *Bifidobacterium* | F CGC GTC YGG TGT GAA AG<br>R CCC CAC ATC CAG CAT CCA | *Bifidobacterium longum* subsp. Infantis ATCC 15697 | 244 | 50 |
| *Clostridium leptum* subgroup | F GCA CAA GCA GTG GAG T<br>R CTT CCT CCG TTT TGT CAA | *Clostridium leptum* | 239 | 45 |
| Firmicutes | F ATG TGG TTT AAT TCG AAG CA<br>R AGC TGA CGA CAA CCA TGC AC | *Lactobacillus gasseri* ATCC 33323 | 126 | 45 |
| Bacteroidetes | F CAT GTG GTT TAA TTC GAT GAT<br>R AGC TGA CGA CAA CCA TGC AG | *Bacteroides vulgatus* ATCC 8482 | 126 | 45 |
| Bacteroides | F ATA GCC TTT CGA AAG RAA GAT<br>R CCA GTA TCA ACT GCA ATT TTA | *Bacteroides vulgatus* ATCC 8482 | 495 | 45 |
| *Prevotella* | F CAC RGT AAA CGA TGG ATG CC<br>R GGT CGG GTT GCA GAC C | *Prevotella nigrescens (DSMZ 13386)* | 513 | 45 |
| *Enterococcus* | F CCC TTA TTG TTA GTT GCC ATC ATT<br>R ACT CGT TGT ACT TCC CT TGT | *Enterococcus gilvus (DSMZ 15689)* | *144* | 55 |

F- forward primer; R- reverse primer

### 7.2.4. Short-chain fatty analysis

Short-chain and branched-chain fatty analysis were determined in supernatants resulted from the faecal fermentation (Gullon *et al.*, 2015). The analyses were performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to IR and UV detector using Aminex 37-H column (Bio-rad, Berkeley, USA) at 55 °C and 35 mmol $L^{-1}$ $H_2SO_4$ as mobile phase (flow rate: 0.5 mL/min). The identification and quantification were achieved by comparing the relative retention times of sample peaks with standards and using a calibration curve (0.2-2.0 mg/mL).

### 7.2.5. Phenolic compounds analysis

#### 7.2.5.1. Total phenolic compounds and antioxidant activity

The total phenolic content (TPC) of supernatants were determined according to the Folin-Ciocalteu method (Oliveira *et al.*, 2016; Singleton and Rossi, 1965). Results were expressed as mg gallic acid equivalents (GAE)/100 g of dry weight (DW).

According to the ORAC method, the AOX of supernatants of each OP powder and controls throughout the faecal fermentation was achieved (Costa *et al.*, 2019) using a multidetection plate reader (Synergy H1, Vermont, USA). The radical stock solutions were freshly prepared. All analyses were performed in triplicate and expressed in mmol $L^{-1}$ of Trolox-equivalents (TE)/mg DW.

#### 7.2.5.2. Identification of phenolic compounds by LC-ESI-UHR-QqTOF-MS

The complete profile of phenolic compounds and its derivatives in supernatants of faecal fermentation of three donors were analysed in an LC-ESI-UHR-QqTOF-MS following the methodology of Monforte *et al.*, (2018). Identification of main olive phenolic compounds was based

on the retention time, UV-Vis and mass spectra with those obtained from the standard solutions, when available. The other peaks were tentatively identified comparing the information with available data reported in the literature (Ribeiro *et al.*, 2020a). Data from Phenol-Explorer 3.6 listed under "polyphenol metabolites" (http://phenol-explorer.eu/metabolites) was used as a reference for compound identification. The elemental composition for compounds was confirmed according to accurate mass (5 mDa), and isotope rate calculations designated mSigma (<20) (Bruker Daltonics).

Phenolic metabolites that passed the mass accuracy and frequency of detection thresholds, which had plausible chromatogram peak features, and showed significantly different trends from the control (faeces only), were considered potential phenolic fermentation markers.

### 7.2.6. Evaluation of the antiadhesion ability in mucin

The Gram-positive food contaminant/pathogenic bacteria (*Bacillus cereus* ATCC 2599 and *Listeria monocytogenes* 13562) and Gram-negative food contaminant/pathogenic bacteria (*Yersinia enterocolitica* NCTC 10406 and *Escherichia coli* ATCC 25922) were used to study the antiadhesion ability of OP powders digested. Suspensions of overnight cultures were adjusted to a final concentration of $10^8$ colony forming units (CFU)/mL on a DEN-1 McFarland densitometer (Biosan, Latvia). The antiadhesion ability was estimated according to a modified method of Valeriano, Parungao-Balolong and Kang (2014) and Vunduk *et al.* (2019). Antiadhesion assays were performed in flat bottomed 96-well polystyrene microtitre plates (Tissue Culture Testplate; SPL Life Sciences, Pocheon, Korea) using pig ileal mucin (1 mg $mL^{-1}$ and) as a matrix. Approximately 100 µL of mucin solution (1 mg $mL^{-1}$) in 10 mmol L-1 phosphate-buffered saline (PBS) buffer was immobilised on the plate wells for 1 h and subsequently incubated overnight at 4 °C. Wells were washed twice with 200 µL PBS buffer and incubated with 100 µL (20 mg $mL^{-1}$) bovine serum albumin (BSA) (Sigma) for 2 h at 4 °C. Wells were again washed twice with 200 µL of PBS buffer to remove unbound BSA. Approximately 50 µL of bacterial suspension (approx. $10^8$ – $10^9$ CFU $mL^{-1}$) was washed and suspended in 10 mmol $L^{-1}$ PBS buffer and added to the wells. Additionally, in control samples, 50 µL of PBS and to OP samples 50 µL of each OP powder (2% m/v) were added. Then plates were incubated at 37 °C for 1 h. After incubation, wells were washed five times with 200 µL sterile citrate buffer to remove unbound bacteria. Another 200 µL of 0.5% (v/v) Triton X-100 was then added to isolate attached bacteria. The viable cell count expressed as CFU $mL^{-1}$ was determined in all cases by plating on specific media. Each assay was performed in triplicate with each trial having more than two replicates each. The percentage of inhibition of bacterial adhesion in the presence of OP powders was calculated as:

$$Antiadhesion\ ability\ (\%) = \frac{(CFU_{Control} - CFU_{Sample})}{CFU_{Control}} \times 100 \qquad \text{Equation (1)}$$

Where $CFU_{Control}$ was the average for control samples, and $CFU_{Sample}$ was the average for treated samples.

### 7.2.7. Statistical Analyses

The statistical analyses were carried out using R software. The significance of the differences between samples after *in vitro* colonic fermentation was determined by one-way analysis of variance (ANOVA) or Kruskal-Wallis according to the normality of data distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Test multiple comparisons were made at those statistically significant variables using the Tukey's posthoc test or Dunn's method at the $p < 0.05$ significance level.

Supervised cluster analysis (Partial Least Squares Discriminant Analysis (PLS-DA)) and hierarchical clustering analysis (HCA) were applied to evaluate the metabolite patterns of OP powders detected (relative intensity) as a function of time using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) on log-transformed data after autoscaling (mean-centred and divided by the standard deviation of each variable). The HCA was applied with Euclidean distance measure and Ward clustering algorithm.

## 7.3. Results and Discussion

### 7.3.1. Changes in faecal microbial communities during in vitro fermentation

The real-time PCR investigation on the microbial groups' *Firmicutes*, *Lactobacillus* spp., *Enterococcus* spp., *Clostridium leptum*, *Bacteroidetes*, *Bacteroides* spp., *Prevotella* spp., and *Bifidobacterium* spp. were used to assess the OP powders effect on gut microbiota. For comparison, FOS was used as a positive control, i.e., compound with a prebiotic effect on the gut microbiota. As seen in Table 7.3, the most significant effects of OP powders were verified in Firmicutes and Bacteroidetes groups ($p < 0.05$). In healthy adults, the Gram-positive *Firmicutes* and the Gram-negative *Bacteroidetes* are the main phyla in the human gut microbiome (Abenavoli *et al.*, 2019). During the time of fermentation, the highest amounts of *Firmicutes* and *Bacteroidete*s were verified after 12 h of fermentation of FOS and OP powders. After 24 and 48 h, OP powders showed that they could maintain the growth of these two major phyla without decreasing their amounts significantly. On the other hand, FOS influenced more positively the amount of *Firmicutes* and *Bacteroidetes* until 12 h of fermentation, but cell numbers decreased significantly after that ($p < 0.05$). These decreases could be related to the fact that this work's *in vitro* faecal fermentation was performed without pH control. OP powders seemed to have a more prolongated positive effect on *Firmicutes* and *Bacteroidetes* than FOS. Indeed, POPP increased significantly ($p < 0.05$) the amount of *Firmicutes* compared to the FOS, principally after the 48 h of fermentation. This result was expected due to the higher amount of dietary fibre of POPP. Previous studies showed that diets low in fat and high in dietary fibre were associated with higher *Firmicutes* amount (Simpson and Campbell, 2015). Regarding *Bacteroidetes*, OP powders and FOS had a positive and similar effect on this group ($p < 0.05$) after 12, 24 and 48 h of *in vitro* colon fermentation.

It is known that more important than the stimulation of the amount of *Firmicutes* and *Bacteroidetes*, it is to maintain the relative abundance of these two prominent phyla (Simpson and Campbell, 2015). Commonly, healthy individuals display a nearly 1:1 ratio of *Firmicutes* to *Bacteroidetes* (F/B)

(Cockburn and Koropatkin, 2016) and its increase (e.g., to 20:1) or decrease have been associated with obesity and weight loss, respectively (Koliada *et al.*, 2017). OP powders and FOS maintained the ratio of F/B stable and near to one without significant differences.

Another important ratio is the *Prevotella* spp. to *Bacteroides* spp. (P/B) ratio. *Bacteroides* spp. and *Prevotella* spp. belong to the *Bacteroidetes* phylum and are among the most abundant anaerobes in the human colon (Flint and Duncan, 2014). If higher levels of *Bacteroides* spp. are related to a superior intake of fat and protein, increased Prevotella spp levels are correlated with high carbohydrates and fibre intake (Jiao *et al.*, 2019; Kovatcheva-Datchary *et al.*, 2015). An increasing number of studies have suggested that *Prevotella* spp. is a microbial group associated with beneficial gut microbiota (Rui *et al.*, 2019), particularly in improving glucose metabolism (Kovatcheva-Datchary *et al.*, 2015). On the other hand, *Bacteroides* spp. had demonstrated potential health benefits for hosts by suppressing intestinal inflammatory responses and promoting intestinal homeostasis (Rui *et al.*, 2019). However, a higher P/B ratio protects against *Bacteroides* spp. - induced glucose intolerance (Kovatcheva-Datchary *et al.*, 2015). FOS and OP powders increased P/B ratio through fermentation. LOPP and FOS improved P/B ratio until 48 h of fermentation without significant differences. Instead, POPP had a similar effect to FOS and LOPP until 24 h. The amount of *Prevotella* spp. between OP powders was very similar throughout fermentation. The higher amount of *Prevotella* spp. throughout fermentation could be explained by the high amount of dietary fibre and soluble sugars (mainly the polyol mannitol, which is considered a prebiotic substance) on POPP and LOPP, respectively (Jiao *et al.*, 2019; Kovatcheva-Datchary *et al.*, 2015). On the other hand, the higher amount of *Bacteroides* spp. after 24 and 48 h of POPP fermentation could be related to its higher fat content.

Regarding the other bacteria of *Firmicutes* phylum evaluated, OP powders did not negatively affect the *Lactobacillus* spp., *Clostridium leptum* and *Enterococcus* spp. genus compared to the blank. However, FOS exhibited a significant positive effect on Lactobacillus spp. group's growth compared to OP powders ($p < 0.05$). In the *Actinobacteria* phylum, the same behaviour with FOS and OP powders was verified when compared to the *Bifidobacterium* spp. Group, associated with health positive effects.

Regarding the time of fermentation, OP powders were fermented more slowly than FOS. Most *in vitro* fermentation studies suggest that less soluble substrates (e.g., insoluble arabinoxylan, cellulose) and longer chain lengths are fermented more slowly. As reported in our previous work, POPP was mainly a source of insoluble cellulose and arabinoxylan, which explain its slower fermentation rates by microbiota. On the other hand, LOPP is mostly a source of mannitol and phenolics, which are metabolised by gut microbiota, but less than oligosaccharides such as FOS and inulin. Mannitol effect as potential prebiotic was reported upon its use as a substrate on the acrylic pathway, which is observed only in some *Clostridium* spp. and *Prevotella* spp., such such as *C. propionicum* and *P. ruminicola* (Maekawa *et al.*, 2009). In turn, as shown by Parkar, Trower, & Stevenson (2013), in order to increase the gut microbiota in the same degree than oligosaccharides, phenolics needed to be supplied at higher concentrations (at least ten times more). Nevertheless,

the slow fermentation of OP powders could be an advantage in terms of health benefits, e.g. to avoid the production of gases that can result in discomfort, such as bloating and flatulence, and will also allow the production of SCFAs throughout the colon preventing colon cancer, which mainly occurs in the distal colon (Seong *et al.*, 2019).

Until now, the influence of OP on the composition of human gut microbiota was only assessed by incorporating the OP into a biscuit formulation (Conterno *et al.*, 2019). In the Conterno *et al.*, (2019) study, the potential of an olive pomace-enriched biscuit formulation delivering 17.1 ± 4.01 mg/100 g of HYD and its derivatives to modulate the composition and metabolic activity of the human gut microbiota was measured by a double-blind, controlled parallel dietary intervention during 8 weeks. The 16S rRNA metagenomics analysis of dominant bacterial phyla revealed a change in relative abundance at the genus level for *Bifidobacterium* spp., *Ruminococcus* spp. and *Lactobacillus* spp., while quantitative analysis using fluorescent *in situ* hybridisation coupled with flow cytometry did not confirm these results. Besides, qPCR showed a slight increase in *Bifidobacterium* spp.

As in the study of Conterno *et al.*, (2019), the analysis of the 16S rRNA gene results (Table 7.3**)** allowed to validate that OP powders did not negatively impact the diversity of the faecal microbiota.

**Table 7.3. Values of log 16S rRNA gene copies/ng of DNA of gut bacterial population sizes after 12, 24 and 48 h of the in vitro faecal fermentation of OP powders.**

| Samples | Time (h) | Gut microbiota | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Phylum *Firmicutes* | | | | Phylum *Bacteroidetes* | | | Phylum *Actinobacteria* | *Firmicutes*/ *Bacteroidetes* | *Prevotella* spp. / *Bacteroides* spp. |
| | | Total | *Lactobacillus* spp. | *Enterococcus* spp. | *Clostridium leptum* | Total | *Prevotella* spp. | *Bacteroides* spp. | *Bifidobacterium* spp. | | |
| Blank | *0* | 7.12 ± 0.41 $^{a}$ | 2.06 ± 0.42 $^{a}$ | 2.84 ± 0.66 $^{a}$ | 5.52 ± 0.16 $^{a}$ | 5.64 ± 0.11 $^{b}$ | 4.11 ± 0.88 $^{a}$ | 2.73 ± 0.61 $^{b}$ | 3.96 ± 0.34 $^{a}$ | 1.44 ± 0.07 $^{a, A}$ | 1.57 ± 0.44 $^{a, A}$ |
| | *12* | 7.20 ± 0.46 $^{a, A}$ | 2.34 ± 0.24 $^{a, A}$ | 3.52 ± 0.78 $^{a, A}$ | 4.91 ± 0.39 $^{a, A}$ | 6.60 ± 0.18 $^{a, A}$ | 4.77 ± 0.35 $^{a, A}$ | 5.08 ± 0.46 $^{a, A}$ | 3.82 ± 0.37 $^{a, B}$ | 1.13 ± 0.02 $^{b, A}$ | 0.94 ± 0.07 $^{a, A}$ |
| | *24* | 7.64 ± 0.23 $^{a, AB}$ | 1.96 ± 0.39 $^{a, AB}$ | 2.84 ± 0.69 $^{a, A}$ | 4.89 ± 0.14 $^{a, A}$ | 5.42 ± 0.23 $^{b, A}$ | 3.82 ± 0.62 $^{a, A}$ | 2.63 ± 0.37 $^{b, A}$ | 3.72 ± 0.13 $^{a, B}$ | 1.44 ± 0.06 $^{a, A}$ | 1.48 ± 0.33 $^{a, A}$ |
| | *48* | 7.56 ± 0.28 $^{a, A}$ | 1.54 ± 0.31 $^{a, AB}$ | 2.79 ± 0.61 $^{a, A}$ | 5.07 ± 0.25 $^{a, A}$ | 5.49 ± 0.20 $^{b, A}$ | 3.57 ± 0.14 $^{a, A}$ | 2.54 ± 0.11 $^{b, AB}$ | 3.37 ± 0.13 $^{a, A}$ | 1.38 ± 0.03 $^{a, A}$ | 1.41 ± 0.05 $^{a, B}$ |
| FOS | *12* | 7.46 ± 0.38 $^{a, A}$ | 2.41 ± 0.24 $^{a, A}$ | 4.39 ± 0.66 $^{a, A}$ | 4.94 ± 0.51 $^{a, A}$ | 6.68 ± 0.37 $^{a, A}$ | 5.22 ± 0.79 $^{a, A}$ | 4.71 ± 0.17 $^{a, A}$ | 4.87 ± 0.30 $^{a, A}$ | 1.12 ± 0.06 $^{b, A}$ | 1.10 ± 0.13 $^{b, A}$ |
| | *24* | 7.12 ± 0.18 $^{ab, A}$ | 2.49 ± 0.40 $^{a, A}$ | 4.21 ± 0.28 $^{a, A}$ | 4.71 ± 0.23 $^{a, AB}$ | 4.94 ± 0.49 $^{b, A}$ | 3.92 ± 0.67 $^{a, A}$ | 2.41 ± 0.39 $^{b, A}$ | 4.94 ± 0.50 $^{a, A}$ | 1.50 ± 0.13 $^{a, A}$ | 1.65 ± 0.29 $^{ab, A}$ |
| | *48* | 6.44 ± 0.52 $^{b, B}$ | 1.91 ± 0.33 $^{a, A}$ | 3.16 ± 0.39 $^{a, A}$ | 4.31 ± 0.35 $^{a, A}$ | 4.52 ± 0.37 $^{b, A}$ | 3.49 ± 0.22 $^{a, A}$ | 1.86 ± 0.27 $^{b, B}$ | 3.92 ± 0.57 $^{a, A}$ | 1.40 ± 0.15 $^{ab, A}$ | 2.00 ± 0.11 $^{a, A}$ |
| POPP | *12* | 7.36 ± 0.44 $^{a, A}$ | 1.42 ± 0.23 $^{a, B}$ | 3.61 ± 0.77 $^{a, A}$ | 4.50 ± 0.25 $^{a, A}$ | 6.65 ± 0.97 $^{a, A}$ | 4.70 ± 0.17 $^{a, A}$ | 4.40 ± 0.29 $^{a, A}$ | 3.50 ± 0.41 $^{a, B}$ | 1.13 ± 0.20 $^{a, A}$ | 1.07 ± 0.10 $^{b, A}$ |
| | *24* | 7.74 ± 0.31 $^{a, A}$ | 1.01 ± 0.18 $^{a, B}$ | 3.50 ± 0.61 $^{a, A}$ | 4.41 ± 0.38 $^{a, AB}$ | 5.70 ± 0.45 $^{a, A}$ | 4.31 ± 0.16 $^{a, A}$ | 2.83 ± 0.27 $^{b, A}$ | 3.39 ± 0.38 $^{a, B}$ | 1.36 ± 0.10 $^{a, A}$ | 1.60 ± 0.13 $^{a, A}$ |
| | *48* | 7.70 ± 0.19 $^{a, A}$ | 0.83 ± 0.15 $^{a, B}$ | 3.39 ± 0.63 $^{a, A}$ | 4.50 ± 0.20 $^{a, A}$ | 5.65 ± 0.26 $^{a, A}$ | 4.13 ± 0.37 $^{a, A}$ | 2.89 ± 0.20 $^{b, A}$ | 3.15 ± 0.35 $^{a, A}$ | 1.36 ± 0.07 $^{a, A}$ | 1.43 ± 0.08 $^{a, B}$ |
| LOPP | *12* | 7.08 ± 0.85 $^{a, A}$ | 0.69 ± 0.08 $^{a, c}$ | 2.90 ± 0.70 $^{a, A}$ | 4.18 ± 0.43 $^{a, A}$ | 7.01 ± 1.01 $^{a, A}$ | 4.51 ± 0.18 $^{a, A}$ | 4.80 ± 0.27 $^{a, A}$ | 3.22 ± 0.24 $^{a, B}$ | 1.03 ± 0.22 $^{b, A}$ | 0.96 ± 0.06 $^{b, A}$ |
| | *24* | 7.71 ± 0.47 $^{a, A}$ | 0.81 ± 0.20 $^{a, B}$ | 3.52 ± 0.60 $^{a, A}$ | 4.27 ± 0.21 $^{a, B}$ | 4.92 ± 0.86 $^{a, A}$ | 3.45 ± 0.25 $^{b, A}$ | 2.51 ± 0.09 $^{b, A}$ | 3.57 ± 0.30 $^{a, B}$ | 1.60 ± 0.23 $^{a, A}$ | 1.38 ± 0.16 $^{a, A}$ |
| | *48* | 7.52 ± 0.62 $^{a, A}$ | 1.07 ± 0.19 $^{a, AB}$ | 3.40 ± 0.70 $^{a, A}$ | 4.46 ± 0.31 $^{a, A}$ | 5.01 ± 0.83 $^{a, A}$ | 3.81 ± 0.10 $^{b, A}$ | 2.33 ± 0.41 $^{b, AB}$ | 3.21 ± 0.49 $^{a, A}$ | 1.52 ± 0.16 $^{ab, A}$ | 1.59 ± 0.33 $^{a, AB}$ |

Results are the means of five determinations ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters indicate the differences among Blank, FOS, and OP powders for the population of same microbial genus at the same time, and small letters indicate the differences for the same sample among time for the population of the same microbial genus.

### 7.3.2. Short-chain fatty acids production during *in vitro* faecal fermentation

The changes in the concentration of SCFAs after the fermentation of OP powders (2%) and FOS (2%) with human faeces in basal media analysed by HPLC are presented in Figure 7.1. SCFAs are volatile fatty acids produced by the gut microbiota in the colon as fermentation products from food components that are unabsorbed/undigested in the small intestine, such as acetate, propionate, butyrate and valerate (Ríos-Covián *et al.*, 2016). These SCFAs can be produced during growth phases (initial *vs* late), be present in medium composition (presence and absence of amino acids) and be produced by metabolic cross-feeding (e.g. consumption of lactate or formate to produce acetate) (Seong *et al.*, 2019). Butyrate and propionate derive exclusively from bacterial metabolism, but acetate could also have an endogenous origin (Pouteau *et al.*, 1998; Tabernero and Gómez de Cedrón, 2017).

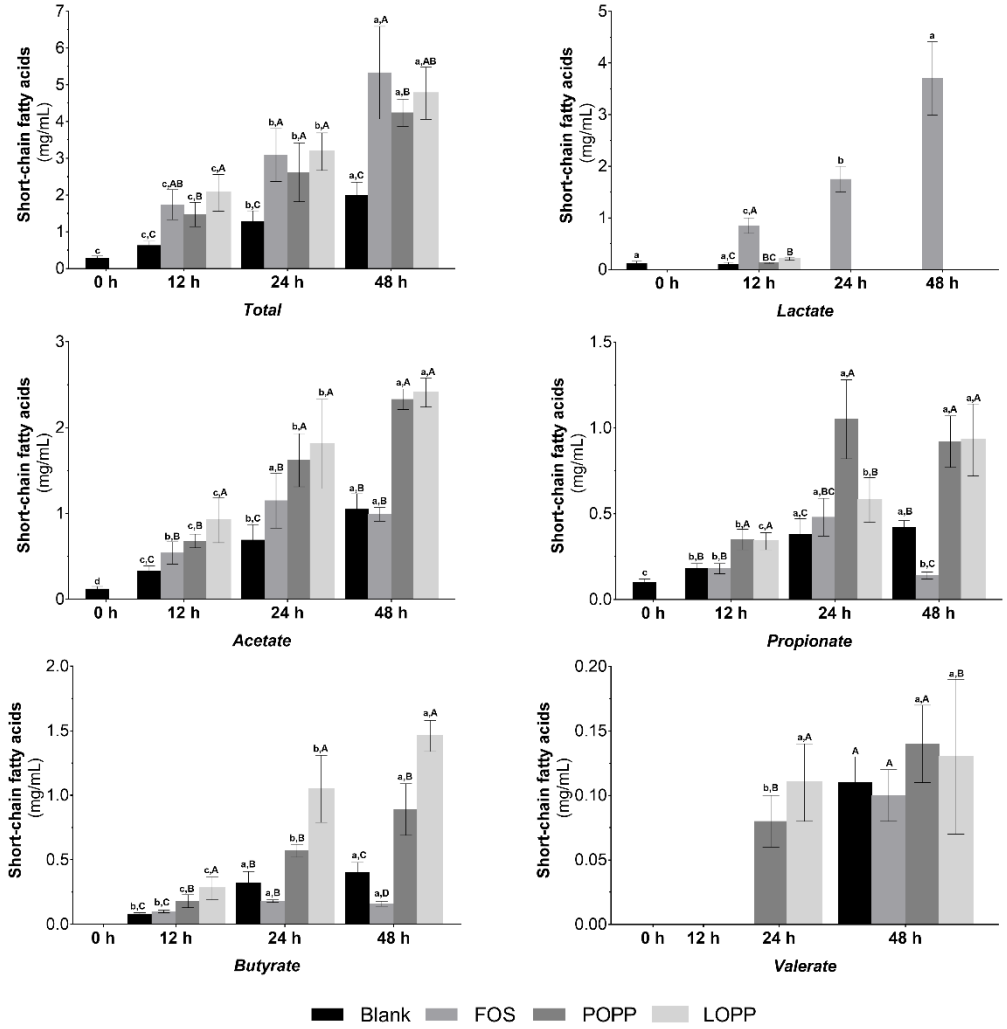



**Figure 7.1. Concentrations of short-chain fatty acids (SCFAs) produced after 12, 24 and 48 h *in vitro* faecal fermentation of OP powders.**
Results are the means of five determinations ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The capital letters indicate the differences among Blank, FOS, and OP powders for short-chain fatty acids concentration at the same time, and small letters indicate the differences for the same sample among time for short-chain fatty acids concentration.

The OP powders and FOS showed to substantially enhance total SCFAs' production without significant differences between each other. However, different production profiles of SCFAs were detected for FOS and OP powders. In FOS fermentation, the main metabolite produced during the whole fermentation was lactate. On the other hand, lactate production was nearly null during OP powders faecal fermentation. The low stimulation of *Lactobacillus* spp. and *Bifidobacterium* spp. group by OP powders explained this limited production of lactate (Dominika *et al.*, 2011), validated above with 16S rRNA gene analysis.

The OP powders enhanced the production of the acetate, propionate and butyrate principally in a ratio of 3:1:1, as reported in previous works (Cummings, 1981; Scott *et al.*, 2013; Topping and Clifton, 2001). The SCFA production stimulated by OP powders and FOS increased throughout fermentation time. Nevertheless, only the fermentation of OP powders showed an increase in the amount of acetate, propionate and butyrate that could be considered significantly different from the blank sample ($p < 0.05$). This higher production of SCFAS was reported in other *in vitro* batch fermentation investigations using human faecal inocula with slow fermentation rate like OP powders (Wang *et al.*, 2019).

Comparing the OP powders, POPP led to a higher rise of propionate production than LOPP after 24 h of faecal fermentation. This high amount of propionate resulted from the higher abundance of *Bacteroidetes* detected in POPP compared to LOPP after 24 and 48 h. *Bacteroidetes*, as members of the polysaccharide-degrading consortia, contribute to the release of energy from DF, which is the main component of POPP and consequent propionate formation (Koh *et al.*, 2016). *Bacteroidetes* are likely to be the main contributors to propionate production in the colon (Flint and Duncan, 2014).

Compared to POPP, only the amount of butyrate was significantly higher in LOPP than in POPP after 24 and 48 h of fermentation ($p < 0.05$). The main butyrate-producing groups belong to *Firmicutes* phylum (De Vuyst and Leroy, 2011; Parkar *et al.*, 2013), more specifically *Coprococcus comes*, *C. eutactus*, *C. catus*, *E. rectale*, *E. hallii*, *F. prausnitzii*, *Anaerostipes* spp., *Roseburia* spp (Koh *et al.*, 2016). These genera were not measured in the present work, and no significant differences were observed in phylum *Firmicutes* between LOPP and POPP. However, a typical cross-feeding effect could occur among the genus of intestinal bacteria enumerated (except *C. comes*, *C. eutectic)* using acetate to synthesise butyrate via the butyryl-CoA: acetate CoA-transferase route (De Vuyst and Leroy, 2011; Koh *et al.*, 2016), which could explain the higher amount of butyrate in LOPP fermentation. The LOPP rich composition in sugar and formic acid could enhance acetate production by enteric bacteria fermentation or by acetogenic bacteria fermentation via the Wood–Ljungdahl pathway, respectively (Seong *et al.*, 2019). In an acetate-rich colon ecosystem, previous studies reported that butyryl-CoA: acetate CoA transferase activity has been more common among butyrate-producing groups (De Vuyst and Leroy, 2011; Louis *et al.*, 2007, 2004). This makes LOPP acetate a key intermediate for colon butyrate. On the other hand, as Maekawa *et al.* (2009) reported, mannitol, present at a substantial amount in LOPP, was associated with the stimulation of the lactate-utilising butyrate producers and propionate producers. Other reason for the higher increase of butyrate in LOPP fermentation could be linked to its higher phenolics content. Caffeic acid and its derivative

caffeoyl-6'-secologanoside were detected in higher amounts in LOPP undigested fraction, and its metabolisation might be responsible for the enhanced production of butyrate in the fermentation of these OP powders. In previous studies, at concentrations of 10 and 100 mg/mL, the caffeic acid standard exhibited the highest increase in butyrate production after 24 h of faecal fermentation when compared to other phenolics (chlorogenic acid, caffeic acid, rutin and quercetin) and inulin (Parkar *et al.*, 2013).

The common cross-feeding effect among intestinal bacteria of acetate, propionate or butyrate production as the final metabolite using the lactate as substrate (Koh *et al.*, 2016; Seong *et al.*, 2019) was not evident during FOS fermentation. Indeed, lactate was accumulated throughout the fermentation time, and its utilisation was not verified, once the other SCFAs concentrations were maintained or decreased after 24 h. So, the prevention of lactate accumulation to stabilise the intestinal environment was not verified in FOS fermentation, which could result in further acidosis related to certain gut disorders or dietary intakes and lead to adverse consequences (Kowlgi and Chhabra, 2015). However, the low pH value of the faecal fermentation (the *in vitro* faecal fermentation experiment of this work performed without pH control) of FOS could be responsible for the lactate accumulation (Belenguer *et al.*, 2007).

The SCFAs as metabolites of gut microbiota are distributed throughout the body, having diverse functions such as signalling molecules and/or energy substrates. In the colon, SCFAs could inhibit pathogenic microorganisms, increase the absorption of some nutrients and seem to play an essential role in the maintenance of the gut barrier function (Ríos-Covián *et al.*, 2016). During its intestinal absorption, butyrate may be metabolised (energy source) by colonocytes. At the same time, propionate may be transported to the liver where it has a role in gluconeogenesis, and acetate enters the systemic circulation and is used in lipogenesis (Scott *et al.*, 2013). SCFAs might play a vital role in several organs' metabolic pathways, modulating different processes including cell proliferation and differentiation, hormones secretion (e.g., leptin and peptide YY) and activation of immune/inflammatory responses (Gullon *et al.*, 2015; Koh *et al.*, 2016). Many biological effects seem to be mediated by SCFAs, namely (1) acetate and propionate might affect satiety and intestinal transit; (2) butyrate could exert anti-inflammatory effects; (3) propionate could lead to satiety and decreased hepatic glucose production; (4) small amounts of SCFAs (mostly acetate and possibly propionate) reach the circulation and can also directly affect the adipose tissue, brain and liver, inducing overall beneficial metabolic effects and (5) SCFAs can also reduce inflammation and tumorigenesis (Koh *et al.*, 2016; Ríos-Covián *et al.*, 2016). The role of SCFAs in the prevention and treatment of several diseases such as metabolic syndrome, bowel disorders and cancer has also been reported in several studies (Gullon *et al.*, 2015). Still, excessive SCFAs concentrations might induce adverse effects on gastrointestinal and colonic motility and sensitivity in certain diseases such as inflammatory bowel disease and gastro-oesophageal reflux disease, and the conclusive proof is not available for many of the health claims made for SCFAs (Gullon *et al.*, 2015; Ríos-Covián *et al.*, 2016).

Despite the higher total SCFAs production by FOS, OP powders exhibited a profile of SCFAs with a higher concentration of acetate, propionate and butyrate throughout fermentation time. These SCFAs could exert interesting beneficial health properties not only in the colon and gut microbiota but also in other organs. So, OP powders could be considered prebiotics as inducers of the SCFAs production activity by gut microorganisms.

### 7.3.3. Phenolic compounds throughout *in vitro* faecal fermentation

#### 7.3.3.1. Total phenolic compounds and antioxidant activity

The total phenolic content (TPC), as assayed according to the Folin-Ciocalteau method, along with *in vitro* antioxidant capacity (ORAC radical scavenging), was investigated to support the following evaluation of phenolic profile through LC-ESI-UHR-QqTOF-MS. Figure 7.2 displays the TPC and the *in vitro* antioxidant capacity (ORAC radical scavenging) during *in vitro* faecal fermentation process (8, 24, and 48 h) in comparison to FOS and blank, but also the TPC and ORAC of the digested OP powder previously evaluated and throughout the *in vitro* faecal fermentation process (8, 24, and 48 h) expressed by DW of OP powder. Comparing TPC results of OP powders with FOS, OP powders showed significant higher TPC values ($p < 0.05$) due to their richness in phenolic compounds. Indeed, TPC values were constant during FOS faecal fermentation time (3-5 mg GAE/g DW), which corroborates that FOS as polysaccharide did not contain phenolics to liberate after microbial fermentation.

Concerning OP powders, LOPP and POPP revealed similar TPC values after 12 and 24 h of faecal fermentation. Indeed, both fermented OP powders exhibited the highest TPC after 24 h of *in vitro* colonic fermentation (POPP: 8.92 ± 1.12 mg GAE/g DW; LOPP: 9.78 ± 1.89 mg GAE/g DW). Nevertheless, after 48 h of fermentation both OP powders decreased their TPC values, even though LOPP kept a significantly higher TPC value than POPP ($p < 0.05$), with values of 5.63 ± 0.82 mg GAE/g DW and 4.03 ± 0.35 mg GAE/g DW, respectively. The highest TPC values of LOPP throughout *in vitro* colon fermentation might be linked to its higher phenolic richness**.** As LOPP comprise more phenolics than POPP, it could originate higher amounts of phenolic metabolites during the faecal fermentation process as reviewed by Duda-Chodak, Tarko, Satora, & Sroka, (2015). A similar increase in TPC after 24 h of fermentation was reported for a goji berries mix (Rocchetti *et al.*, 2018) and black mulberry (Bao *et al.*, 2019). However, Bao *et al.* (2019) also described that TPC of black mulberry decreased from 24 to 48 h of fermentation time as shown to happen for LOPP, while TPC of white mulberry decreased until 48 h of fermentation time, as observed for POPP.

Considering the TPC results of the previous works regarding the digested OP powders, a significant increase of phenolic compounds occurred after faecal fermentation for both OP powders (Figure 7.2 c). An increase in TPC value from 93 to 255% was exhibited from digested to fermented OP powders. Similar levels of increase were reported for black mulberry (Bao *et al.*, 2019) after 12, 24 and 48 h. This effect has been associated with gut microbiota's ability to transform larger phenolic molecules

into smaller ones during colonic fermentation (Rocchetti *et al.*, 2018) using the free and bound phenolic fraction of OP powders (Rocchetti *et al.*, 2019). The higher amount of POPP bound phenolics could explain the significant increase of TPC just after 12 h of fermentation ($p < 0.05$) compared to digested POPP. In the case of LOPP, only after 24 h of fermentation, the TPC value was significantly different from the digested LOPP.

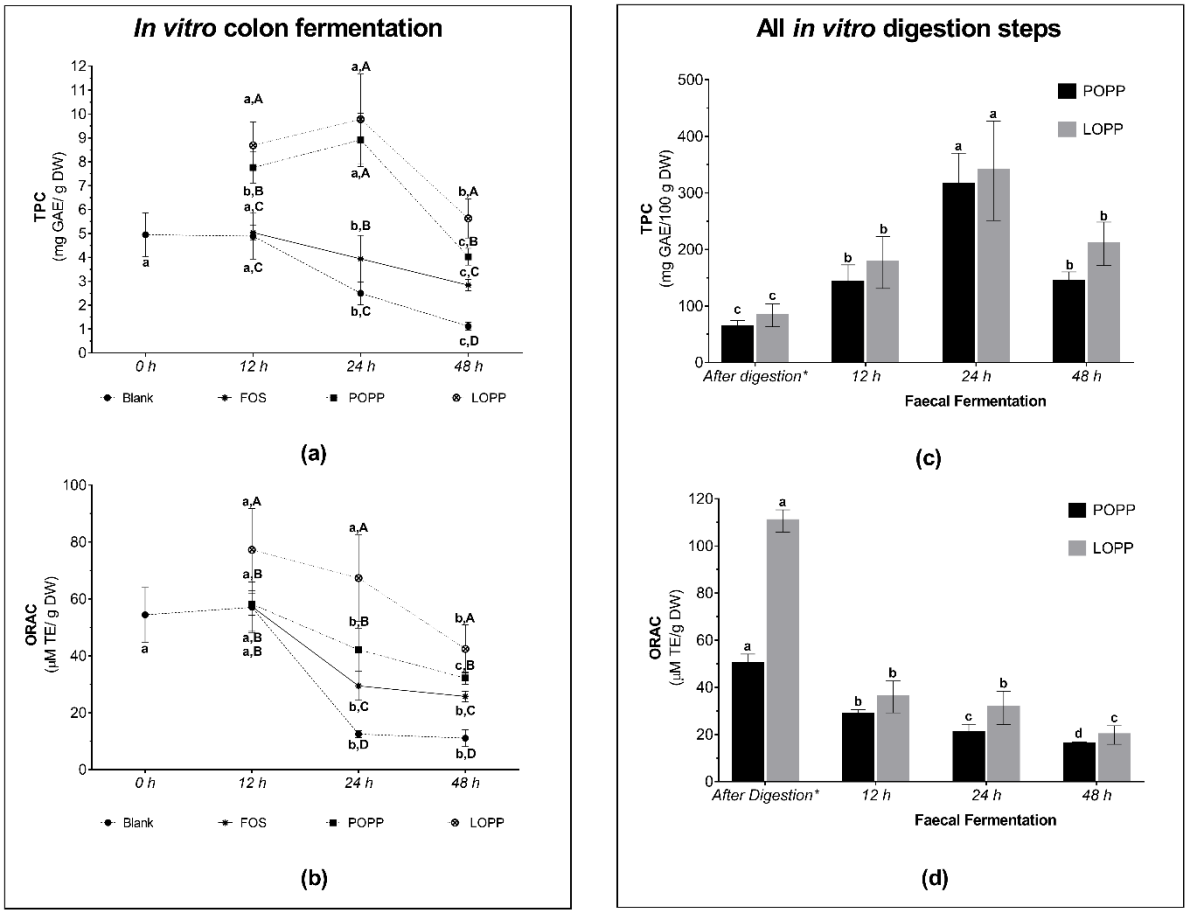



**Figure 7.2. Total phenolic content (TPC) expressed as gallic acid equivalents (GAE), and antioxidant capacity (ORAC), expressed as trolox equivalents (TE) after 12, 24 and 48 h of the *in vitro* faecal fermentation of olive pomace (OP) powders. (a) OP powders TPC (mg GAE/100 g DW fermented sample) during the faecal fermentation in comparison to FOS and Blank. (b) OP powders ORAC (μmol $L^{-1}$ TE/g DW fermented sample) during the faecal fermentation in comparison to FOS and Blank. (c) OP powders TPC (mg GAE/ 100 g DW sample) in the digested OP powders and during 12, 24 and 48 h of the *in vitro* faecal fermentation process. (d) OP powders ORAC (μM TE/g DW sample) in the digested OP powders and during 12, 24 and 48 h of the *in vitro* faecal fermentation process.**
*In vitro* faecal fermentation results are the means of five determinations ± standard deviation. *Samples analysed in previous works. Different letters indicate significant differences ($p < 0.05$). The capital letters indicate the differences among Blank, FOS, and OP powders at the same time, and small letters indicate the differences for the same sample among the digestion phases.

The antioxidant capacity (AOX) of the fermented OP powders was determined using the ORAC assay. This method is extensively used to assess the chain-breaking antioxidant capacity at physiological pH values (Burgos-Edwards *et al.*, 2018).

As shown in Figure 7.2 b, OP powders exhibited higher AOX than FOS and LOPP revealed the highest AOX ($p < 0.05$), following the trend of the TPC values reported. LOPP showed similar AOX

between 12 and 24 h of faecal fermentation, decreasing its AOX value significantly after 48 h ($p < 0.05$). Previous studies exhibited similar behaviour on the evolution of ORAC. Burgos-Edwards *et al.* (2018) and Rocchetti *et al.* (2019) also reported similar ORAC values after 8 and 24 h of faecal fermentation of a phenolic-enriched extract of *Ribes punctatum* currant and pigmented gluten-free flours of red quinoa and black chickpea, respectively. On the other hand, POPP revealed a continuous decline of AOX through *in vitro* fermentation. Burgos-Edwards *et al.* (2018) and Rocchetti *et al.* (2019) also reported a similar decrease in ORAC values throughout *in vitro* colon fermentation of a phenolic-enriched extract of *Ribes magellanicum* currant and pigmented gluten-free flours of teff and amaranth, respectively. Therefore, LOPP revealed to be a more effective source of antioxidants in the colon than POPP.

By comparing the ORAC values obtained in previous works for digested OP powders and those obtained for fermented OP powders, it could be assumed that significant losses of AOX occur during faecal fermentation. Similar results were verified in previous investigations (Burgos-Edwards *et al.*, 2018; Rocchetti *et al.*, 2019). However, other matrices where ORAC values (Rocchetti *et al.*, 2019) were evaluated after 6 and 8 h of fermentation exhibited increased ORAC values for a phenolic-enriched extract of Chilean currants (*Ribes punctatum* and *Ribes magellanicum*). For that reason, the TPC and ORAC assessment in more fermentation time points might be crucial for a superior understanding of the phenolics evolution in a food matrix and its metabolites in the colon. The different trend of ORAC and TPC values observed between digested and fermented OP powders could be explained by the occurrence of different reaction mechanisms of the various antioxidants present in the samples, and has been reported in previous works (Tabart *et al.*, 2010)

In conclusion, OP powders provided potentially higher health benefits linked to the creation of an antioxidant environment in the gut than FOS. OP powders showed to be a valuable source of phenolics with AOX through *in vitro* colon fermentation. Future studies in cell lines and human clinical experiments will be required to quantify these antioxidant effects accurately.

#### 7.3.3.2. Identification of phenolic compounds and its metabolites (LC-ESI-UHR-QqTOF-MS)

This study was performed to identify the main molecules generated by human microbiota action on different OP powders phenolics classes. The phenolic profile pattern obtained to OP powders is in agreement with similar works with OP (Conterno *et al.*, 2019) and olive oil (López de las Hazas *et al.*, 2017; Mosele *et al.*, 2014).

Identification of phenolic precursors and metabolites derived from *in vitro* microbial colonic fermentation of the OP powders based on high-performance LC-ESI-UHR-QqTOF-MS analyses and a summary of mass spectrometric specific molecular patterns in negative ionisation are summarised in Table 7.4. Each reported metabolite was detected in at least three of the five donors after 12, 24 or 48 h of fermentation.

PLS-DA of the phenolic metabolite patterns measured in the samples derived from OP powders fermentation revealed separate clusters for each time of fermentation and sample (Figure 7.3 a). The

scores plot indicates that the first two principal components account for 61.9% of the total variance (PC1 = 47.1% and PC2 = 14.8%). If on the one hand the three clusters of fermentation time of LOPP were positioned close to each other in the III quadrant, on the other hand, the 12 h fermentation-cluster of POPP was located on the I quadrant, and the other clusters of POPP fermentation (24 and 48 h) were very close to each other in the opposite quadrant (II). Therefore, POPP and LOPP exhibited significant differences in phenolics throughout faecal fermentation, being separated by the most critical factor (PC1), which contributes with 47.1% of the total variance. Nevertheless, after 48 h of fermentation, LOPP exhibited a phenolic pattern more similar to that of POPP after 24 and 48 h of fermentation (LOPP - 48 h cluster closer to POPP – 24 and 48 h clusters).

Variable importance in projection (VIP) was obtained (Figure 7.3 b) in order to understand better the differences observed between the different clusters of phenolics from OP powders. Markers assigned a VIP score > 0.8 were counted as the 15 most significative compounds, which define the phenolics patterns of the OP powders trough faecal fermentation. LOPP, as expected, exhibited a higher relative concentration of almost all the phenolic compounds. HYD glucoside ($C_{14}H_{20}O_8$ *mz* 315.1035), a hydroxylated product of the dialdehydic form of decarboxymethyl-elenolic acid ($C_9H_{12}O_5$ *mz* 199.0564), a decarboxylated form of hydroxyl-elenolic acid ($C_{10}H_{14}O_5$ *mz* 213.0718) and 3-(3,4 -dihydroxyphenyl)-propionic acid ($C_9H_{10}O_4$ *mz* 181.0452) were the most significant and at higher relative amounts until 12 h of LOPP and POPP fermentation (VIP >1.3). These phenolics decreased throughout in vitro fermentation in both OP powders. On the other hand, oleuropein aglycone derivative ($C_{16}H_{26}O_{10}$ *mz* 377.1353), 3-(4-hydroxyphenyl) propionic acid ($C_9H_{10}O_3$ *mz* 165.0505) and oxidised HYD ($C_8H_8O_3$ *mz* 151.0352) increased after 24 h of LOPP fermentation (VIP >1.2). However, in POPP after 24 h of fermentation, only oxidised HYD increased, while the oleuropein aglycone derivative and 3-(2-hydroxyphenyl) propionic acid decreased. Other phenolic acids metabolites identified in LOPP after 24 h of fermentation at higher relative concentration were 3-hydroxy-4-methoxyphenyllactic acid ($C_{10}H_{12}O_5$ *mz* 211.0560) (VIP > 1.1) and 3-(3-hydroxyphenilpropionic acid) ($C_9H_{10}O_3$ *mz* 165.0489) (VIP >0.95). The 3-(3-hydroxyphenilpropionic acid) also increased in POPP fermentation until 48 h of fermentation, and even at a higher rate than in fermented LOPP. Vanillyl alcohol ($C_8H_{10}O_3$ *mz* 153.0493) was another metabolite identified (VIP > 0.97) at higher concentration in fermented POPP after 48 h. Other phenolic acids' metabolites were also identified in POPP and LOPP with low VIP (between 0.3 and 0.1), namely homovanillic alcohol ($C_9H_{12}O_3$ *mz* 167.0655) and homovanillic acid ($C_9H_{10}O_4$ *mz* 181.0464). All these phenolic acids were reported in previous works with OP biscuits (Conterno *et al.*, 2019) and olive oil (López de las Hazas *et al.*, 2017; Mosele *et al.*, 2014) as colonic metabolites of olive phenolics and precursors of the phenylacetic and phenylpropionic acids. Phenylacetic and phenylpropionic acids have been reported as products from the gut microbial metabolisation of phenolics with more strong potential health effects, such as anti-inflammatory and antioxidant effects (López de las Hazas *et al.*, 2016), as well as acting as prebiotics inhibiting pathogenic bacteria and stimulating the beneficial bacteria on the colon (Karković Marković *et al.*, 2019).

**Table 7.4. Detection of polyphenols and its metabolites after 12, 24 and 48 h of the *in vitro* faecal fermentation of the OP powders.**

| | Compound | Molecular formula | RT (min) | m/z calcd | m/z exptl | Err [mDa] | mSigma | Major fragments ESI negative MS/MS ions | *In vitro* faecal fermentation time (h) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | POPP | | | LOPP | | |
| | | | | | | | | | *12* | *24* | *48* | *12* | *24* | *48* |
| **M1** | **Oxidised hydroxytyrosol**[*2] | $C_6H_{12}O_6$ | 1.5 | 179.0561 | 179.0511 | 0.3 | 12.2 | 179.0563; 161.0457; 89.0242; 75.0086 | D | D | D | D | D | D |
| **M2** | **Hydroxytyrosol glucoside**[*2] | $C_{14}H_{20}O_8$ | 7.1 | 315.1088 | 315.1085 | 0.0 | 9.1 | 315.1092; 153.0556; 123.0451 | D | D | D | D | D | D |
| **M3** | **Hydrated product of the dialdehydic form of decarboxymethyl-elenolic acid**[*2] | $C_9H_{14}O_5$ | 7.2 | 201.077 | 201.0768 | 0.1 | 6.5 | 201.0405; 153.0553; 123.0449; 95.0502 | D | D | D | D | D | D |
| **M4** | **3,4 - Dihydroxybenzoic acid** [*3] | $C_7H_6O_4$ | 7.3 | 153.0193 | 153.0198 | 0.3 | 9.2 | 153.0198; 109.0295 | D | D | ND | D | D | D |
| **M5** | **Vanillyl alcohol** [*3] | $C_8H_{10}O_3$ | 7.4 | 153.0557 | 153.0493 | 0.2 | 5.2 | 153.0557; 123.0447 | ND | ND | D | ND | ND | ND |
| **M6** | **Hydroxytyrosol** [*1] | $C_8H_{10}O_3$ | 7.5 | 153.0556 | 153.0557 | 0.1 | 5.7 | 153.0452; 123.0457 | D | D | D | D | D | D |
| **M7** | **Oleoside derivative isomer**[*2] | $C_{17}H_{28}O_{11}$ | 7.7 | 407.1557 | 407.1559 | -0.5 | 11.7 | 151.0760; 119.0346 | D | D | D | D | D | D |
| **M8** | **Decarboxylated form of hydroxy-elenolic acid** [*2] | $C_{10}H_{14}O_5$ | 7.9 | 213.0765 | 213.0768 | 0.1 | 5.6 | 213.0920; 137.0601; 121.0665; 111.0085 | D | D | D | D | D | D |
| **M9** | **3-(3,4-Dihydroxyphenyl)-propionic acid** [*4] | $C_9H_{10}O_4$ | 8.0 | 181.0506 | 181.0452 | 0.0 | 5.4 | 181.0507; 163.0397; 135.0447 | D | D | ND | D | D | D |
| **M10** | **Tyrosol glucoside**[*3] | $C_{14}H_{20}O_7$ | 8.1 | 299.1139 | 299.1136 | -0.3 | 3.2 | 119.0505; 137.0244; 135.0299 | D | D | D | D | D | D |
| **M11** | **Hydroxylated product of the dialdehydic form of decarboxymethyl-elenolic acid** [*2] | $C_9H_{12}O_5$ | 8.2 | 199.0612 | 199.0564 | 0.0 | 9.5 | 199.0616; 155.0709; 111.0812 | D | D | D | D | D | D |
| **M12** | **Oleoside**[*2] | $C_{16}H_{22}O_{11}$ | 8.4 | 389.1093 | 389.1089 | -0.4 | 4.3 | 389.1092; 183.0664; 165.0556; 121.0654 | D | D | D | D | D | D |
| **M13** | **p-Coumaroyl-D-glucose**[*3] | $C_{15}H_{18}O_8$ | 8.7 | 325.0925 | 325.0929 | 0.4 | 0.9 | 326.0976; 163.0402; 119.0501 | D | D | D | D | D | D |
| **M14** | **Oleuropein aglycone derivative** [*3] | $C_{16}H_{26}O_{10}$ | 9.6 | 377.1453 | 377.1353 | 0.3 | 9.4 | 377.1452; 197.0825; 153.0918 | D | D | D | D | D | D |
| **M15** | **Tyrosol** [*1] | $C_8H_{10}O_2$ | 9.8 | 137.0608 | 137.0608 | 0.5 | 4.5 | 111.0084; 95.0510 | D | D | D | D | D | D |
| **M16** | **Aldehydic form of decarboxymethyl elenolic acid** [*2] | $C_{10}H_{16}O_5$ | 10.8 | 215.0925 | 215.0873 | 0.1 | 7.6 | 215.0925; 153.0918; 171.1026 | D | D | D | D | D | D |
| **M17** | **Elenolic acid** [*2] | $C_{11}H_{14}O_6$ | 12.5 | 241.0720 | 241.0718 | -0.2 | 0.5 | 241.0737; 139.0035; 127.0398; 111.0086; 95.0551 | D | D | D | D | D | D |
| **M18** | **3 - (4-Hydroxyphenyl) propionic acid** [*4] | $C_9H_{10}O_3$ | 12.6 | 165.0557 | 165.0505 | 0.0 | 7.9 | 165.0557; 147.0451; 119.0499; 103.0552 | D | D | D | D | D | D |
| **M19** | **3-Hydroxyphenilpropionic acid** [*4] | $C_9H_{10}O_3$ | 12.7 | 165.0557 | 165.0489 | 0.1 | 7.0 | 165.0556; 121.0655; 121.0294; 106.0423 | D | D | D | D | D | D |
| **M20** | **3 - hydroxy-4-methoxyphenyllactic acid** [*4] | $C_{10}H_{12}O_5$ | 13.0 | 211.0612 | 211.0560 | 0.5 | 4.0 | 211.0612; 165.0569; 123.0812 | D | D | D | D | D | D |
| **M21** | **Homovanilic alcohol** [*4] | $C_9H_{12}O_3$ | 13.2 | 167.0714 | 167.0655 | 0.3 | 11.1 | 166.0585; 139.0412; 136.9341; 109.0273; 121.0517 | D | D | D | ND | D | D |
| **M22** | **Homovanillic acid** [*4] | $C_9H_{10}O_4$ | 13.7 | 181.0506 | 181.0464 | 0.8 | 8.7 | 181.0486; 137.0611; 124.0187; 109.0656 | D | D | D | D | D | D |
| **M23** | **Luteolin** [*1] | $C_{15}H_{10}O_6$ | 15.9 | 285.0406 | 285.0405 | 0.1 | 0.8 | 285.0414; 151.0037 | D | D | D | D | D | D |

[*1] Hydroxytyrosol, tyrosol and luteolin were identified by comparison with the standards. LC-ESI-UHR-QqTOF-MS tentatively identified the other compounds based on accurate mass, isotope rate calculations designated mSigma and literature.[*2] Lozano-Sánchez *et al.*, (2013), [*3] Jerman Klen & Mozetič Vodopivec(2012), [*4] Phenol-Explorer 3.6. D – Compound detected; ND – Compound not detected.

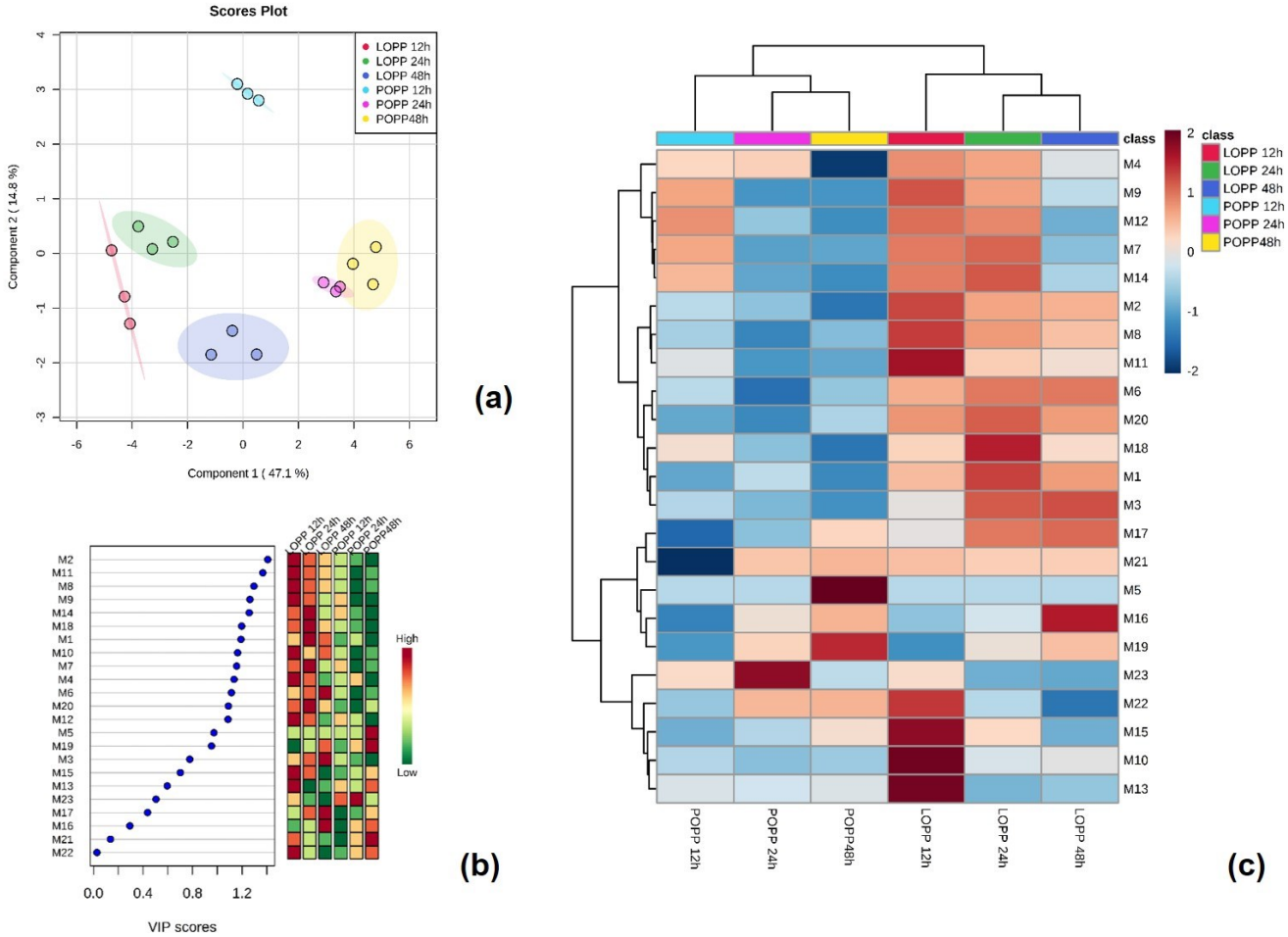



**Figure 7.3. Chemometrics and cluster analysis of the phenolics and its metabolites identified in olive pomace (OP) powders after 12, 24 and 48h of the in vitro faecal fermentation.** (a) Partial Least Squares - Discriminant Analysis (PLS-DA) of OP powders faecal metabolite profiles. The relative intensity of the metabolites detected in the faecal samples collected by LC-ESI-UHR-QqTOF-MS was used for a supervised clustering analysis by a partial least squares discriminant analysis. (b) VIP (Variable Importance in Projection) of phenolic compounds identified following the PLS-DA model, during faecal fermentation of OP powders. VIP allowed to measure the variable's importance in the PLS-DA model. Green and red tiles, respectively, indicate a lower or higher intensity of metabolite concentration in the mean of all fermented samples. (c) Hierarchical clustering analysis heatmap of phenolic metabolites identified during faecal fermentation of OP powders. Blue and red tiles, respectively, indicate a lower or higher intensity of metabolite concentration in the mean of all fermented samples.

Hierarchical clustering (HCA) of the relative intensity of the 23 phenolic metabolites analysed following the faecal fermentation of OP powders is presented in Figure 7.3 (c). The heatmap shows the clustering of phenolic metabolites according to their detection in faecal fermentation samples of OP powders. The clustering of the different phenolic metabolites could be divided into two main principal clusters. These clusters allowed to understand the possible colonic transformations of OP dietary phenolics into phenolic acids. For example, oleuropein aglycone (M14), hydroxytyrosol (M6), 3-(3,4-dihydroxyphenyl)propionic acid (M9), 3,4-dihydroxybenzoic acid (M4) and 3-(4'-hydroxyphenyl) propionic acid (M18) were found in the same principal cluster.

The formation of aglycones by gut microbiota has been reported before as affecting positively or negatively, their activities and functional effects (Laparra and Sanz, 2010). In this work, oleuropein aglycon showed to be a key compound in the colonic phenolic pathway of OP powders. Most of the identified phenolic metabolites could be obtained by the proposed colonic pathway of oleuropein (Mosele *et al.*, 2014). The oleuropein aglycone (M14) through hydrolysis could generate 3-(3',4'-dihydroxyphenyl)propionic acid (M9) and HYD (M6), and then 3-(3',4'-dihydroxyphenyl) propionic acid (M9) could be transformed into 3,4-dihydroxybenzoic acid (M4) or 3-(4'-hydroxyphenyl)propionic acid (M18) by β oxidation and dihydroxylation, respectively. Instead, the colonic pathway of HYD proposed by Mosele *et al.*, (2014) could also enlighten the detection of the 4-dihydroxybenzoic acid metabolite, which could be found after two successive oxidations reactions: HYD (HYD (M6) oxidation into 2-(3',4'-dihydroxyphenyl)acetic acid which produces 3,4-dihydroxybenzoic acid (M4) by α-oxidation. The higher relative concentration and VIP of 3-(4'-hydroxyphenyl)propionic acid (M18) compared to 3,4-dihydroxybenzoic acid (M4) allowed to hypothesise that the dihydroxylation of 3-(4'-hydroxyphenyl)propionic acid (M18) into 3-(3',4'-dihydroxyphenyl) propionic acid (M9) was privileged during colonic fermentation of OP powders.

In the other principal cluster, homovanillic acid (M22), homovanillic alcohol (M21), vanillyl alcohol (M5) and 3-hydroxyphenilpropionic acid (M19) metabolites were detected in growing relative concentrations throughout in vitro faecal fermentation, mostly in fermented POPP. These compounds could result from the metabolisation of HYD. In previous works, HYD was converted to homovanillic alcohol (M21) in Caco-2 cells, and homovanillic acid (M22) and homovanillic alcohol (M21) were detected in urine after metabolisation of HYD supplied by olive oil consumption (Naczk and Shahidi, 2003). Homovanillic acid (M22) increase was also reported in previous work, after ingestion of OP biscuits (in plasma) (Conterno *et al.*, 2019). On the other hand, the vanillyl alcohol (M5) and 3-hydroxyphenilpropionic acid (M19) could result from biotransformation of vanillin and caffeic acid, respectively.

The metabolites compounds identified in LOPP and POPP derived mainly from the oleuropein aglycon and HYD's breakdown pathways present in significant amount in these OP powders, principally in LOPP. Therefore, LOPP exhibited a higher relative concentration of phenolic acids metabolites than POPP, in agreement with TPC and ORAC results. Other metabolic pathways could also occur during POPP colonic fermentation, considering the metabolites identified in these samples. All steps of the potential metabolic pathways identified during in vitro fermentation of OP

powders intermediated by the gut microbiota and its catabolites and similar small phenolic acids have been reported to be excreted following ingestion of olive or olive fractions in previous studies (Conterno *et al.*, 2019).

### 7.3.4. Antiadhesion ability in mucin

The antiadhesion ability of OP powders (2% m/v) relative to food contaminant/pathogenic bacteria is reported in Table 7.5. However, it is essential to clarify that the inhibition of bacterial adhesion can be the result of various mechanisms such as cell surface modification, quorum sensing, or changes in the cell structure that are not directly connected with the antibacterial activity of the tested substance (Vunduk *et al.*, 2019).

**Table 7.5. Antiadhesion activity of OP powders after gastrointestinal digestion.**

| Pathogenic strain | Sample | | | | |
|---|---|---|---|---|---|
| | Blank | POPP | | LOPP | |
| | Log (Cfu/mL) | Log (Cfu/mL) | Antiadhesion activity (%) | Log (Cfu/mL) | Antiadhesion activity (%) |
| *Bacillus cereus* | 5.23 ± 0.17 | 4.26 ± 0.35 | 22.03 ± 2.45 | 5.14 ± 0.15 | 2.86 ± 0.04 |
| *Listeria monocytogenes* | 6.76 ± 0.30 | 5.42 ± 0.35 | 20.01 ± 1.93 | 6.46 ± 0.26 | 4.54 ± 1.12 |
| *Escherichia coli* | 6.06 ± 0.03 | 6.19 ± 0.04 | Not detected | 5.93 ± 0.07 | 1.43 ± 0.27 |
| *Yersinia enterocolitica* | 6.44 ± 0.14 | 6.22 ± 0.15 | 3.51 ± 0.29 | 6.06 ± 0.07 | 5.98 ± 1.03 |

The results are expressed as % of adhesion prevention in comparison with the control sample (bacteria adhered without the presence of tested samples). Results are the means of three determinations ± standard deviation.

Our study showed that OP powders exhibited antiadhesion activity towards almost all bacteria, varying between 1.4 – 22%. The most pronounced effect was detected for POPP against Gram-positive bacteria. *Bacillus cereus* (22.03 ± 2.45%) and *Listeria monocytogenes* (20.01 ± 1.93) adhesions were inhibited at least at 20%. POPP is mostly composed by IDF, but it also contains a considerable amount of free and bound phenolic compounds, which may act as anti-adhesion agents. For example, luteolin is a reported antimicrobial agent against several bacterial species that was retained in higher amounts in POPP undigested fraction (Aziz *et al.*, 2018; Sousa *et al.*, 2006).

LOPP displayed a weaker antiadhesion activity against *Gram*-positive bacteria (2.86 – 4.54%) than POPP but demonstrated a higher capacity to inhibit *Gram*-negative bacteria's adhesion. LOPP exhibited a low capacity to inhibit the adhesion of *Escherichia coli* (1.43 ± 0.27%) and the double of the capacity of POPP from inhibiting the adhesion of *Yersinia enterocolitica* (5.98 ± 1.03%). Its phenolic profile could explain the lower antiadhesion activity of LOPP despite its higher phenolics content. When comparing LOPP and POPP phenolic profile, it can be concluded that LOPP retained higher amounts of tyrosol and tyrosol glucoside in the colon after digestion. However, tyrosol was reported as the least antimicrobial active phenolic molecule from secoiridoid breakdown in *Olea europaea* (Thielmann *et al.*, 2017). On the other hand, the low but higher LOPP capacity to inhibit

*Gram*-negative bacteria could result from synergistic effects of phenolic compounds and other non-phenolics such as minerals (Costa *et al.*, 2019) and organic acids (Östling and Lindgren, 1993).

In the present study, only one concentration was tested (2% m/v) to simultaneously evaluate the anti-adhesion capacity and potential prebiotic effect of OP powders. However, other concentrations should be tested to clarify a possible dose-dependent inhibition activity.

## 7.4. Conclusion

Prebiotics are defined as substances that induce the growth or metabolic activity of microorganisms that contribute to their host's well-being. In this sense, olive pomace powders showed to confer health benefits to the gastrointestinal tract as promoters of short-chain fatty acid production by gut microbiota in a higher degree than fructooligosaccharides, thus contributing to the improvement of several health factors. Equally, olive pomace powders were shown to be a significant source of phenolics metabolites after LC-ESI-UHR-QqTOF-MS detection. Supervised cluster and hierarchical clustering analysis allowed to evaluate the metabolite patterns of olive pomace powders phenolics throughout *in vitro* faecal fermentation, explaining the potential antioxidant activity and antiadhesion ability against food pathogens discovered to these powders. Other potential gut health benefits could also be expected to olive pomace powders.

The development of new prebiotics and/or gut-health-benefits powders from olive pomace could be an opportunity of adding value to the higher by-product stream from the modern olive oil production. However, before developing added value secondary product lines, future studies using pH control, cell lines or even clinical studies are needed to clarify the potential prebiotic health benefits of olive pomace powders.

# PART IV

## *Prototype Application in a Food Matrix*

# Chapter 8.

# Incorporation of olive pomace ingredients into yoghurts as a source of fibre and hydroxytyrosol: Antioxidant activity and stability throughout gastrointestinal digestion

## Abstract

The liquid-enriched powder (LOPP) and pulp-enriched powder (POPP) obtained from olive pomace were incorporated into yoghurt to increase its dietary fibre content hydroxytyrosol and unsaturated fatty acids, but also to understand the lipids-phenolics interaction by simultaneous incorporation of olive oil. POPP (2%) and LOPP (1%) addition to yoghurt allowed fulfilling the condition on being a "source of fibre" and provides 5 mg of hydroxytyrosol and derivatives in a standard yoghurt (120 g), respectively. Yoghurts' unsaturated fatty acids profile was positively influenced by the addition of only POPP and olive oil +LOPP or +POPP. All OP powder-fortified yoghurts exhibited higher total phenolic content and antioxidant activity than control ($p$ < 0.05). After *in vitro* digestion, the bioaccessibility of total phenolics (more 25.58%) and hydroxytyrosol (more 68.71%) in LOPP-yoghurts were improved by the addition of olive oil. In conclusion, OP powders´ incorporation given additional and essential healthy properties to yoghurt.

**Keywords:** Olive pomace; Yoghurt; Hydroxytyrosol; Dietary fibre; Unsaturated fatty acids; Bioaccessibility.

## 8.1. Introduction

The current consumers' awareness about the importance of diet to health fostered the development of functional and/or fortified foods (Hashemi Gahruie *et al.*, 2015). Food fortification, defined as the addition of one or more essential nutrients to food to levels higher than usual with the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients, is also a way of enhancing the nutritional value and potential health benefits of food (Gahruie *et al.*, 2015).

The consumption of fortified foods has increased in the last decade, principally in dairy products (Hashemi Gahruie *et al.*, 2015; Helal and Tagliazucchi, 2018). Fortified dairy products, such as yoghurt, have overrun traditional products' appeal (Baba *et al.*, 2018). Yoghurt is highly consumed and appreciated for its nutritional value and positive health benefits mainly associated to the presence of relevant nutrients (e.g. protein, calcium) combined with functional ingredients such as bioactive peptides, prebiotics and probiotics (Helal and Tagliazucchi, 2018; Oliveira and Pintado, 2015). The global yoghurt market was valued at USD 99,553.38 million in 2019, and it is estimated to reach USD 141,829.95 million by 2025 (CAGR of 6.25%, from 2020 to 2025). The yoghurt market has viewed significant growth due to the rise of health-conscious consumers. Consequently, players in the yoghurt market are coming up with various healthy and flavour options to satisfy consumer preferences (Mordor Intelligence, 2019).

Among the food components that yoghurt does not contain, the dietary fibre (DF), phenolic compounds and unsaturated fatty acids (UFAS) can be highlighted. Diets with high DF, phenolics and UFAs content play a significant role in preventing several diseases (Román *et al.*, 2019). As a result, natural sources such as fruits and cereals have been used to fortify yoghurts with phenolics (Helal and Tagliazucchi, 2018) and DF (Hashemi Gahruie *et al.*, 2015). However, limited research concerning the fortification of yoghurt UFAS such as monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) to improve the lipid profile of yoghurts has been reported (Baba *et al.*, 2018; Dal Bello *et al.*, 2015). Among the few studies reported to improve fatty acids (FAs) profile of yoghurts, vegetable oils and fish oils were usually used (Baba *et al.*, 2018; Dal Bello *et al.*, 2015; Van Nieuwenhove *et al.*, 2019).

More recently, sustainability concerns have stimulated the food industry to develop added-value food ingredients from their by-products and use them to fortify yoghurts. Some examples are the incorporation of pomegranate peel powder (Kennas *et al.*, 2020) to boost the antioxidant activity (AOX) of yoghurt, as well as the addition to yoghurt of hazelnut skins (Bertolino *et al.*, 2015) and wine grape pomace powder (Tseng and Zhao, 2013) as a simultaneous source of DF and phenolics. More recently, pomegranate seeds obtained from the juice industry were incorporated into yoghurt for its enrichment in conjugated linolenic acid and antioxidant compounds (Van Nieuwenhove *et al.*, 2019).

Olive pomace (OP) powders from the olive oil (OO) industry can be an attractive source of the same bioactive compounds reported above. OP is a semi-solid by-product obtained from the widely implemented 2-phase, being the most abundant and relevant by-product of the modern OO industry (Dermeche *et al.*, 2013; Nunes *et al.*, 2018). This by-product is a combination of olive husk and pulp,

crushed olive stone and olive mill wastewater (moisture content of 65%) and it is associated with severe environmental problems and waste management costs (Moreno-Maroto *et al.*, 2019). According to different authors, one hectare of olive tree originates about 2500 kg of olives (Rodrigues *et al.*, 2015) and approximately 40-70 kg of OP per 100 kg of olives are produced (AGAPA, 2015; Nunes *et al.*, 2016; Romero-García *et al.*, 2014; Ruiz *et al.*, 2017). Nowadays, OP is mainly applied in the energy sector (electricity generation or cogeneration, and thermal uses). Other typical applications are the direct incorporation of OP into the soil, composting and finally, as animal feed (AGAPA, 2015). However, these traditional OP treatments waste a significant amount of high value-added bioactive compounds as DF, MUFAs, PUFAs and phenolic compounds, associated with several health benefits and potential economic incomings to OO producers (Dermeche *et al.*, 2013; Nunes *et al.*, 2018). So, it is preeminent to adopt valorisation approaches as the development of food ingredients to increase the OP value and consequently enhance the olive oil sector's economic and environmental sustainability.

In our previous works, we have developed two biologically safe food powdered ingredients: the liquid-enriched olive pomace powder (LOPP) and the pulp-enriched olive pomace powder (POPP) (Ribeiro *et al.*, 2020b). The production of powdered ingredients has been proposed as a more feasible and low environmental impact approach in comparison to the traditional (involve the use of organic solvents) and emergent technologies (possess higher operational costs) with the advantage of retaining all the bioactive compounds of food by-products without any extraction step (García-Lomillo *et al.*, 2014). Indeed, a ‘‘whole by-product valorisation” could be attained producing these multifunctional powdered ingredients (Crizel *et al.*, 2016; Gouw *et al.*, 2017; Saura-Calixto, 1998).

POPP was characterised to be a potential antioxidant dietary fibre (ADF) source that can deliver the physiological effects of both DF and antioxidants (Ribeiro *et al.*, 2020b). Besides, POPP also contains significant MUFA (mainly oleic acid corresponding to 70% of POPP total FAs) and PUFA amounts (mainly linoleic acid which corresponds to 6% of POPP total FAs) (Supplementary material 1). The liquid-enriched olive pomace powder (LOPP) exhibits high hydroxytyrosol (HYD) and derivatives levels (Ribeiro *et al.*, 2020a). HYD and its derivatives are well-known antioxidant compounds. Indeed, a daily intake of 5 mg of HYD and derivatives protects low-density lipoprotein (LDL) particles from oxidative damage, according to the health claim approved by the EFSA (until now only allowed in OO) (EFSA, 2011). The significant content of LOPP and POPP in antioxidants could also be an opportunity to protect other UFAs sources added to yoghurt. UFAs are easily prone to oxidation, and its incorporation in food with AOX such as those containing phenolics may have an adjuvant effect against lipid oxidation (Román *et al.*, 2019). Besides that, both OP powders demonstrated to be biologically safe and demonstrated adequate functional properties for food applications (Ribeiro *et al.*, 2020b).

OP has been incorporated in food products, principally in the formulation of bakery products as biscuits and bread (Conterno *et al.*, 2019; Di Nunzio *et al.*, 2020) Regarding yoghurt formulations, other olive-derived powders from olive green (Cho *et al.*, 2017) or extracts from the olive leaf (Cho *et al.*, 2020; Peker and Arslan, 2017; Zoidou *et al.*, 2017), olive-mill wastewater (phenolic

concentrate) (Servili *et al.*, 2011) and three-phase oil extraction process (Aliakbarian *et al.*, 2015) have been tested in the preparation of functional milk beverages or yoghurts without interfering with the fermentation process and probiotic counts (Aliakbarian *et al.*, 2015; Servili *et al.*, 2011). The yoghurt matrix can be a great carrier of phenolic compounds. Proteins or large peptides present in yoghurts have been reported to have the capacity to maintain phenolics integrity during digestion (Helal and Tagliazucchi, 2018). Lipids were also described as protectors of phenolics, improving their stability during digestion (Jakobek, 2015). So, yoghurts' components could increase phenolics protection and bioaccessibility (Helal and Tagliazucchi, 2018). The bioaccessibility definition comprises the release of compounds from food matrices and their stability under the gastrointestinal condition (Helal and Tagliazucchi, 2018). However, to the author's knowledge until now, ingredients obtained from OP have never been applied to yoghurt formulations to improve its DF simultaneously, UFAs and phenolics compound content. Yoghurts fortified with OP powders are not only an excellent way to improve the daily intake of DF, phenolic compounds and UFAs but also an opportunity to dairy industry achieve new "sustainable" products - which is a new and growing food category at the same time that OP was valorised (Coderoni and Perito, 2020). This study brings new insights to help spread the circular bioeconomy concept in the whole food sector.

In this context, the present study's main objective was to evaluate the potential of OP powders to enhance the nutritional and functional value of yoghurt as a source of DF, UFAs and phenolic compounds, and to evaluate the bioaccessibility of phenolics and the AOX during in vitro SGD. The potential interaction of phenolics-lipids was also analysed by the simultaneous incorporation of OO and OP powders.

## 8.2. Materials and methods

### 8.2.1. Chemicals and reagents

The ABTS diammonium salt (2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), methanol, potassium sorbate and sodium carbonate were purchased from Sigma-Aldrich (Sintra, Portugal). Folin-Ciocalteu's reagent and potassium persulfate were purchased from Merck (Algés, Portugal). Standards of Trolox, gallic acid, p-coumaric acid, vanillin, protocatechuic acid, caffeic acid and quercetin were obtained from Sigma-Aldrich (Sintra, Portugal), whereas HYD, tyrosol, luteolin were purchased from Extrasynthese (Lyon, France).

### 8.2.2. Preparation of olive pomace powders

OP was collected from an olive mill from Oliveira do Hospital, Portugal. Homogenous samples of OP were packed in polyethene flasks and kept in a freezer at - 80 ºC until use to avoid the phenolics damage.

OP was fractionated by centrifugation (10,000 ×*g* for 10 min). The liquid fraction was freeze-dried (Telstar Lyo Quest HT 40) with 2% of mannitol (as a cryoprotectant and to prevent aggregation), and

the powder obtained was denominated liquid-enriched olive pomace powder (LOPP). The solid fraction was oven-dried (90 °C, water activity < 0.4, 90 min), milled using a coffee grinder and sieved (mesh 40). All the stones pieces (potential physical hazard) were removed to obtain a food-grade ingredient denominated as pulp-enriched olive pomace powder (POPP).

### 8.2.3. Fortification of yoghurts with the OP powders

Yoghurts were prepared using homogenised (180 bar), pasteurised, and whole milk (supplemented with 3% milk powder (w/v)). The starter culture (fresh yoghurt) was added at 2% to the milk after cooling down to 44 °C. The mixture was packed and then fermented at 42 °C (oven) until the final pH of 4.6 (about 4.5 h). Six yoghurt formulations were obtained, including a control yoghurt (Y-control) and yoghurt supplemented with OO (Y-OO) both without OP powders. Supplementation of yoghurts with 5% of OO (w/v) was added before homogenisation step. LOPP and POPP were incorporated before pasteurisation (90 °C) in order to obtain OP-fortified yoghurts without OO (Y-LOPP and Y-POPP) and with OO (Y-LOPP-OO and Y-POPP-OO). LOPP was added at 1% to provide the amount of HYD (5 mg) that would be needed to protect LDL particles from oxidative damage, according to the health claim (EFSA, 2011). The amount of LOPP was added in excess due to possible losses unintentionally caused by the thermal and mechanical procedures during yoghurt preparation. On the other hand, the Y-POPP (2% POPP) was formulated to fulfil the condition of being a “source of fibre” (> 1.5 g of fibre per 100 kcal) (European Commission, 2006b). Supplementation with 5% of OO was used in OP-fortified yoghurts formulation with the aim that at least 45% of FAs present in the yoghurt derive from MUFAs. A flow diagram of the development of OP powders and the fortified yoghurts is present in Figure 8.1.

### 8.2.4. Chemical characterisation of yoghurts

#### 8.2.4.1. Proximate composition

The crude protein content was determined using the Kjeldahl method, with a conversion factor of 6.25. The lipid content was obtained using an automated Soxhlet Soxtec™ 8000 (Foss, Spain) for 4 h using *n*-hexane as a solvent. The ash content was determined in a muffle furnace (AOAC No. 942.05) according to the AOAC (1990). DF (TDT, SDF and IDF) was measured using the Megazyme Total Dietary analysis kit according to the enzymatic gravimetric method (991.43; AOAC (1990)), with slight modification in obtaining process of SDF as described previously by Ribeiro *et al.*, (2020b). SDF was obtained by dialysis (dialysis tube with 3.5 kDa) to avoid the error caused by ethanol precipitation of SDF. Afterwards, SDF was recovered by dialysate freeze-drying. All measurements were done in triplicate and expressed as g/100 g dry weight (DW).

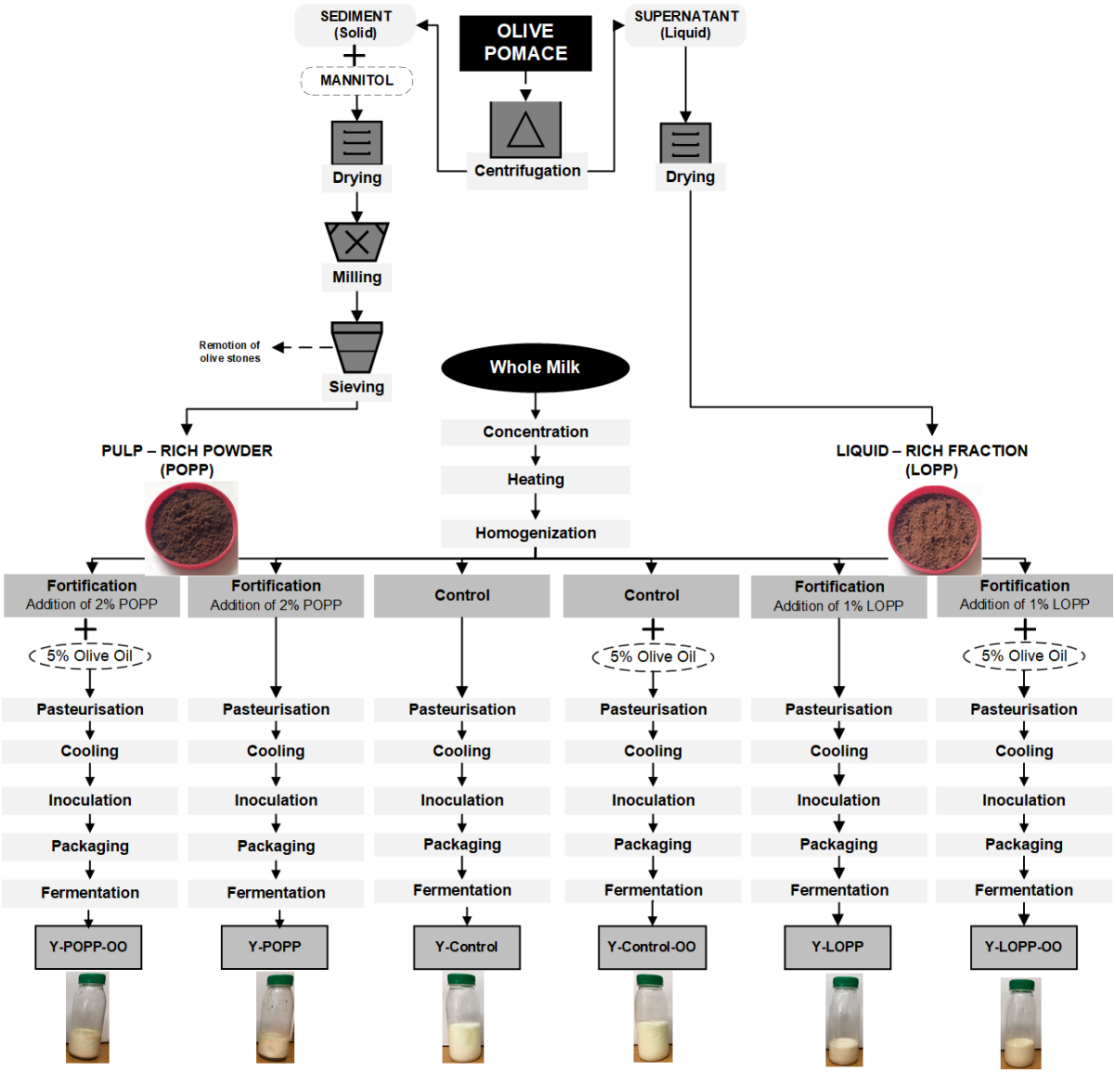



**Figure 8.1. Flow diagram of the development of olive pomace powders (LOPP and POPP), fortified yoghurts, and controls.**

### 8.2.4.2. Analyses of fatty acids and related health lipid indices

The yoghurts' FA profiles were obtained and analysed following the methodology of Pimentel, Fontes, Gomes, & Rodríguez-Alcalá (2015) with some modifications as described previously by Ribeiro *et al.* (2020b).

Nutritional quality indices of all yoghurts' formulations were analysed from FAs composition data. The indices of thrombogenicity (*TI*) and atherogenicity (*AI*) were calculated using Eqs. (1) and (2), respectively. Other nutritional quality indices, namely PUFA/SFA and Saturation Index (*SI*) (Eq. (3)) were also determined (de Alba *et al.*, 2019).

$$TI = \frac{[C14{:}0 + C16{:}0 + C18{:}0]}{\left[0.5 \times (\sum MUFA + \sum n6) + 3 \times \sum n3 + \frac{\sum n3}{\sum n6}\right]} \qquad Equation\ (1)$$

$$AI = \frac{[C12:0 + 4 \times C14:0 + C16:0]}{[\sum MUFA + \sum PUFA]} \qquad Equation\ (2)$$

$$SI = \frac{[C14:0 + C16:0 + C18:0]}{[\sum MUFA + \sum PUFA]} \qquad Equation\ (3)$$

#### 8.2.4.3. Phenolic compounds and antioxidant activity

According to Oliveira & Pintado (2015), phenolic extracts from yoghurts' formulations were obtained with some modifications. This procedure was adopted in order to reduce the interferences from peptides. Each yoghurt formulation (in triplicate) was homogenised with 30 mL of methanol acidified with formic acid (9:1 v/v), using an orbital shaker at 250 RPM, for 1 h. The homogenised sample was centrifuged at 4000 × g, at 4 °C for 10 min, and the supernatant kept at -20 °C overnight, to allow for protein precipitation. Then the slurry was centrifuged again to remove soluble proteins. The extract was evaporated to dryness in a speed- vacuum evaporator at 30 ºC and the residue dissolved in 2 mL of methanol for further analysis.

The total phenolic content (TPC) of extracts was determined according to the Folin-Ciocalteau method. Results were expressed as mg gallic acid equivalents (GAE)/100 g DW.

The HPLC analysis was performed using a Waters e2695 separation module system interfaced with a Photodiode array UV/Vis detector (PDA 190-600 nm) as described by Ribeiro *et al.* (2020a).

The AOX of yoghurts extracts was achieved according to the methods of DPPH, ABTS and ORAC (Costa *et al.*, 2019; Ribeiro *et al.*, 2020a) using a multidetection plate reader (Synergy H1, Vermont, USA). The radical stock solutions were freshly prepared. All analyses were performed in triplicate and expressed in mM of Trolox-equivalents (TE)/g DW.

### 8.2.5. *In vitro* digestion

The *in vitro* simulation of gastrointestinal digestion (SGD) was performed according to the method described previously by Ribeiro *et al.* (2020a), using the dialyses process to simulate the intestinal and blood absorption. At the end of the incubation process, the solution left outside the dialysis tubing was taken as the OUT sample representing material that remained in the gastrointestinal tract (colon-available). The solution managed to diffuse into the dialysis tubing was taken as the IN sample (serum-available).

To screen the release of individual phenolics from yoghurt matrices (100 g) at different stages of digestion, samples of yoghurt were collected from the mouth (ca. 20 mL), gastric (ca. 20 mL), and intestinal digestion step (ca. 20 mL) and used to make extracts to further phenolics analysis. Three replicas from the SGD system were made.

#### 8.2.5.1. Recovery and bioaccessibility index of phenolic compounds

The results of each extract determination (on the sample, after mouth, gastric and intestinal digestion) were reported in 100 g of DW of yoghurt.

Recovery index (RI%) and bioaccessibility index (BI%) were studied to evaluate the effect of the yoghurt composition on the digestion of its phenolic compounds (Lucas-Gonzalez *et al.*, 2016).

The percentage of recovery (RI%) allows the determination of the amount of phenolics on food sample present in the digested sample after oral, gastric and intestinal step, according to Eq. 4:

$$Recovery\ index\ (\%) = \left(\frac{PC_{DF}}{PC_{TF}}\right) \times\ 100 \qquad Equation\ (4)$$

Where $PC_{DF}$ is the phenolic content (mg) in the digested, and $PC_{TF}$ is the phenolic content (mg) quantified in the test matrix.

The bioaccessibility index is defined as the percentage of the phenolic compound that is solubilised after intestinal dialysis step. Thus, this index defines the proportion of the phenolic compound that could become available for absorption into the systematic circulation (Eq. 5):

$$Bioaccessibility\ index\ (\%) = \left(\frac{PC_{S}}{PC_{DFE}}\right) \times\ 100 \qquad Equation\ (5)$$

where: $PC_S$ is the phenolic content (mg) in the digested sample after the dialysis step (IN) and $PC_{DFE}$ is the total phenolic content (mg) in the digested sample after the dialysis step (IN + OUT).

#### 8.2.5.2. Antioxidant activity: ABTS and DPPH

The AOX of yoghurts during the SGD was achieved according to DPPH and ABTS methods as reported above.

### 8.2.6. Statistical analyses

Software R was used to carry out statistical analyses. All experiments were carried out in triplicates, and data were reported as mean ± standard deviation. The Shapiro - Wilk test tested the normality of data distribution. The differences of mean values were analysed by one-way analysis of variance (ANOVA). Tukey's post hoc test was used for comparisons of means; differences were considered significant at $p < 0.05$.

Principal Component Analysis (PCA) and Discriminant Analysis (PLS-DA)) were applied to evaluate the nutrients and bioactives patterns of OP powders-fortified yoghurts using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) on data after autoscaling (mean-centred and divided by the standard deviation of each variable).

## 8.3. Results and Discussion

### 8.3.1. Chemical characterisation of yoghurts

Table 8.1 shows the proximate and FAs composition of the yoghurts. Moisture decreased in all fortified yoghurts in comparison with control (Y-control), but the differences observed were not statistically significant ($p > 0.05$). Regarding ash content, yoghurts fortified with LOPP exhibited statistically significant higher ash amount ($p < 0.05$) than the other yoghurt formulations. On the other hand, POPP's addition decreased the ash content compared to control (Y-control). The protein content of yoghurts was maintained between 23 – 28% (DW) without significant differences ($p < 0.05$). The addition of OO and OP powders did not negatively affect yoghurts' composition as a rich protein source. Total fat content was significantly higher in yoghurt formulations with OO (32-34% DW) than in the other formulations without OO (20-22% DW). LOPP and POPP addition had a similar decreasing effect on yoghurt's total fat content, statistically significant ($p < 0.05$) in yoghurt formulations with OO. Similar results on moisture, ash, protein and lipids content were reported in yoghurts fortified with hazelnut skins as a source of fibre and phenolics (Bertolino *et al.*, 2015) and in yoghurts fortified with omega-3 (ω-3) FAs from vegetable sources (Dal Bello *et al.*, 2015).

The addition of OP powders was associated with an increase in the TDF levels. The higher increase was detected in the POPP-fortified yoghurts, which exhibited TDF amounts of 6.42 ± 0.03 g/100 g (DW) in Y-POPP and 7.57 ± 0.19 g/100 g (DW) in Y-POPP-OO, with no significant differences between both ($p > 0.05$). Similar TDF increase in yoghurt due to added fibre was achieved by Tseng & Zhao (2013) and Bertolino *et al.* (2015) using wine grape pomace and hazelnut skins, respectively. Regarding SDF and IDF content, the highest IDF concentration was observed in Y-POPP and Y-POPP-OO as expected, but the SDF content was similar for all OP powders – fortified yoghurts due to the identical SDF content of both OP powders ($p > 0.05$).

**Table 8.1. Proximate composition of fortified yoghurts and control.**

| | Yoghurt formulations without olive oil | | | Yoghurt formulations with olive oil | | |
|---|---|---|---|---|---|---|
| | **Control** | **LOPP fortified** | **POPP fortified** | **Control** | **LOPP fortified** | **POPP fortified** |
| **Moisture** (g/100 g WW) | 83.39 ± 1.21 $^{a}$ | 82.89 ± 0.30 $^{a}$ | 83.30 ± 0.34 $^{a}$ | 81.23 ± 1.84 $^{a}$ | 81.09 ± 2.89 $^{a}$ | 82.13 ± 1.93 $^{a}$ |
| **Ash** (g/100 g DW) | 5.69 ± 0.01 $^{ab}$ | 5.84 ± 010 $^{a}$ | 5.51 ± 0.24 $^{abc}$ | 4.87 ± 0.23 $^{bc}$ | 6.03 ± 0.16 $^{a}$ | 4.55 ± 0.09 $^{c}$ |
| **Protein** (g/100 g DW) | 28.13 ± 0.83 $^{a}$ | 26.38 ± 0.92 $^{a}$ | 27.40 ± 0.49 $^{a}$ | 23.43 ± 1.52 $^{a}$ | 24.58 ± 0.35 $^{a}$ | 24.17 ± 0.89 $^{a}$ |
| **Dietary fibre** (g/100 g DW) | | | | | | |
| *TDF* | nd | 3.62 ± 0.26 $^{b}$ | 6.42 ± 0.03 $^{a}$ | nd | 2.75 ± 0.50 $^{b}$ | 7.57 ± 0.19 $^{a}$ |
| *IDF* | nd | 2.24 ± 0.07 $^{b}$ | 5.12 ± 0.12 $^{a}$ | nd | 0.93 ± 0.19 $^{c}$ | 5.76 ± 0.22 $^{a}$ |
| *SDF* | nd | 1.38 ± 0.20 $^{a}$ | 1.31 ± 0.09 $^{a}$ | nd | 1.82 ± 0.48 $^{a}$ | 1.81 ± 0.22 $^{a}$ |
| **Fat** (g/100 g DW) | 22.47 ± 0.61 $^{c}$ | 19.87 ± 0.16 $^{d}$ | 21.23 ± 0.26 $^{cd}$ | 34.43 ± 0.53 $^{a}$ | 32.28 ± 0.27 $^{b}$ | 32.12 ± 0.37 $^{b}$ |

nd - non-detected; Results are the means of three determinations ± standard deviation. Values with different letters in the same line are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

### 8.3.2. Fatty acid composition

The FAs profile in yoghurts with different OP powders supplemented with 5 % OO or not, is shown in Table 8.2. Addition of OP powders in yoghurt samples showed to have a lower effect on the amount of SFAs. In all yoghurt formulations, the most abundant SFA was palmitic acid (C16:0) as reported before in several yoghurt studies (Marand *et al.*, 2020; Baba *et al.*, 2018; Van Nieuwenhove *et al.*, 2019). As a poor FAs source, LOPP did not affect yoghurt's FA content, but POPP seems to have enhancer effect in UFAs. However, when LOPP or POPP were incorporated together with OO, a significant UFAs increase occurred. Y-LOPP-OO and Y-POPP-OO showed a significantly higher UFAs content than the Y-control and Y-OO ($p < 0.05$). LOPP and POPP appeared to protect UFAs, principally the MUFAs. Y-LOPP-OO and Y-POPP-OO showed the most significant MUFA amounts. The OP powders´ richness in phenolics seemed to enhance OO stability. Similar lipid protector effect was reported when a cocoa bean husk phenolic extract was added to extra virgin olive jam (Hernández-Hernández *et al.*, 2019).

Oleic acid (C18:1 *c9*) was the most abundant MUFA in all yoghurt formulations, being significantly higher in Y-LOPP-OO and Y-POPP-OO ($p < 0.05$). PUFAs were also positively affected by the simultaneous addition of OO and OP powders ($p < 0.05$) when compared to Y-control and Y-OO. However, the POPP incorporation without OO (Y-POPP) also increased the PUFAs amount significantly in comparison to Y-control. The addition of oleic acid, PUFAs or combinations of both to dairy products has been used to produce healthier products (Lopez-Huertas, 2010).

A normal balance between omega-6 (ω-6) and omega-3 (ω-3) in the range of 4:1 to 10:1 is also crucial in order to obtain healthier products (Ardabilchi Marand *et al.*, 2020). ω-3 and ω-6 are essential FAs not synthesised by mammals and thus must be obtained from the diet. They include the ω-3 linoleic acid (LA, C18:2 *c*9 *c*12), ω-6 α-linoleic acid (ALA, $\alpha$ C18:3 *c*9 *c*12 *c*15) and ω-6 arachidonic acid (ARA, C20:4 c*5* c*8* c*11* c14) (Román *et al.*, 2019). After POPP incorporation, the total ω-3 and ω-6 amount increased significantly ($p < 0.05$) in Y-POPP. Y-LOPP-OO and Y-POPP-OO also exhibited significantly higher content of total ω-3 and ω-6 than controls ($p < 0.05$). For that reason, all fortified yoghurts showed significant lower ω-6/ω-3 ratio than the controls, Y-LOPP-OO (8.53 ± 0.71), Y-POPP-OO (8.39 ± 0.19) against Y-OO control (9.93 ± 0.90) and Y-POPP (10.41 ± 0.44) and Y-LOPP (10.87 ± 0.32) against Y-control (11.56 ± 0.11).

The recommended ω-6/ω-3 ratio 4:1 to 10:1 ratio was achieved in both OO-fortified yoghurts and Y-POPP. OP powders allowed to reduce ω-6/ω-3 ratio ca. 10% in Y-POPP and 26 - 27% in OO formulations (Y-LOPP-OO and Y-POPP-OO). In previous studies the addition of oil (Baba *et al.*, 2018) and powder (Ardabilchi Marand *et al.*, 2020) from flaxseed, which is an oilseed known by its low ω-6/ω-3 ratio, reduced in 29% and 89% the ω-6/ω-3 ratio of yoghurt samples, respectively. However, a ratio lower than 1:1 was obtained, which could compromise ω-6 metabolism (Simopoulos, 2002). Thus, the addition of single POPP and both OP powders with OO increased MUFA, and PUFA content yoghurts also improved the ω-6/ω-3 ratio.

**Table 8.2. Fatty acid composition of fortified yoghurts and control.**

| **Fatty acids** (mg/g DW) | **Yoghurt formulations without olive oil** | | | **Yoghurt formulations with olive oil** | | |
|---|---|---|---|---|---|---|
| | **Control** | **LOPP fortified** | **POPP fortified** | **Control** | **LOPP fortified** | **POPP fortified** |
| *C6:0* | 1.37 ± 0.03 $^{a}$ | 1.17 ± 0.14 $^{abc}$ | 1.18 ± 0.06 $^{abc}$ | 1.27 ± 0.02 $^{ab}$ | 0.92 ± 0.16 $^{bc}$ | 0.89 ± 0.03 $^{c}$ |
| *C8:0* | 1.30 ± 0.02 $^{a}$ | 1.12 ± 0.11 $^{abc}$ | 1.12 ± 0.05 $^{abc}$ | 1.20 ± 0.02 $^{ab}$ | 0.88 ± 0.18 $^{bc}$ | 0.83 ± 0.02 $^{c}$ |
| *C9:0* | 42.99 ± 3.25 $^{a}$ | 34.61 ± 6.57 $^{ab}$ | 36.96 ± 3.76 $^{ab}$ | 39.37 ± 2.08 $^{ab}$ | 25.41 ± 5.13 $^{b}$ | 36.07 ± 2.35 $^{ab}$ |
| *C10:0* | 3.96 ± 0.06 $^{a}$ | 3.52 ± 0.22 $^{abc}$ | 3.48 ± 0.07 $^{abc}$ | 3.65 ± 0.11 $^{ab}$ | 2.79 ± 0.64 $^{bc}$ | 2.52 ± 0.09 $^{c}$ |
| *C12:0* | 5.97 ± 0.08 $^{a}$ | 5.47 ± 0.16 $^{ab}$ | 5.36 ± 0.03 $^{ab}$ | 5.54 ± 0.23 $^{ab}$ | 4.36 ± 1.15 $^{ab}$ | 3.82 ± 0.14 $^{b}$ |
| *C14:0* | 23.67 ± 0.32 $^{a}$ | 22.33 ± 0.10 $^{ab}$ | 21.82 ± 0.43 $^{ab}$ | 22.21 ± 1.13 $^{ab}$ | 17.97 ± 2.70 $^{bc}$ | 15.31 ± 0.59 $^{c}$ |
| *C15:0* | 2.45 ± 0.03 $^{a}$ | 2.35 ± 0.04 $^{a}$ | 2.28 ± 0.07 $^{a}$ | 2.34 ± 0.11 $^{a}$ | 1.91 ± 0.33 $^{ab}$ | 1.61 ± 0.05 $^{b}$ |
| *C16:0* | 81.27 ± 1.05 $^{a}$ | 78.58 ± 1.90 $^{a}$ | 79.23 ± 2.85 $^{a}$ | 93.74 ± 8.94 $^{a}$ | 88.98 ± 14.97 $^{a}$ | 78.30 ± 3.23 $^{a}$ |
| *C17:0* | 1.31 ± 0.02 $^{a}$ | 1.32 ± 0.04 $^{a}$ | 1.32 ± 0.06 $^{a}$ | 1.47 ± 0.06 $^{a}$ | 1.23 ± 0.25 $^{a}$ | 1.14 ± 0.04 $^{a}$ |
| *C18:0* | 26.31 ± 0.41 $^{a}$ | 25.79 ± 1.08 $^{a}$ | 25.68 ± 1.16 $^{a}$ | 28.15 ± 0.10 $^{a}$ | 23.36 ± 5.60 $^{a}$ | 20.92 ± 0.61 $^{a}$ |
| *C10:1 c2* | 0.33 ± 0.00 $^{a}$ | 0.30 ± 0.03 $^{abc}$ | 0.29 ± 0.01 $^{abc}$ | 0.31 ± 0.01 $^{ab}$ | 0.23 ± 0.05 $^{bc}$ | 0.21 ± 0.07 $^{bc}$ |
| *C14:1 c9* | 1.17 ± 0.01 $^{a}$ | 1.12 ± 0.01 $^{ab}$ | 1.08 ± 0.03 $^{ab}$ | 1.10 ± 0.07 $^{ab}$ | 0.93 ± 0.12 $^{bc}$ | 0.76 ± 0.04 $^{c}$ |
| *C15:1 c10* | 0.67 ± 0.01 $^{a}$ | 0.66 ± 0.01 $^{a}$ | 0.64 ± 0.02 $^{a}$ | 0.65 ± 0.05 $^{a}$ | 0.52 ± 0.08 $^{ab}$ | 0.44 ± 0.02 $^{b}$ |
| *C16:1 t9* | 0.17 ± 0.01 $^{a}$ | 0.17 ± 0.01 $^{a}$ | 0.20 ± 0.05 $^{a}$ | 0.22 ± 0.05 $^{a}$ | 0.15 ± 0.02 $^{a}$ | 0.11 ± 0.01 $^{a}$ |
| *C16:1 c7* | 0.53 ± 0.02 $^{a}$ | 0.51 ± 0.01 $^{a}$ | 0.51 ± 0.01 $^{a}$ | 0.60 ± 0.06 $^{a}$ | 0.56 ± 0.04 $^{a}$ | 0.50 ± 0.01 $^{a}$ |
| *C16:1 c9* | 3.82 ± 0.06 $^{a}$ | 3.64 ± 0.02 $^{a}$ | 3.86 ± 0.08 $^{a}$ | 5.55 ± 1.41 $^{a}$ | 5.81 ± 0.62 $^{a}$ | 5.37 ± 0.21 $^{a}$ |
| *C17:1 c10* | 0.53 ± 0.00 $^{ab}$ | 0.50 ± 0.04 $^{b}$ | 0.57 ± 0.07 $^{ab}$ | 0.85 ± 0.17 $^{ab}$ | 0.87 ± 0.12 $^{a}$ | 0.85 ± 0.04 $^{ab}$ |
| *C18:1 t11* | 4.54 ± 0.01 $^{a}$ | 4.25 ± 0.21 $^{a}$ | 4.25 ± 0.15 $^{a}$ | 4.89 ± 0.77 $^{a}$ | 3.31 ± 0.64 $^{ab}$ | 2.60 ± 0.04 $^{b}$ |
| *C18:1 c9* | 54.80 ± 0.77 $^{b}$ | 54.36 ± 0.72 $^{b}$ | 70.01 ± 4.67 $^{b}$ | 87.71 ± 6.73 $^{b}$ | 148.23 ± 30.45 $^{a}$ | 154.09 ± 6.46 $^{a}$ |
| *C18:1 c11* | 2.01 ± 0.04 $^{b}$ | 1.99 ± 0.02 $^{b}$ | 2.65 ± 0.08 $^{b}$ | 8.27 ± 0.40 $^{a}$ | 7.46 ± 0.64 $^{a}$ | 7.09 ± 0.27 $^{a}$ |
| *C18:1 c12* | 0.85 ± 0.04 $^{a}$ | 0.85 ± 0.05 $^{a}$ | 0.80 ± 0.04 $^{a}$ | 0.84 ± 0.05 $^{a}$ | 0.60 ± 0.06 $^{b}$ | 0.53 ± 0.04 $^{b}$ |
| *C18:1 c13* | 0.27 ± 0.02 $^{a}$ | 0.28 ± 0.02 $^{a}$ | 0.26 ± 0.01 $^{a}$ | 0.29 ± 0.00 $^{a}$ | 0.22 ± 0.05 $^{a}$ | 0.20 ± 0.00 $^{a}$ |
| *C18:1 c14+t16* | 0.74 ± 0.06 $^{a}$ | 0.76 ± 0.02 $^{a}$ | 0.70 ± 0.04 $^{a}$ | 0.76 ± 0.05 $^{a}$ | 0.51 ± 0.07 $^{b}$ | 0.49 ± 0.02 $^{b}$ |
| *C20:1 c9* | 0.19 ± 0.02 $^{c}$ | 0.20 ± 0.01 $^{c}$ | 0.27 ± 0.02 $^{bc}$ | 0.55 ± 0.14 $^{ab}$ | 0.60 ± 0.14 $^{a}$ | 0.62 ± 0.04 $^{a}$ |
| *C18:2 t9 t12* | 0.79 ± 0.04 $^{a}$ | 0.77 ± 0.04 $^{a}$ | 0.73 ± 0.04 $^{ab}$ | 0.78 ± 0.04 $^{a}$ | 0.59 ± 0.03 $^{bc}$ | 0.50 ± 0.02 $^{c}$ |
| *C18:2 c9 t12* | 0.35 ± 0.01 $^{ab}$ | 0.30 ± 0.10 $^{abc}$ | 0.34 ± 0.06 $^{abc}$ | 0.42 ± 0.02 $^{a}$ | 0.20 ± 0.05 $^{bc}$ | 0.14 ± 0.02 $^{c}$ |
| *C18:2 c9 c12* | 4.32 ± 0.08 $^{c}$ | 4.24 ± 0.09 $^{c}$ | 5.59 ± 0.18 $^{bc}$ | 9.72 ± 1.29 $^{a}$ | 9.18 ± 2.22 $^{ab}$ | 10.44 ± 0.27 $^{a}$ |
| *C18:2 c9 t11* | 1.44 ± 0.02 $^{ab}$ | 1.39 ± 0.04 $^{ab}$ | 1.43 ± 0.03 $^{ab}$ | 1.85 ± 0.30 $^{a}$ | 1.41 ± 0.40 $^{ab}$ | 1.03 ± 0.08 $^{b}$ |
| *C18:3 t9 t12 c15* | 0.37 ± 0.03 $^{a}$ | 0.39 ± 0.05 $^{a}$ | 0.40 ± 0.05 $^{a}$ | 0.45 ± 0.01 $^{a}$ | 0.34 ± 0.08 $^{a}$ | 0.27 ± 0.04 $^{a}$ |
| $\alpha$ *C18:3 c9 c12 c15* | 0.40 ± 0.01 $^{c}$ | 0.41 ± 0.02 $^{c}$ | 0.56 ± 0.00 $^{bc}$ | 1.02 ± 0.22 $^{ab}$ | 1.11 ± 0.28 $^{ab}$ | 1.27 ± 0.04 $^{a}$ |
| *C20:4 c5 c8 c11 c14* | 0.28 ± 0.01 $^{a}$ | 0.26 ± 0.01 $^{a}$ | 0.26 ± 0.01 $^{a}$ | 0.27 ± 0.01 $^{a}$ | 0.22 ± 0.06 $^{a}$ | 0.18 ± 0.02 $^{a}$ |
| ∑*SFA* | 190.57 ± 5.21 $^{a}$ | 176.27 ± 4.38 $^{a}$ | 178.44 ± 3.34 $^{a}$ | 198.96 ± 9.57 $^{a}$ | 167.81 ± 27.36 $^{a}$ | 154.42 ± 12.52 $^{a}$ |
| ∑*UFA* | 78.57 ± 1.11 $^{c}$ | 77.33 ± 0.85 $^{c}$ | 95.37 ± 5.34 $^{c}$ | 127.09 ± 11.02 $^{bc}$ | 183.05 ± 35.38 $^{ab}$ | 187.71 ± 7.28 $^{a}$ |
| ∑*MUFA* | 70.63 ± 1.00 $^{b}$ | 69.58 ± 0.78 $^{b}$ | 86.09 ± 5.01 $^{b}$ | 112.58 ± 9.31 $^{b}$ | 170.01 ± 32.74 $^{a}$ | 173.87 ± 7.06 $^{a}$ |
| ∑*PUFA* | 7.94 ± 0.11 $^{c}$ | 7.76 ± 0.10 $^{c}$ | 9.28 ± 0.32 $^{bc}$ | 14.51 ± 1.71 $^{a}$ | 13.04 ± 2.67 $^{ab}$ | 13.84 ± 0.30 $^{ab}$ |
| ∑*Omega-3 (*ω-3) | 0.40 ± 0.01 $^{c}$ | 0.41 ± 0.02 $^{c}$ | 0.56 ± 0.00 $^{bc}$ | 1.02 ± 0.22 $^{ab}$ | 1.11 ± 0.28 $^{ab}$ | 1.27 ± 0.04 $^{a}$ |
| ∑*Omega-6 (*ω-6) | 4.60 ± 0.06 $^{c}$ | 4.50 ± 0.10 $^{c}$ | 5.83 ± 0.18 $^{bc}$ | 9.99 ± 1.28 $^{a}$ | 9.39 ± 2.25 $^{ab}$ | 10.63 ± 0.28 $^{a}$ |
| ω-3/ ω-6 *ratio* | 11.56 ± 0.11 $^{a}$ | 10.87 ± 0.32 $^{a}$ | 10.41 ± 0.44 $^{ab}$ | 9.93 ± 0.90 $^{ab}$ | 8.53 ± 0.71 $^{b}$ | 8.39 ± 0.19 $^{b}$ |
| *Thrombogenic index (TI)* | 3.37 ± 0.00 $^{a}$ | 3.30 ± 0.06 $^{a}$ | 2.66 ± 0.13 $^{b}$ | 2.24 ± 0.09 $^{b}$ | 1.41 ± 0.17 $^{c}$ | 1.12 ± 0.13 $^{c}$ |
| *Atherogenic index (AI)* | 2.32 ± 0.00 $^{a}$ | 2.24 ± 0.00 $^{a}$ | 1.81 ± 0.09 $^{b}$ | 1.49 ± 0.12 $^{c}$ | 0.91 ± 0.09 $^{d}$ | 0.76 ± 0.00 $^{d}$ |
| *Saturation index (SI)* | 1.67 ± 0.00 $^{a}$ | 1.64 ± 0.02 $^{a}$ | 1.33 ± 0.07 $^{b}$ | 1.14 ± 0.06 $^{b}$ | 0.72 ± 0.08 $^{c}$ | 0.57 ± 0.07 $^{c}$ |

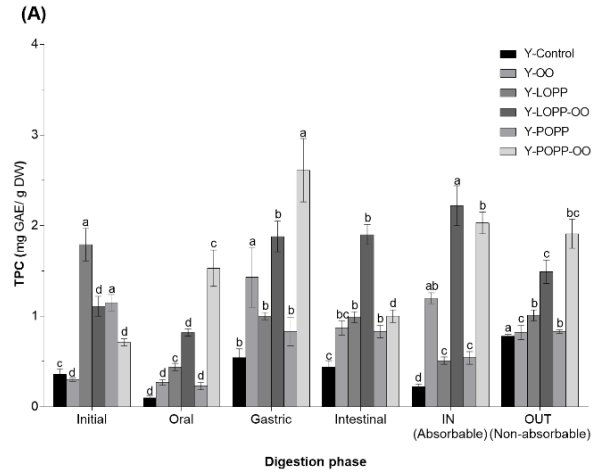
SFA - Saturated fatty acids; UFA - unsaturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. C6:0 – Caproic acid; C8:0 – Caprylic acid; C9:0 – Pelargonic acid; C10:0 – Capric acid; C12:0 – Lauric acid; C14:0 – Myristic acid; C15:0 – Pentadecylic acid; C16:0 – Palmitic acid; C17:0 – Margaric acid, C18:0 – Estearic acid; C10:1 c2 – Decenoic acid; C14:1 *c9* – Myristoleic acid; C15:1 *c10* – Pentadecanoic acid; C16:1 *t9* - *trans*-palmitoleic acid; C16:1 *c7* – *cis*-7-hexadecenoic acid; C16:1 *c9* – Palmitoleic acid; C17:1 *c10* - *cis*-10-heptadecenoic acid; C18:1 *t11* – *trans*-11-octadecenoic acid; C18:1 *c9* – Oleic acid; C18:1 *c11* – *cis*-Vaccenic acid; C18:1 *c12* – *cis*-12-Oleic acid; C18:1 *c13* – *cis*-13-Oleic acid; C18:1 *c14+t16* – *c14+t16*-octadecenoic acid; C20:1 *c9* – *cis*-Eicosanoid acid; C18:2 *t9 t12* – *trans-9-trans-12*-Octadecadienoic; C18:2 *c9 t12* – *cis-9-trans*-12-Octadecadienoic acid; C18:2 *t9 c12* – *trans-9-cis-12*-Octadecadienoic acid; C18:2 *c9c12* – Linoleic acid; C18:2 *c9 t11* – *cis-9-trans-11*-Octadecadienoic acid; C18:3 *t9 t12 c15* – *trans-9-trans-12-cis-15*-octadecatrienoic acid; $\alpha$ C18:3 *c9c12c15* – $\alpha$-Linolenic acid; C20:4 *c5 c8 c11 c14* - Arachidonic acid. Results are the means of three determinations ± standard deviation. Values with different letters in the same line are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

Nutritional quality indices regarding the FA profile of yoghurt samples were calculated, namely AI, TI and SI (Table 8.2). TI and AI measure the influence of the different FAs ingested on coronary heart disease (de Alba *et al.*, 2019). TI values relate to the tendency to form clots in the blood vessels, defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenicity acids (MUFAs, ω-6 and ω-3 PUFAs). AI correlates the risk of atherosclerosis, i.e., the increase of blood cholesterol level with the increase of the SFAs (C12:0, C14:0 and C16:0) or the decrease of the ∑MUFA, and ∑PUFA. The UFAs C12:0, C14:0 and C16:0 are considered pro-atherogenic and MUFAs and PUFAs, antiatherogenic. Low values for AI and TI are recommended (Ardabilchi Marand *et al.*, 2020). In the present study, Y-POPP, Y-LOPP-OO and Y-POPP-OO exhibited significant lower TI and AI than Y-control ($p < 0.05$). TI and AI were reduced in around 20 % in Y-POPP, but OO's simultaneous addition with OP powders (Y-LOPP-OO and Y-POPP-OO) allowed reducing TI and AI in 60 - 67%. Another good indicator of the nutritional value of dietary fat is the SI. The SI indicates the relationship between the sum of SFAs (pro-thrombogenic) and UFAs (anti-thrombogenic). There are no numerical values assigned to SI, but food with lower values of C14:0, C16:0 and C18:0 compared to UFAs would be considered healthier food (de Alba *et al.*, 2019). Y-POPP, Y-LOPP-OO and Y-POPP-OO presented significantly lower SI values than Y-control ($p < 0.05$). The reduction of TI, AI and SI by the POPP addition and simultaneous fortification with OO and OP powders could be used as an innovative strategy to increase the health appeal of high-fat yoghurts.

### 8.3.3. Phenolic compounds and antioxidant activity

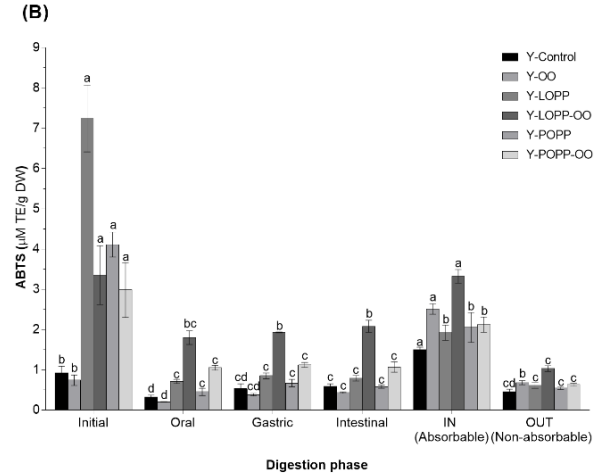
OP powders-fortified yoghurts exhibited significant higher TPC content than the plain yoghurt (Y-control) ($p < 0.05$) (Figure 8.2). The TPC detected in Y-control is probably due to the presence of low molecular weight antioxidants in milk (Chouchouli *et al.*, 2013), as well as to the presence of milk compounds such as lactose, free amino acids, peptides, proteins or reducing compounds, which respond to the Folin–Ciocalteau photometric measurement (Oliveira & Pintado, 2015).

The addition of LOPP powder to yoghurts increased more significantly ($p < 0.05$) TPC values than POPP powder, following the trend observed in powders composition. From all the formulations, the higher TPC was exhibited by Y-LOPP (179.38 ± 18.05 mg GAE/100 g DW**)**, which resulted in a value of 143.42 mg GAE/100 g DW when corrected with the contribution from control yoghurt (35.96 ± 5.94 mg GAE/100 g DW). Besides being a DF source, the Y-POPP formulation also contains a significant TPC content (114.74 mg ± 9.27 GAE/100 g DW), which is three times higher than the Y-control. On the other hand, the yoghurts formulated with 5% of OO and OP powders showed the lower TPC values (half of the TPC values assessed to Y-LOPP and Y-POPP). These lower TPC values assessed in Y-LOPP-OO (110.81 ± 11.44 mg GAE/100 g DW) and Y-POPP-OO (71.07 ± 3.82 mg GAE/100 g DW) could be a consequence of the interactions between the higher amount of MUFAs/ PUFAs and phenolics during yoghurt production. Phenolic compounds can protect lipids from lipid peroxidation reacting with the hydrophilic radicals and are eventually lost in preventing UFAs' oxidation, explaining the lower TPC amount in yoghurts with 5% of OO (Gorelik *et al.*, 2013; Jakobek, 2015).



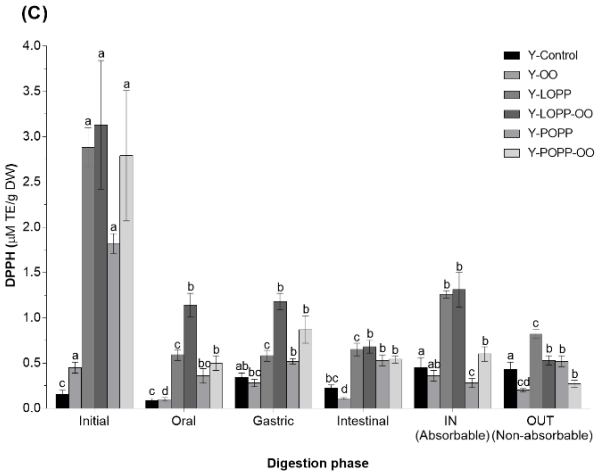

**Recovery index (RI %) and bioaccessibility index (%) of OP powders-fortified yoghurts throughout SGD**

| Total phenolic compounds | | | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO |
|---|---|---|---|---|---|---|
| Without yoghurt correction | RI (%) | *Oral* | 24.70 | 74.08 | 20.42 | 215.15 |
| | | *Gastric* | 55.48 | 169.45 | 60.08 | 357.02 |
| | | *Intestinal* | 54.98 | 171.10 | 72.72 | 102.77 |
| | | *IN* | 28.94 | 200.68 | 47.05 | 286.07 |
| | | *OUT* | 28.49 | 134.89 | 72.58 | 214.73 |
| | BI (%) | | 33.49 | 59.06 | 39.21 | 61.60 |
| With yoghurt correction | RI (%) | *Oral* | 24.82 | 67.80 | 16.47 | 306.17 |
| | | *Gastric* | 27.07 | 54.26 | 36.15 | 309.78 |
| | | *Intestinal* | 37.96 | 127.83 | 49.83 | 39.47 |
| | | *IN* | 20.08 | 125.97 | 40.23 | 201.63 |
| | | *OUT* | 16.09 | 82.90 | 6.99 | 263.86 |
| | BI (%) | | 33.57 | 59.15 | 86.51 | 44.32 |




**Recovery index (RI %) and remained activity (%) of OP powders-fortified yoghurts throughout SGD**

| Total phenolic compounds | | | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO |
|---|---|---|---|---|---|---|
| Without yoghurt correction | RI (%) | *Oral* | 9.84 | 53.63 | 10.87 | 35.34 |
| | | *Gastric* | 11.79 | 57.59 | 16.26 | 37.35 |
| | | *Intestinal* | 10.87 | 61.99 | 14.20 | 35.82 |
| | | *IN* | 26.58 | 99.00 | 49.81 | 71.27 |
| | | *OUT* | 8.46 | 30.82 | 13.05 | 21.21 |
| | Remained activity (%) | | 75.87 | 59.06 | 78.80 | 77.02 |
| With yoghurt correction | RI (%) | *Oral* | 6.06 | 61.27 | 5.06 | 38.25 |
| | | *Gastric* | 5.11 | 59.37 | 5.66 | 33.32 |
| | | *Intestinal* | 3.39 | 63.01 | 0.00 | 28.43 |
| | | *IN* | 7.63 | 30.87 | 20.90 | 0.00 |
| | | *OUT* | 2.59 | 13.57 | 2.14 | 0.00 |
| | Remained activity (%) | | 70.79 | 69.38 | 83.02 | 0.00 |




**Recovery index (RI %) and remained activity (%) of OP powders-fortified yoghurts throughout SGD**

| Total phenolic compounds | | | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO |
|---|---|---|---|---|---|---|
| Without yoghurt correction | RI (%) | *Oral* | 20.58 | 36.54 | 19.94 | 18.01 |
| | | *Gastric* | 20.00 | 37.77 | 28.72 | 31.16 |
| | | *Intestinal* | 22.71 | 21.82 | 29.06 | 19.30 |
| | | *IN* | 43.67 | 41.89 | 15.38 | 20.72 |
| | | *OUT* | 28.61 | 16.95 | 28.78 | 9.61 |
| | Remained activity (%) | | 60.44 | 70.98 | 36.74 | 74.22 |
| With yoghurt correction | RI (%) | *Oral* | 18.19 | 38.90 | 15.96 | 17.16 |
| | | *Gastric* | 8.65 | 33.80 | 10.97 | 25.36 |
| | | *Intestinal* | 15.99 | 21.35 | 18.67 | 18.28 |
| | | *IN* | 29.62 | 35.39 | 0.00 | 11.21 |
| | | *OUT* | 14.45 | 10.45 | 7.61 | 2.77 |
| | Remained activity (%) | | 65.32 | 71.54 | 0.00 | 78.13 |

**Figure 8.2. Effect of *in vitro* gastrointestinal digestion on OP fortified-yoghurts total phenolics and antioxidant properties after each step of *in vitro* gastrointestinal digestion (oral, gastric, intestinal, after dialysis IN and OUT). (A) Total phenolic compounds (TPC) (mg GAE/g DW); (B) Antioxidant activity measured by ABTS (µM TE/ g DW). (C) Antioxidant activity measured by DPPH (µM TE/ g DW).** Results are the means of three determinations ± standard deviation. Values with different letters above are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

**Note:** *Without yoghurt correction* - The amount of phenolic compounds or antioxidant activity in OP-fortified yoghurt was not corrected for the contribution of control yoghurt (Y-Control) and control yoghurt with olive oil (Y-OO*; With yoghurt correction* - The amount of phenolic compounds or antioxidant activity in OP-fortified yoghurt was corrected for the contribution of control yoghurt (Y-Control) to Y-LOPP and Y-POPP, and the contribution of control yoghurt with olive oil (Y-OO) to Y-LOPP-OO and Y-POPP-OO.

The most representative phenolic compounds were identified and quantified using HPLC in the methanolic extracts of OP-fortified yoghurts (Y-LOPP, Y-POPP, Y-LOPP-OO and Y-POPP-OO) (Table 8.3). No phenolic compounds were found in the control yoghurt (Y-control), nor in the yoghurt fortified with OO (Y-OO). The most typical phenolic compounds in the OP-fortified yoghurts were HYD and derivatives, in agreement with OP powders composition. The results obtained by HPLC were also in line with TPC results. LOPP fortified yoghurts showed higher TPC plus higher HYD content than the formulations where POPP was added.

**Table 8.3. Amount of individual phenolic compounds determined by HPLC (mg/100 g DW) in OP powders-fortified yoghurts supernatants.**

| Phenolic compound | Yoghurt formulation | Phenolic amount | Theoretical amount expected (TAE) [*1] | Maximum theoretical amount expected (MTAE)[*2] | Recovery in yoghurt formulation based in TAE (%) | Recovery in yoghurt formulation based in MTAE (%) |
|---|---|---|---|---|---|---|
| ***Hydroxytyrosol glucoside*** | **Y-LOPP** | 0.91 ± 0.11 [b] | 10.79 | 10.79 | 8.43 | 8.43 |
| | **Y-LOPP-OO** | 2.33 ± 0.24 [a] | 11.01 | 11.01 | 21.16 | 21.16 |
| | **Y-POPP** | 0.91 ± 0.06 [b] | 3.17 | 3.17 | 28.74 | 28.74 |
| | **Y-POPP-OO** | nd | 3.37 | 3.37 | 0 | 0 |
| ***Hydroxytyrosol*** | **Y-LOPP** | 12.72 ± 0.98 [b] | 68.97 | 83.62 | 18.44 | 15.21 |
| | **Y-LOPP-OO** | 16.46 ± 2.65 [a] | 70.36 | 85.3 | 23.39 | 19.3 |
| | **Y-POPP** | 1.47 ± 0.33 [d] | 4.94 | 42.82 | 29.8 | 3.44 |
| | **Y-POPP-OO** | 5.47 ± 0.53 [c] | 5.26 | 45.63 | 103.91 | 11.99 |
| ***Tyrosol glucoside*** | **Y-LOPP** | 4.90 ± 0.33 [a] | 25.69 | 25.69 | 19.07 | 19.07 |
| | **Y-LOPP-OO** | 4.10 ± 0.88 [a] | 26.21 | 26.21 | 15.64 | 15.64 |
| | **Y-POPP** | 0.80 ± 0.05 [b] | 6.51 | 6.51 | 12.28 | 12.28 |
| | **Y-POPP-OO** | 1.37 ± 0.30 [b] | 6.94 | 6.94 | 19.74 | 19.74 |
| ***Tyrosol*** | **Y-LOPP** | 3.72 ± 0.15 [a] | 16.17 | 16.17 | 23.01 | 23.01 |
| | **Y-LOPP-OO** | 2.34 ± 0.62 [b] | 16.49 | 16.49 | 14.19 | 14.19 |
| | **Y-POPP** | 1.00 ± 0.10 [c] | 6.73 | 6.73 | 14.85 | 14.85 |
| | **Y-POPP-OO** | nd | 7.18 | 7.18 | 0 | 0 |
| ***Caffeic acid*** | **Y-LOPP** | 0.48 ± 0.05 [a] | 2.59 | 10.95 | 18.56 | 4.38 |
| | **Y-LOPP-OO** | 0.06 ± 0.01 [c] | 2.64 | 11.17 | 2.27 | 0.54 |
| | **Y-POPP** | 0.35 ± 0.08 [b] | 1.35 | 20.62 | 25.83 | 1.7 |
| | **Y-POPP-OO** | 0.08 ± 0.02 [c] | 1.44 | 21.97 | 5.54 | 0.36 |
| ***p - Coumaric acid*** | **Y-LOPP** | 0.30 ± 0.04 [a] | 1.18 | 0 | 25.44 | 7.68 |
| | **Y-LOPP-OO** | nd | 1.2 | 0 | 0 | 0 |
| | **Y-POPP** | 0.32 ± 0.08 [a] | 2.13 | 7.15 | 15 | 4.54 |
| | **Y-POPP-OO** | 0.04 ± 0.01 [b] | 2.27 | 7.62 | 1.76 | 0.53 |
| ***Luteolin*** | **Y-LOPP** | nd | 0 | 0 | 0 | 0 |
| | **Y-LOPP-OO** | nd | 0 | 0 | 0 | 0 |
| | **Y-POPP** | 1.82 ± 0.24 [a] | 5.14 | 7.15 | 35.38 | 25.45 |
| | **Y-POPP-OO** | 0.59 ± 0.13 [b] | 5.48 | 7.62 | 10.76 | 7.74 |

nd – not detected. Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$). [*1] Based on the amount of free compounds quantified in OP powders. [*2] Based on the amount of free and bound phenolic compounds quantified in OP powders.

Other phenolics as luteolin, caffeic and *p*-coumaric acid were also detected in OP powders, but in the yoghurts, only luteolin was detected between 0.59 – 1.82 mg/100 g DW in Y-POPP-OO and Y-POPP, respectively.

Comparing the formulations with the same OP powder without and with OO, it was evident that OO reduced TPC values in the yoghurts (Y-LOPP-OO and Y-POPP-OO), which could be related to the detection of significantly lesser amounts of luteolin, caffeic and *p*-coumaric acids ($p < 0.05$) in these yoghurt formulations. However, OO addition did not affect the content in HYD and derivatives negatively. Y-LOPP-OO showed a statistically significant higher amount of HYD and HYD glucoside than Y-LOPP ($p < 0.05$).

Despite OP fortified-yoghurts, formulations exhibited considerable amounts of individual phenolics, and TPC, only a part of added phenolics by OP powder addition remained in the final products. A higher loss of total phenolics content occurred in Y-LOPP-OO (about 54%) than in Y-LOPP (about 13%). This higher loss was linked mainly to the lower recovery of caffeic and *p*-coumaric acid in Y-LOPP-OO formulation than in Y-LOPP (Table 8.3). However, it is vital to stand out that the OP powders were added to homogenised milk, followed by pasteurisation, inoculation with yoghurt culture and fermentation, which could explain the loss of phenolic compounds. Chouchouli *et al.* (2013) verified equivalent losses when supplemented yoghurts with grape seed extract; indeed, in this study, control and fortified yoghurt revealed similar TPC and individual phenolics amount. As with other studies on yoghurt fortification, Helal & Tagliazucchi (2018) and Chouchouli *et al.* (2013) designed the study with the direct fortification of plain yoghurts, producing stirred fortified yoghurts. The main reasons to justify the reduction or absence of phenolics in fortified yoghurts were linked to phenolics-protein interactions, which were enhanced by the yoghurt mixture's heating/pasteurisation before inoculation (Chouchouli *et al.*, 2013). The acidification of milk during yoghurt production, which results in gel formation (charge neutralisation of the protein particles in milk) decreases the binding capacity of milk proteins, and a fraction of added phenolics was unbound to be detected (Najgebauer-Lejko *et al.*, 2011).

On the other hand, an increase (about 39%) and decrease (about 23%) of TPC loss were verified in Y-POPP and Y-POPP-OO, respectively when compared to Y-LOPP-OO. The protection of bound phenolics by POPP fibre could explain these higher TPC values during the yoghurt production (Supplementary material). Indeed, the RI (%) of almost all individual phenolics in POPP-fortified formulations was higher than in LOPP-fortified formulations (Table 8.3).

The AOX of plain yoghurt and OP-fortified yoghurts are shown in Table 8.4. Fortified yoghurts exhibited significantly higher radical scavenging activity than the plain yoghurt (Y-control) both in the ABTS and in DPPH assay ($p < 0.05$). The free radicals quenching activity by hydrogen donation measured by ORAC was also higher in OP-fortified yoghurts than in Y-control ($p < 0.05$). The AOX observed in Y-control and Y-OO is mainly due to the formation of bioactive peptides with AOX because of the proteolytic activity of the starter lactobacilli used in yoghurt production (Helal and Tagliazucchi, 2018). Following the higher AOX of LOPP compared to POPP, LOPP-fortified yoghurts revealed statistically significant superior AOX values than POPP-fortified yoghurts ($p < 0.05$) for all

the methodologies tested. Nevertheless, the AOX retained in fortified yoghurts using ABTS methodology from the OP powders was similar to Y-LOPP (86%) and Y-POPP (85%), probably due to the role of fibre as protector of phenolics (Jakobek and Matić, 2019). A lower ABTS retention was verified in OP-fortified yoghurts when OO was incorporated. The Y-POPP-OO still contained 61% of the expected ABTS from the supplementation with 2% of POPP, but Y-LOPP-OO only retained 37% of the ABTS value assessed previously to LOPP (1%). The higher loss of TPC and OP phenolics reported above in Y-LOPP-OO, and Y-POPP-OO formulations was probably linked to the higher AOX losses measured by ABTS in these yoghurts' samples. Although DPPH showed lower values than ABTS and higher AOX losses when LOPP (24 %) was incorporated in yoghurt compared with POPP incorporation (33-50%). On the other hand, the yoghurt formulations with and without OO showed similar DPPH retention values from LOPP and POPP. For example, Y-LOPP and Y-LOPP-OO showed similar retention values of DPPH from LOPP (around 24%). The addition of OO did not affect so negatively the DPPH values as observed for ABTS, possibly due to the higher efficiency of DPPH in measuring the AOX of less polar compounds (Sadeer *et al.*, 2020; Schaich *et al.*, 2015). This superior DPPH's capacity to measure polar compounds could also explain the higher retention of DPPH from POPP addition (33%) and without OO (50%). At least ORAC values showed similar behaviour to ABTS, with higher AOX losses in formulations with (50 – 90%) than without OO (37 – 76%). ORAC assay is based on the reaction of water and lipid-soluble substances with peroxyl free radical from ROS generator AAPH ((2,2′-azobis(2-methylpropionamidine) dihydrochloride)). The higher reactivity of AAPH with soluble compounds explained the higher ORAC recovery attained after incorporation of LOPP (49 – 62%) than POPP (10 – 24%) since LOPP is water-soluble.

**Table 8.4. Antioxidant activity measured by ABTS, DPPH and ORAC (μM TE/g DW) and potential nutrition and health claims of fortified yoghurts.**

| | Yoghurt formulations without olive oil | | | Yoghurt formulations with olive oil | | |
|---|---|---|---|---|---|---|
| | **Control** | **LOPP fortified** | **POPP fortified** | **Control** | **LOPP fortified** | **POPP fortified** |
| **Antioxidant activity** | | | | | | |
| *ABTS* | 0.92 ± 0.17 $^{d}$ | 7.24 ± 0.83 $^{a}$ | 4.11 ± 0.31 $^{b}$ | 0.74 ± 0.13 $^{d}$ | 3.35 ± 0.73 $^{bc}$ | 2.98 ± 0.68 $^{c}$ |
| *DPPH* | 0.16 ± 0.04 $^{c}$ | 2.88 ± 0.22 $^{a}$ | 1.82 ± 0.11 $^{b}$ | 0.45 ± 0.06 $^{c}$ | 3.13 ± 0.71 $^{a}$ | 2.79 ± 0.72 $^{a}$ |
| *ORAC* | 6.68 ± 1.03 $^{d}$ | 31.06 ± 6.42 $^{ab}$ | 27.04 ± 5.82 $^{b}$ | 17.79 ± 2.50 $^{c}$ | 36.47 ± 4.62 $^{a}$ | 26.40 ± 2.61 $^{b}$ |
| **Total dietary fibre** | | | | | | |
| *g/100 WW* | nd | 0.61 ± 0.04 $^{b}$ | 1.07 ± 0.01 $^{a}$ | nd | 0.52 ± 0.09 $^{b}$ | 1.35 ± 0.03 $^{a}$ |
| *g/100 kcal* $^{*1}$ | nd | 1.01 ± 0.07 $^{b}$ | 1.76 ± 0.01 $^{a}$ | nd | 0.84 ± 0.15 $^{b}$ | 2.22 ± 0.06 $^{a}$ |
| **Hydroxytyrosol and derivatives** | | | | | | |
| *mg/100 g WW* | nd | 3.83 ± 0.20 $^{b}$ | 0.70 ± 0.07 $^{c}$ | nd | 4.79 ± 0.48 $^{a}$ | 1.23 ± 0.13 $^{c}$ |
| *mg/120 g WW* $^{*2}$ | nd | 4.60 ± 0.24 $^{b}$ | 0.83 ± 0.08 $^{c}$ | nd | 5.75 ± 0.58 $^{a}$ | 1.48 ± 0.15 $^{c}$ |
| **Potential claims** | | *Consumption of olive oil polyphenols contributes to the protection of blood lipids from oxidative damage* | Source of fibre | | *Consumption of olive oil polyphenols contributes to the protection of blood lipids from oxidative damage* | *Source of fibre* |

nd- non-detected; $^{*1}$ A yoghurt plain - whole milk contains 61 kcal/100 g. $^{*2}$ A regular dose of solid yoghurt is 120 g. Results are the means of three determinations ± standard deviation. Values with different letters in the same line are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

In conclusion, all methodologies used showed AOX losses compared to the expected values to added OP powders. Similar results were previously obtained, where the AOX of strawberry-fortified yoghurt was reduced due to the phenolic-protein interaction (Oliveira and Pintado, 2015). However, it is necessary to mention that only AOXs of free or unbounded phenolics were quantified in yoghurt samples' supernatant. The action of the digestive enzymes could liberate the phenolics bound to proteins and also to DF and thus become available to being absorbed by the human intestine and exert its AOX potential (Jakobek, 2015).

### 8.3.4. Nutritional and antioxidant activities properties of the yoghurt formulations obtained by addition of OP powders and olive oil

Regarding nutritional claims, the Y-POPP and Y-POPP-OO (2% POPP) fulfilled the condition of being a “source of fibre” (> 1.5 g of fibre per 100 kcal) (European Commission, 2006b) (Table 8.4). Y-POPP-OO (2.22 ± 0.06 g/100 kcal) and Y-POPP (1.76 ± 0.01), exhibited a TDF amount of about 2.00 g/100kcal. On the other hand, in the LOPP case, to achieve the fibre content required to bear the claim “source of fibre”, it would be necessary an amount of at least 3%.

Additionally, the objective of obtaining a yoghurt with a healthier FA profile, i.e., a ratio between SFAs and UFAs more equilibrated, was also attained by the addition of POPP (34% UFAs which 31% are MUFAs) and both OP powders together with OO (LOPP: 47% UFAs/ MUFAs; POPP: 54% UFAs which 51% are MUFAs). The MUFA and PUFA content was increased in Y-POPP, Y-LOPP-OO and Y-POPP-OO. Besides that, these yoghurt formulations exhibited ω-6/ω-3 ratios improved to a healthier range (≤ than 10).

The aim of achieving a yoghurt rich in HYD and derivatives was attained in the two formulations with LOPP. Both formulations with LOPP could provide the amount of HYD and derivatives (5 mg) in a regular dose of set yoghurt (120 g) that would be needed to protect LDL particles from oxidative damage, according to the health claim approved by the EFSA until now only to OO (Table 8.3). Indeed, LOPP-OO (5.75 ± 0.58 mg/120 g WW yoghurt) exhibited a higher amount than Y-LOPP (5.44 ± 0.33 mg/120 g WW yoghurt). Despite the higher recovery of phenolics from POPP than LOPP due to the liberation of the bound phenolics present in DF of POPP during yoghurt fermentation, the yoghurts fortified with POPP supply only about 1 mg of HYD and derivatives in a regular dose of a standard yoghurt (120 g). This is explained by the fact that POPP has a lower amount of HYD and derivatives. POPP and POPP-OO contained 0.83 ± 0.33 mg/120 g WW yoghurt and 1.48 ± 0.15 mg/120 g WW yoghurt, respectively.

The positive effect of OO addition, together with OP powders, was supported by the chemical and bioactives analyses. PCA and PLS-DA were applied to reduce the multidimensional structure of the data and provide a two-dimensional map to understand the nutritional and antioxidant variance of yoghurt formulations after the addition of OP powders OO. The scree plot of PCA analysis and scores plot of the yoghurt formulations are presented in Figure 8.3. (A). The scree plot indicates that the first two principal components account for 70% of the total variance (PC1 = 37.7% and PC2 = 32.3%).

PCA revealed separate clusters for each yoghurt formulation. Clusters of control yoghurts (Y-control and Y-OO) were close to each other in quadrant II separated from OP fortified – yoghurts. According to the scores plot, the main difference between control and OP fortified – yoghurts was its content in total UFAs. The OP - fortified yoghurts without OO (Y-LOPP and Y-POPP) were in quadrant III and IV. On the other hand, OP - fortified yoghurts with OO (Y-LOPP-OO and Y-POPP-OO) were positioned in quadrant I. The main difference between OP-fortified yoghurts with and without OO was the UFAs content (UFA, MUFA, PUFA, ω-6 and ω3).

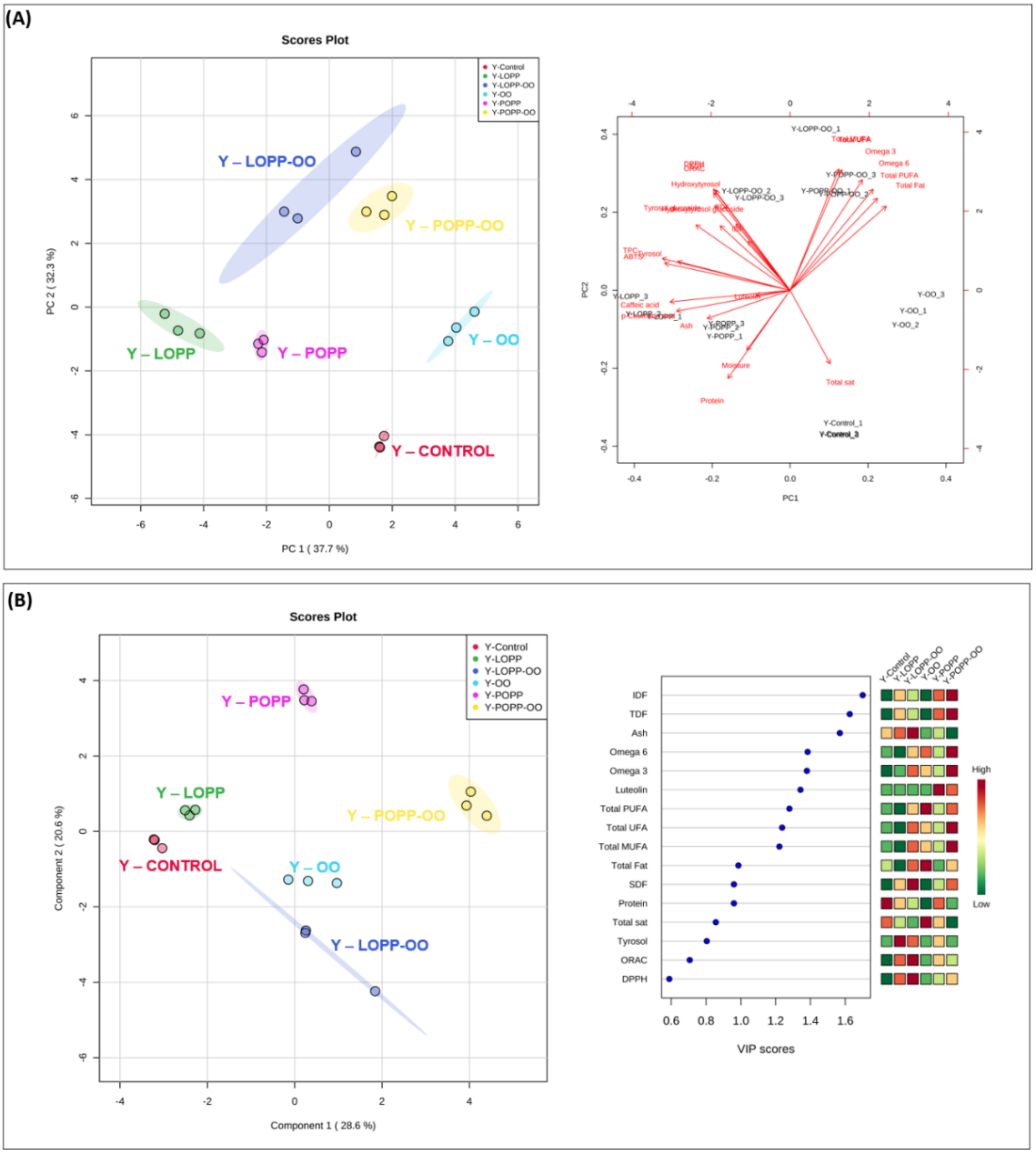



**Figure 8.3.PCA and PLS-DA of chemical and bioactivity characterisation of yoghurt formulations. (A) Scree plot of the principal component analysis and scores plot of chemical compounds and bioactivities identified in yoghurts formulations. (B) Partial Least Squares - Discriminant Analysis (PLS-DA) and VIP (Variable Importance in Projection) for component 1 of chemical compounds and bioactivities identified in yoghurts formulations following the PLS-DA model.** VIP allowed to measure the variable's importance in the PLS-DA model. Green and red tiles, respectively, indicate a lower or higher intensity of chemical compounds and bioactivities amount in the mean of all yoghurt sample.

PLS-DA of the chemical components and bioactivities also revealed separate clusters for each yoghurt formulation, as shown in Figure 8.3. (B). PLS-DA maximises the covariance between X (data) and Y (group). Variable importance in projection (VIP) was obtained (Figure 8.3. B) to understand better the differences observed between the different clusters of OP powders – yoghurt formulations. Markers assigned a VIP score > 0.6 were counted as the 16 most significant compounds, which define the differences in terms of nutritional and bioactive properties of yoghurt formulations in component 1 and component 2. IDF, TDF, ash, luteolin and UFA content (ω-3, ω-6, total UFA, total MUFA, total PUFA) were the most significant variants (VIP > 1.2) associated to both components. These relevant variants explain the separation of yoghurts fortified with POPP (Y-POPP and Y-POPP-OO) from the other yoghurt formulations.

After analyses of PCA and PLS-DA, yoghurt formulations with POPP (Y-POPP and Y-POPP-OO) were substantially different from the other formulations due to their content TDF, IDF and luteolin. Yoghurts with LOPP added (L-POPP and Y-LOPP-OO) distinguished from the other yoghurt formulations by their HYD content and its derivatives and AOX. At least, the UFA content was the main reason for the main differences observed between OP-fortified yoghurts with controls (Y-control and Y-OO).

It is also important to refer that OP-fortified yoghurts could be considered an excellent example of the newly emerging food category – “sustainable food products”. “Sustainable food products” are new value-added foods with higher nutritional properties formulated using ingredients developed from by-products generated during other foods' manufacturing. Nowadays, consumers' preferences for this new emerging food category are rising, not only for its environmentally sustainable character but also by health concerns related to consumers' preferences for natural food products (Coderoni and Perito, 2020).

In the future, sensorial analyses of the developed yoghurts should be performed to validate if the levels of LOPP (1%), POPP (2%) and OO (5%) used to achieve the nutritional and health claims influence the sensory properties of the yoghurts negatively. Some studies reported adverse effects as very bitter and spicy taste after 10% (w/w) OP incorporation into bread and spaghetti. However, no significative sensorial negative effects were reported when olive mill wastewater was used to replace the water in the bread and spaghetti formulations (Cedola *et al.*, 2020). On the other hand, Di Nunzio *et al.* (2020) established a limit of organoleptic acceptance in a consumer preference test using 2.5 or 4% of OP into biscuits and bread. To be noted, in our study, lower LOPP and POPP concentrations for the enrichment of yoghurt were chosen to guarantee a most promising organoleptic acceptance in a future consumer preference test. Moreover, the incorporation of sweeteners and other bitterness masking ingredients showed to be a viable option to reduce the potential negative sensorial impact of olive phenolics into fruit smoothies (Kranz *et al.*, 2010). This option could be explored in the future to improve potential negative effects on organoleptic attributes of OP-fortified yoghurts.

### 8.3.5. Evolution of phenolic compounds and antioxidant activity throughout the gastrointestinal tract

The alterations in TPC in the yoghurt samples during the SGD is shown in Figure 8.2. Regarding TPC content, all yoghurt formulations were significantly affected by SGD ($p < 0.05$). The OP-yoghurt formulations showed the highest TPC values in all SGD phases compared to its yoghurt controls (Y-control and Y-OO). The TPC values measured in OP-fortified yoghurts during SGD can be explained by the presence of individual phenolic compounds, as reported in Table 8.5**.** However, both control yoghurts (Y-control and Y-OO) also showed substantial TPC amount in all SGD phases, principally the Y-OO formulation. Since no phenolic compounds were detected in Y-control and Y-OO by HPLC after and before SGD, its TPC values possibly reflect phenolic compounds related to milk protein breakdown. For example, the amino acid tyrosine has a phenolic side chain suggested to give rise to TPC reading (Amirdivani and Baba, 2011).

Between all digestion phases, oral steps affected more negatively TPC content for all OP fortified-yoghurt formulations except the Y-POPP-OO. In mouth, the recovery indexes (RI%) after control yoghurt correction varied from 16.47% (Y-POPP) < 24.82% (Y-POPP) < 67.80% (Y-LOPP-OO) < 306.17% (Y-POPP-OO). During the gastric step, TPC values increased and then in the intestine increased or were maintained to all OP-fortified yoghurts, except to Y-POPP-OO. The TPC increase during gastric digestion in yoghurts fortified with phenolics. Previous studies explained that this increase could be mainly attributed to the acidic pH and enzymatic activity during the gastric phase, which can induce the hydrolysis of some phenolic compounds bound to proteins or even to lipids of the yoghurt matrix (Helal and Tagliazucchi, 2018; Oliveira and Pintado, 2015).

The free form of phenolics after stomach usually turns these compounds more sensitive to degradation in the intestine due to the intestinal alkaline conditions (Oliveira and Pintado, 2015). However, the negative effect of the alkaline pH of the intestine was only verified in Y-POOP-OO.

During Y-POPP-OO digestion, high TPC values were reported in the mouth (RI = 306.17%) and stomach (RI = 309.78%) followed by a significant decrease in the intestine (RI = 39.47%). A higher liberation of caffeic and p-coumaric acids occurred during the oral and gastric phase, decreasing during the intestinal phase. HYD and tyrosol glucoside amount in Y-POPP-OO also decreased during the intestinal step. Another factor that could decrease TPC value in the intestine could be related to the higher fat content of Y-POPP-OO. Fat digestion occurs mainly in the duodenum where emulsions formed during mastication are exposed to several surface active-components and lipases carry out a process of lipolysis, i.e. a breakdown of lipids into smaller particles which can then be absorbed (Jakobek, 2015). Several studies supported the inhibition of the lipase activity and fat absorption process by phenolic compounds (Paz-Yépez *et al.*, 2019). Lipid-phenolics interaction might also help deliver phenolics in the lower parts of the gastrointestinal tract (Jakobek, 2015). An increase of TPC was observed during intestinal absorption phase to Y-POPP-OO.

**Table 8.5. Amount of Individual phenolic compounds determined by HPLC (mg/100 g DW) in OP powders-fortified yoghurts throughout simulated gastrointestinal digestion (SGD).**

| SGD phase | Hydroxytyrosol glucoside | | | | Hydroxytyrosol | | | | Tyrosol glucoside | | | | Tyrosol | | | | Caffeic acid | | | | p-coumaric acid | | | | Luteolin | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP- | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO | Y-LOPP | Y-LOPP-OO | Y-POPP | Y-POPP-OO | Y-LOPP | Y-LOPP- | Y-POPP | Y-POPP-OO |
| **Initial** | 0.91 ± 0.11 $^{c}$ | 2.33 ± 0.24 $^{b}$ | 0.91 ± 0.06 $^{c}$ | nd | 12.72 ± 0.98 $^{a}$ | 16.46 ± 2.65 $^{a}$ | 1.47 ± 0.33 $^{a}$ | 5.47 ± 0.53 $^{a}$ | 4.90 ± 0.33 $^{a}$ | 4.10 ± 0.88 $^{a}$ | 0.80 ± 0.05 $^{b}$ | 1.37 ± 0.30 $^{b}$ | 3.72 ± 0.15 $^{a}$ | 2.34 ± 0.62 $^{a}$ | 1.00 ± 0.10 $^{a}$ | nd | 0.48 ± 0.05 $^{a}$ | 0.06 ± 0.01 $^{c}$ | 0.35 ± 0.08 $^{a}$ | 0.08 ± 0.02 $^{bc}$ | 0.30 ± 0.04 $^{a}$ | nd | 0.32 ± 0.08 $^{a}$ | 0.04 ± 0.01 $^{b}$ | nd | nd | 1.82 ± 0.24 $^{a}$ | 0.59 ± 0.13 $^{a}$ |
| **Oral** | 0.15 ± 0.04 $^{d}$ | 0.57 ± 0.12 $^{c}$ | 0.15 ± 0.04 $^{b}$ | 0.22 ± 0.11 $^{d}$ | 2.02 ± 0.10 $^{b}$ | 5.35 ± 1.25 $^{bc}$ | 0.23 ± 0.06 $^{b}$ | 1.11 ± 0.22 $^{b}$ | 0.88 ± 0.05 $^{bc}$ | 1.23 ± 0.18 $^{b}$ | 0.53 ± 0.16 $^{a}$ | 0.97 ± 0.10 $^{a}$ | 0.56 ± 0.03 $^{b}$ | 1.88 ± 0.38 $^{a}$ | 0.31 ± 0.09 $^{c}$ | nd | 0.08 ± 0.00 $^{b}$ | nd | 0.04 ± 0.02 $^{b}$ | 0.13 ± 0.03 $^{a}$ | 0.05 ± 0.01 $^{b}$ | nd | 0.03 ± 0.01 $^{b}$ | 0.12 ± 0.02 $^{a}$ | nd | nd | 0.32 ± 0.04 $^{b}$ | 0.41 ± 0.06 $^{ab}$ |
| **RI (%)** | **17.48** | **24.26** | **16.10** | **na** | **15.84** | **32.49** | **15.38** | **20.25** | **17.98** | **30.05** | **70.44** | **23.58** | **15.17** | **80.17** | **32.91** | **na** | **15.98** | **0.00** | **11.66** | **168.16** | **13.08** | **0.00** | **8.09** | **293.11** | **na** | **na** | **17.70** | **69.24** |
| **Gastric** | nd | 0.57 ± 0.02 $^{c}$ | nd | 0.38 ± 0.18 $^{cd}$ | 1.89 ± 0.15 $^{b}$ | 5.35 ± 0.25 $^{b}$ | 0.29 ± 0.02 $^{b}$ | 1.11 ± 0.03 $^{b}$ | 0.82 ± 0.09 $^{bc}$ | 1.21 ± 0.12 $^{b}$ | 0.70 ± 0.08 $^{a}$ | 0.99 ± 0.10 $^{a}$ | nd | 1.88 ± 0.18 $^{a}$ | nd | nd | 0.07 ± 0.01 $^{b}$ | 0.16 ± 0.01 $^{a}$ | 0.04 ± 0.01 $^{b}$ | 0.11 ± 0.02 $^{ab}$ | nd | 0.11 ± 0.01 | 0.05 ± 0.01 $^{b}$ | 0.12 ± 0.02 $^{a}$ | nd | nd | 0.30 ± 0.05 $^{b}$ | 0.42 ± 0.09 $^{ab}$ |
| **RI (%)** | **0.00** | **24.49** | **0.00** | **na** | **14.85** | **36.06** | **19.58** | **20.22** | **16.78** | **29.56** | **87.15** | **24.14** | **0.00** | **80.47** | **0.00** | **na** | **14.56** | **270.89** | **11.97** | **141.87** | **14.21** | **na** | **14.40** | **293.56** | **na** | **na** | **16.43** | **71.42** |
| **Intestinal** | 2.01 ± 0.17 $^{b}$ | 5.16 ± 0.40 $^{a}$ | 2.42 ± 0.18 $^{b}$ | 4.28 ± 0.24 $^{a}$ | 1.53 ± 0.08 $^{b}$ | 2.47 ± 0.22 $^{cd}$ | 0.14 ± 0.02 $^{b}$ | 0.53 ± 0.05 $^{bc}$ | 0.93 ± 0.11 $^{bc}$ | 0.73 ± 0.09 $^{b}$ | 0.50 ± 0.17 $^{a}$ | nd | nd | nd | nd | nd | 0.04 ± 0.00 $^{b}$ | 0.09 ± 0.01 $^{b}$ | 0.02 ± 0.00 $^{b}$ | 0.06 ± 0.00 $^{c}$ | 0.02 ± 0.00 $^{b}$ | nd | 0.02 ± 0.00 $^{b}$ | 0.06 ± 0.00 $^{b}$ | nd | nd | 0.22 ± 0.01 $^{b}$ | 0.22 ± 0.01 $^{bc}$ |
| **RI (%)** | **221.28** | **221.32** | **266.04** | **na** | **12.06** | **15.00** | **9.34** | **9.75** | **19.03** | **17.90** | **62.83** | **0.00** | **0.00** | **0.00** | **0.00** | **na** | **7.60** | **19.18** | **6.11** | **75.84** | **0.00** | **0.00** | **5.54** | **147.78** | **na** | **na** | **12.13** | **37.94** |
| **IN** | 0.78 ± 0.23 $^{c}$ | nd | 1.10 ± 0.10 $^{c}$ | 2.02 ± 0.09 $^{b}$ | 0.43 ± 0.08 $^{c}$ | 6.75 ± 0.35 $^{b}$ | nd | 0.89 ± 0.04 $^{bc}$ | 0.48 ± 0.00 $^{c}$ | nd | nd | nd | nd | nd | 0.62 ± 0.06 $^{b}$ | nd | nd | nd | nd | nq | nd | nd | nd | nq | nd | nd | nd | nd |
| **RI (%)** | **80.22** | **0.00** | **120.67** | **na** | **3.35** | **40.99** | **0.00** | **16.13** | **9.69** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **62.36** | **na** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **na** | **na** | **0.00** | **0.00** |
| **OUT** | 3.13 ± 0.02 $^{a}$ | 0.72 ± 0.10 $^{c}$ | 2.93 ± 0.11 $^{a}$ | 0.62 ± 0.10 $^{c}$ | 1.37 ± 0.12 $^{bc}$ | 0.61 ± 0.05 $^{d}$ | 0.56 ± 0.00 $^{b}$ | 0.15 ± 0.03 $^{c}$ | 1.08 ± 0.25 $^{b}$ | 0.23 ± 0.02 $^{b}$ | nd | nd | nd | nd | nd | nd | 0.04 ± 0.01 $^{b}$ | nd | 0.03 ± 0.00 $^{b}$ | nq | 0.02 ± 0.01 $^{bc}$ | nd | 0.03 ± 0.00 $^{b}$ | nq | nd | nd | 0.23 ± 0.00 $^{b}$ | 0.15 ± 0.00 $^{c}$ |
| **RI (%)** | **343.85** | **31.11** | **322.08** | **na** | **10.77** | **3.72** | **37.80** | **2.88** | **22.02** | **5.53** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **na** | **8.66** | **0.00** | **8.26** | **0.00** | **9.41** | **0.00** | **8.39** | **0.00** | **na** | **na** | **12.57** | **24.96** |
| **BI (%)** | **18.79** | **0.00** | **26.98** | **76.67** | **22.95** | **91.66** | **0.00** | **0.42** | **31.18** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **100.00** | **na** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **0.00** | **na** | **na** | **0.00** | **0.00** |

nd – not detected; nq – not quantifiable; na – not applicable. Results are the means of three determinations ± standard deviation. Values with different letters are significantly different, as determined by one-way ANOVA test ($p < 0.05$).

**Note:** The initial amount before digestion ($BC_{TF}$) and the amounts detected in the digested sample for each digestion step ($BC_{DF}$) expressed in this table were used to calculate the Recovery Index (RI%) for each phenolic compound. On the other hand, to calculate the Bioaccessibility Index (BI%) of each phenolic compound, the $BC_S$ which is the amount detected in the digested sample after the duodenal dialysis step (IN) and $BC_{DFE}$ content which is the sum of the amounts after the duodenal step (IN + OUT) detected in this figure were used.

On the other hand, OP-fortified yoghurt (Y-LOPP, Y-LOPP-OO, Y-POPP) exhibited an increase of TPC values after intestinal digestion, which could be related to the increase of HYD glucoside amount in all these yoghurt formulations. A similar increase was obtained in cinnamon-fortified yoghurts at the end of the intestinal phase (Helal and Tagliazucchi, 2018). The main reason for this increase could be associated with the hydrolysis of caseins during the intestinal phase, which allowed the release of the bound phenolic compounds in the intestine. However, a decrease in TPC occurred in all these yoghurt formulations during intestinal absorption.

Comparing OP powders yoghurt formulations at the end of intestinal digestion, Y-LOPP (0.54 ± 0.05 mg GAE/g DW) exhibited slightly higher TPC value than Y-POPP (0.39 ± 0.07 mg GAE/g DW) after yoghurt correction. Nevertheless, as a lower source of phenolic compounds, POPP exhibited a higher RI than LOPP after the intestinal step and during intestinal absorption. These higher releases of phenolic compounds could be justified by the higher liberation of the glucosidic form of phenolics and p-coumaric linked to DF, as reported in Table 8.5. The higher release of HYD glucoside, tyrosol glucoside and p-coumaric during intestinal absorption could be linked to the action of α-amylase present in the pancreatin extract used in SGD. This pancreatin is an extract from porcine pancreas composed by different enzymes, which can be classified as proteolytic, lipolytic, amylolytic, and nucleic acid splitting enzymes. α-Amylase (EC 3.2.1.1), the main amylolytic enzyme in pancreatin, is an endohydrolase specific for α-(1→4) glycosidic bonds.

Despite the importance of the recovery in each digestion phase, phenolics will need to be released from their food matrix and reach the intestine in order to be bioavailable, so they can become then absorbable (bioaccessible), meaning that they can be absorbed by intestinal cells and be metabolised. Between all OP- fortified yoghurt formulations, Y-POPP also showed the highest bioaccessibility index (BI %) of TPC (86.51%), but also the highest BI for tyrosol (100%). In comparison, POPP-OO showed higher TPC values in the absorbable fraction (IN) than Y-POPP, but a higher amount of phenolics were retained in non-absorbable (OUT) of Y-POPP-OO, which decreased its BI to 44.32%. Among LOPP-fortified yoghurts, OO incorporation seems to increase the bioaccessibility of phenolics. Y-LOPP-OO showed not only higher BI values (59.15%) than Y-LOPP for TPC but also higher BI values for HYD. Y-LOPP-OO (91.66%) showed a BI four times higher for HYD than Y-LOPP (22.95%). This positive effect of OO in HYD absorption was reported before in a rat model study, where HYD absorption from a lipid-rich matrix (OO) was higher (≈ 25%) than that from an aqueous solution (Bohn, 2014) or low-fat yoghurt (Visioli *et al.*, 2003). The metabolization of these bioaccessible olive phenolics could exert several biological properties and potential role in the prevention of various inflammatory diseases. Recent studies with OP-enriched water extracts (Di Nunzio *et al.*, 2018) and bakery-enriched products (Di Nunzio *et al.*, 2020) demonstrated a significant anti-inflammatory effect, significantly reducing IL-8 secretion in Caco-2 cells. Futures studies about the potential anti-inflammatory activity of OP-fortified yoghurts with olive oil need to be assessed.

Despite the low BI of Y-LOPP, not only for HYD (22.95%) and TPC (33.57%) but also for HYD glucoside (18.79%) and tyrosol glucoside (31.18%), a significant amount of phenolics were available

in the non-absorbable fraction (OUT) to be metabolised by the microbiota. This may increase the amount of phenolics metabolites and their potential biological activities on the gut as promotors of the growth of healthy bacteria (Liu *et al.*, 2019), as anti-inflammatory agents and as protectors of the Caco-2 intestinal mucosal cells against the cytostatic and cytotoxic effect of oxidised LDL (Bonechi *et al.*, 2019). On the other hand, Y-POOP and Y-POPP-OO have also shown to contain a significant amount of phenolics in OUT fraction per 100 g DW, which includes HYD glucoside (Y-POPP: 2.93 ± 0.11 mg; Y-POPP-OO: 0.62 ± 0.10), HYD (Y-POPP: 0.56 ± 0.10 mg; Y-POPP-OO: 0.15 ± 0.03 mg) and luteolin (Y-POPP: 0.23 ± 0.00 mg; Y-POPP-OO: 0.15 ± 0.00 mg). As mentioned above, HYD is a potent antioxidant agent with several health benefits and luteolin has been pointed out as a potent intestinal anti-inflammatory agent by different mechanisms using in vitro gut inflammation models (Mizuno and Nishitani, 2013). Recently the ingestion of OP-enriched biscuits showed not only to increase the metabolic output of the gut microbiota significantly but also to boost the homovanillic acid and DOPAC levels involved in reducing oxidative LDL cholesterol (Conterno *et al.*, 2019). Moreover, the administration of OP as feed supplemented showed potential to be used to aimed to produce meat or dairy products enriched in functional lipids through the modification of gut microbiota composition(Romani *et al.*, 2019). These potential gut health benefits of POPP-fortified yoghurts need to be explored in more detail in future studies.

Changes in radical scavenging activity were also evaluated during the in vitro digestion, and the data are presented in Figure 8.2. The radical scavenging activity of OP-fortified yoghurts decreased after mouth phase in both the assays during digestion because of the loss of individual phenolic compounds reported in this digestion phase. After the oral phase, the AOX values to both methods were maintained until the intestinal phase, with no significant differences ($p < 0.05$). During intestinal absorption, a significant increase of AOX was observed for all OP-fortified yoghurts in the absorbable fraction (IN) ($p < 0.05$), even after yoghurt control correction, except for Y-POPP in DPPH method and Y-POPP-OO in ABTS method. These differences were also expressed in the percentage of the remained AOX attained in absorbable fraction (IN). After SGD, according to ABTS method, Y-POPP revealed to have 83.03% of AOX that reach intestine accessible in absorbable fraction, but Y-POPP-OO had no AOX accessible. However, when AOX was measured using DPPH, a counter behaviour was verified: Y-POPP did not have accessible AOX, and 78.13% of the AOX of Y-POPP-OO that reached intestine was accessible to be metabolised. The main reason for such distinct AOX values in the different methodologies applied seems to be linked to the IN fraction's phenolic composition. From the phenolics identified in yoghurts, Y-POPP revealed to contain HYD glucoside and tyrosol in fraction IN, and Y-POPP-OO contained only a small amount of HYD. The higher percentage of the remaining ABTS in Y-POPP could relate to their affinity with more polar compounds like tyrosol and HYD glucoside.

On the other hand, the HYD that is bioaccessible in Y-POPP-OO was probably delivered by a fat fraction, which explains the higher AOX measured by DPPH. Another critical feature of tyrosol and its derivatives is its lower AOX compared to HYD (González *et al.*, 2019). The AOX of tyrosol is only as hydroxyl radical scavenger or at most α-tocopherol regenerator (Bonechi *et al.*, 2019). None of the mechanisms of action of tyrosol was individually evaluated by the AOX assays used. This lower

AOX of tyrosol explained the reduced reactivity of IN fraction of Y-POPP with DPPH since DPPH radicals are lesser reactive than ABTS radicals (Hsu *et al.*, 2011). However, it is essential to highlight that the conversion of tyrosol into HYD reported *in vivo* in humans allowed expecting an AOX higher than the one reported (Boronat *et al.*, 2019).

Regarding yoghurt formulations with LOPP, in Y-LOPP-OO (ABTS: 69.38%; DPPH: 71.54%) a higher percentage of AOX was attained in the absorbable fraction (IN) for both AOX methodologies than in Y-LOPP (ABTS: 70.79%; DPPH: 65.32%). This result was intrinsically linked to the higher BI verified for HYD in Y-LOPP-OO. The AOX variations throughout the SGD allowed understanding that phenolic compounds bioaccessibility has an essential role in AOX.

OP powder-fortified yoghurt showed substantial phenolic content with high BI after SGD and therefore may be used to deliver HYD and its derivatives into the human diet. Even though the reduction of AOX verified after SGD, the AOX values attained for OP-fortified yoghurts permitted to considerer these yoghurt formulations as good candidates to create an antioxidant environment in the gastrointestinal tract. Some approaches, like the application of nanoemulsions or acidified milk gels, could be useful strategies for improving stability and AOX of OP powder phenolics after SGD (Villalva *et al.*, 2020).

## 8.4. Conclusion

Olive pomace powders were successfully employed as functional ingredients into the fortification of yoghurt. By consuming a standard yoghurt (120 g) the Y–POPP formulation fulfilled the condition of being a “source of fibre”, and the Y-LOPP formulation provided the 5 mg of hydroxytyrosol and derivatives needed to protect low-density lipoprotein particles from oxidative damage. POPP also allowed the improvement of the quality of the fatty acid profile of yoghurts, increasing the amount of monounsaturated and polyunsaturated fatty acids. Olive oil addition together with olive pomace powders, enhanced its action as a source of unsaturated fatty acids and made hydroxytyrosol more stable after yoghurt fermentation, and also more bioaccessible after *in vitro* digestion. Therefore, olive pomace powders can be considered a key source of dietary bioaccessible phenolics, fibre and unsaturated fatty acids. Taking into account the significative amount of olive pomace produced annually, the incorporation of its powders into dairy products could be a straightforward way to increase the economic and environmental sustainability of olive oil sector, but also to dairy sector offers to its consumers “sustainable food products” with the benefits of dietary fibre, unsaturated fatty acids and of the health claimed olive oil phenolics – hydroxytyrosol and derivatives. This study brings new insights to help spread the circular bioeconomy concept through the whole food sector.

The results obtained in this study should be compared with additional *in vivo* studies to correlate the bioaccessibility of bioactive compounds between *in vivo* and *in vitro* methodologies, but also to validate the health claimed benefits of hydroxytyrosol and derivatives in other food matrices than olive oil. Sensorial analyses of the developed yoghurts should also be performed.

# PART V

# *Final Remarks*

# Chapter 9.
# Final conclusions

The experimental work presented in this PhD thesis is an effort to find a scientific basis for the integral valorisation of the most significant by-product of the olive oil industry – olive pomace. The sustainable exploitation of this by-product is of significant relevance in economic and environmental terms, with the potential to have relevance in consumers' health too. We expect to contribute towards an integral and sustainable valorisation of olive pomace through the development of differentiated added-value ingredients with multi-applicability in the food industry, but also in bioenergy and cosmetic sectors prioritising the high value uses of biological resources, and thus contributing to a circular bioeconomy implementation in the olive oil sector.

Initially, a low-cost sustainable fractionation approach was developed to explore the different fractions from two olive pomace samples using samples from two olive mills with 2-phase extraction method – liquid, pulp and stones – as sources of new value-added ingredients. The wet fractionation (first step) led to the production of a liquid fraction rich in minerals, sugars, and phenolic compounds mainly hydroxytyrosol in the concentration of 513.61 – 625.76 mg/100 g dry weight. This first step also has the advantage of improving the solid fraction's subsequent drying process from olive pomace. The following dry fractionation of olive pomace attaining a stones fraction with significant calorific power (18.65-18.94 MJ/kg) and a pulp fraction rich in antioxidant dietary fibre. Pulp fraction as a source of antioxidant dietary fibre revealed a high fibre content (53-59% dry weight) with a relevant antioxidant activity associated with the free and bound phenolics. Experimental results demonstrated that it would be advantageous to exploit the stones fraction as solid biofuel, but also to turn the liquid and pulp fractions in new potential food ingredients. Overall, this work used the fractionation approach in a biorefinery context, as a new, sustainable, and promising route for converting olive pomace into multiple value-added products prioritising the high over low added-value products. This integral valorisation approach seems to be a feasible methodology to achieve the circularity in the olive oil sector.

After the validation of the potential of the liquid and solid fractions from olive pomace as a source of new food ingredients, the next step was to develop new food powdered ingredients from liquid and pulp fractions obtained from OP. The development of powders without any extraction step has been proposed as a more feasible and sustainable alternative than the non-environmental friendly conventional extraction methods or the novel eco-friendly techniques with high operational costs. Powdered products have the advantage to retain different nutrients and bioactive compounds with potential complementary/synergetic benefits in terms of health benefits and technological properties (antioxidant, antimicrobial and enhancer of aroma/taste) and reduce logistic costs. The production and characterisation of OP powders have been scarcely studied and exhibit a reasonable potential as multifunctional powdered ingredients. The previous liquid and pulp fractions were processed to obtain two stable and biologically safe powdered ingredients, namely a liquid-enriched powder (LOPP) and a pulp-enriched powder (POPP). To guarantee the economic feasibility, stability and

safety of the OP powders some procedures were taken, including: (1) selection of a more cost-effective drying process taking in consideration the stability of the bioactive compounds and microbiological safety; (2) addition of food additives to improve their stability and (3) removal of physical hazards such as small pieces of stones. These powders were characterized chemically, and their bioactivity (antioxidant capacity and antimicrobial activity), functionality (solubility, water-holding and oil-holding capacity) and biological safety were assessed. LOPP could be used simultaneously as a source of mannitol (141 g/kg), potassium (54 g/kg) and hydroxytyrosol (5 mg/g), and POPP as a source of antioxidant dietary fibre (620 g/kg of fibre with a significant amount of bound phenolics - 7.41 mg GAE/g fibre DW) and oleic/linoleic acid (76% of total fatty acids). The high antioxidant and antimicrobial potential exhibited by LOPP could be explored not only as source potential health benefits but also as a potential food preservative. On the other hand, the richness of POPP in antioxidants mainly carried by fibre and its simultaneous richness in unsaturated fatty might give rise to additive or synergic effects as gut health benefits, but also lower lipid peroxidation. Olive pomace powders biological safety was confirmed, and they demonstrated adequate functional properties for food applications.

Although LOPP and POPP composition and demonstration of its bioactive properties support this ingredient's potential application as a functional ingredient, to guarantee their health benefits, the digestion impact on phenolics, minerals, fatty acids, and sugars needs to be evaluated to guarantee bioactives bioaccessibility and related health benefits. The bioaccessibility and stability of LOPP bioactive compounds throughout the different phases of *in vitro* gastrointestinal digestion were assessed. Phenolics and minerals were the most affected LOPP compounds by the gastrointestinal tract. Its low stability was reflected in the significantly lower values of LOPP antioxidant activity throughout the gastrointestinal tract. Even though notable bioaccessibility indexes (> 50%) were obtained in potassium and hydroxytyrosol derivatives, which could exert its potential cardiovascular health benefits. Furthermore, substantial antihypertensive activity and α-glucosidase inhibitory activity were maintained (50% of the initial inhibitory activities). Additionally, a significant amount of soluble sugars and formic acid, and a considerable content of phenolics were retained in the non-bioaccessible fraction (OUT), which could be interesting to study in the future to understand its possible benefits in the gut health.

The content and interaction of dietary fibre, lipids and phenolics throughout the *in vitro* gastrointestinal digestion of POPP were also studied. As a multifunctional powder, POPP compounds seemed to interact with each other during *in vitro* digestion. For example, POPP's dietary fibre and possibly lipids acted as phenolic carriers throughout *in vitro* tract with positive effects on the antioxidant potential of absorbable fraction and non-absorbable fraction. Besides that, the release in the stomach of the bound phenolics linked mainly to fibre allowed to recover a significant amount of the antioxidant compounds lost in the mouth. However, dietary fibre and lipids also exhibited a positive synergetic interaction, decreasing the bioaccessibility of saturated fatty acids and facilitating the absorption of the unsaturated fatty acids. PCA analysis allowed to validate the negative impact of digestion principally in phenolic compounds and associated antioxidant activity. Notwithstanding the negative effect of digestion on POPP bioactive properties, bioaccessibility of phenolics and

unsaturated fatty acids were substantial and could be expected that POPP antioxidant benefits will occur after its ingestion. Besides that, in colon fraction (OUT), a significant retention of dietary fibre and phenolic compounds was also verified, which could potentially exert potential gut health benefits.

A significant amount of fibre and phenolics, but also soluble sugars as mannitol and glucose were retained in the non-bioaccessible fraction (OUT) for both olive pomace powders. In order to disclose the potential beneficial effect on the gut of dietary fibre and phenolics (free and bound form) retained in significant amounts in the colon fraction, the potential prebiotic effect of OP powders and their potential antioxidant/ antimicrobial effects on gut health were assessed by *in vitro* fermentation with faecal inocula. Olive pomace powders showed to impart health benefits to the gastrointestinal tract as promoters of the production of SCFAs (mainly acetate > butyrate > propionate) by gut microbiota in a higher degree than fructooligosaccharides, without negative impact in the gut microbiota diversity. Both powders also showed significant total phenolic content and oxygen radical absorbance capacity during faecal fermentation until 48 h. Besides that, these powders showed mucin-adhesion inhibition ability against pathogens, principally POPP against *Bacillus cereus* (22.03 ± 2.45%) and *Listeria monocytogenes* (20.01 ± 1.93%). Equally, olive pomace powders were shown to be a significant source of phenolics metabolites after LC-ESI-UHR-QqTOF-MS detection. Supervised cluster and hierarchical clustering analysis allowed to evaluate the metabolite patterns of olive pomace powders phenolics throughout *in vitro* faecal fermentation, explaining the potential antioxidant activity and antiadhesion ability against food pathogens discovered to these powders.

The food matrix may play a crucial role in bioactives' bioaccessibility of the food ingredients, affecting its absorption positively or negatively. In this sense, the effect of yoghurt matrix in phenolics' bioaccessibility of olive pomace powders was investigated. The potential of olive pomace powders as multifunctional ingredients to increase yoghurt content in dietary fibre, hydroxytyrosol and unsaturated fatty acids was validated. Additionally, LOPP and POPP's potential to protect other unsaturated fatty acid sources added to yoghurt were also studied by the simultaneous incorporation of olive oil with olive pomace powders into yoghurt. POPP (2%) and LOPP (1%) addition to yoghurt allowed fulfilling the condition on being a "source of fibre" and provides 5 mg of hydroxytyrosol and derivatives in a standard yoghurt (120 g), respectively POPP also allowed the improvement of the quality of the fatty acid profile of yoghurts, increasing the amount of monounsaturated and polyunsaturated fatty acids. Olive oil addition together with olive pomace powders enhanced its action as a source of unsaturated fatty acids, and made hydroxytyrosol more stable after yoghurt fermentation, and also more bioaccessible after *in vitro* digestion. Therefore, olive pomace powders can be considered a vital source of dietary bioaccessible phenolics, fibre and unsaturated fatty acids. Considering the significant amount of olive pomace produced annually, the incorporation of its powders into dairy products could be a straightforward way to consumers obtain the benefits of the health claimed olive oil phenolics – hydroxytyrosol and derivatives, but also of the other olive pomace phenolics.

Finally, olive pomace valorisation by developing new powdered food ingredients could add value to the higher by-product stream from the modern olive oil industrial line production. Considering the

high nutritional value and potential health benefits identified in this work for olive pomace powders, the valorisation approach proposed in this work could be a key option for the olive oil industry to increase its environmental and economic sustainability towards a circular bioeconomy.

# Chapter 10.
# Future prospects

The studies proposed in this PhD thesis encompasses a novelty on their own, in what concerns the validation of the multifunctionality of the new add-value powders from olive pomace as food ingredients obtained by a feasible and sustainable fractionation process, without compromising the primary goal of achieving a “zero waste food system” towards a circular bioeconomy concept and leading to new research lines.

Several scientific, technological, and regulatory issues should be comprehensively addressed if the primary goal is the development of new bioactive ingredients to be incorporated in the food chain in human nutrition and health and even more in the case of food ingredients rich in hydroxytyrosol and derivatives which are one of the few phenolic compounds with health claims approved by EFSA, but until now only for olive oil, excluding other food matrices. Thus, considering the conclusions obtained within this PhD thesis, additional studies should be performed and, in this section, we propose some ideas that might be interesting and rewarding to explore in the future.

Regarding Chapter 3, a lab-scale experiment, centrifugation without refrigeration and freeze-drying was adopted. However, simulation and optimisation studies in conjunction with life cycle analysis (LCA) should be performed not only to improve the fractionation approach's sustainability and economics proposed as an integrated biorefinery scheme, but also analyse its environmental impacts *versus* the traditional treatments of olive pomace. A possible future optimisation at a pilot-scale will demand further modifications to use a mechanical pressing and conventional drying techniques to ensure a more affordable and feasible process.

Regarding Chapter 4, could be interesting to explore the modification of POPP (grinding, chemical and enzymatic hydrolysis) to enhance its soluble dietary fibre content, and consequently its functional properties and usefulness in the food industry as a technological or health-benefit ingredient. Micronization, extrusion and sourdough fermentation seem to be interesting options to explore in the future in order to improve the ratio between soluble and insoluble dietary fibre without compromising POPP antioxidant properties severely, and possibly increasing them. Besides that, the fraction rich in olive peel and stones removed to obtain POPP should be tested as a potential cosmetic ingredient, namely as exfoliant agent with antioxidant properties.

Concerning Chapters 5, 6 and 7, new strategies to improve LOPP and POPP bioactive compounds' bioaccessibility should be explored. The addition of ascorbic acid to LOPP as a protective agent of phenolics and enhancer of minerals absorption hroughout the gastrointestinal tract might be explored to improve LOPP antioxidant activity and, its α-glucosidase and ACE inhibitory activities. Other solutions such as the conjugation with other phenolic extracts or the development of micro and nano delivery systems could be considered in the future for the improvement of the bioaccessibility of LOPP bioactive compounds. The addition of ascorbic acid or other phenolics extracts could also be an excellent option to protect fatty acids and phenolics of POPP throughout digestion.

Still, in Chapters 5, 6 and 7*, in vitro* experiments represent a consistent approach to evaluate the health effect of new functional ingredients. However, future studies in cell lines, *in vivo* and clinical trials are needed to validate the findings and implications resulting from the *in vitro* experiments described in the present work for POPP and LOPP. Besides that, more research into *in vitro–in vivo* correlations is required to achieve more realistic *in vitro* models and therefore screen the bioaccessibility and digestibility of foods. Thus, further studies in cell lines, *in vivo* and clinical trials, should be performed.

In particular, concerning Chapter 6, the results showed that digestion decreased POPP phenolics compounds and its related antioxidant activity, so other POPP applications could also be explored. Taking into account the particle size, the composition (a rich source of cellulose, pectic polysaccharides, hemicellulosic polymers, oleic acid and proper source of minerals) and the demonstrated activities of POPP (e.g., antioxidant, antimicrobial) its application as a cosmetic product could be an excellent opportunity. POPP could be explored as a facial exfoliant and as a hydrating agent due to its fatty acid and mineral composition and as preservative because of its antimicrobial and antioxidant capacity. The anti-inflammatory activity and capacity to delay cellular senescence of hydroxytyrosol in UVA-induced cellular ageing model of human dermal fibroblast have been reported, and POPP hydroxytyrosol richness should also be explored in the cosmetic field as anti-inflammatory and anti-ageing provoked by UV damages.

In Chapter 7, tests using an *in vitro* faecal model pH-controlled using human or pig faecal inoculum must be carried out before *in vivo* experiments, to confirm olive pomace's gut health benefits powders. Other studies regarding antimicrobial and anti-inflammatory activity in gut cells should also be performed to explore additional olive pomace powders' additional gut health benefits.

Regarding industrial application in a food matrix (Chapter 8), sensorial analyses of the developed yoghurts should be performed. An *in vitro* faecal fermentation model should also be used to evaluate the yoghurt developed in order to obtain more information about the impact of this functional food in the gastrointestinal health. Besides that, the results obtained in this study should be compared with additional *in vivo* studies to correlate the bioaccessibility of bioactive compounds between *in vivo* and *in vitro* methodologies, but also to validate the health claimed benefits of hydroxytyrosol and derivatives in other food matrices than olive oil. Additionally, olive pomace powders should be tested in other food systems and the assessment of their functional properties, such as rheology, and sensory behaviour is also necessary.

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