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AGRO-FOOD BY-PRODUCTS AS SUSTAINABLE RESOURCES: EXPLORING ANALYTICAL APPROACHES FOR THE CHARACTERIZATION AND DEVELOPMENT OF FUNCTIONAL FOODS AND ACTIVE PACKAGING IN A CIRCULAR ECONOMY PERSPECTIVE

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Agro-Food By-Products as Sustainable Resources: Exploring Analytical Approaches for The Characterization and Development of Functional Foods and Active Packaging in a Circular Economy Perspective

PhD Thesis

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Summary

This research focuses on green-related topics under Axis IV of the PON Research and Innovation 2014-2020 initiative, "Education and research for recovery - REACT-EU." The study aimed at valorizing agro-food by-products using green extraction techniques, analytical characterizations, and exploring their potential applications in functional foods, nutraceuticals, and packaging sectors, all within a circular economy framework to enhance sustainability and reduce environmental impacts.

The agro-food sector generates large volumes of by-products and waste that can be considered a low-cost source of bioactive compounds (BCs) with high nutritional and biotechnological relevance. These BCs offer antioxidant, anti-inflammatory, and antimicrobial properties, making them ideal for reuse in food, pharmaceutical, and cosmetic industries. Specifically, the studies aimed at investigating industrial by-products from artichoke (ABP) and tomato (TBP), supplied by the company partner GRECI Industria Alimentare spa (Ravadese, Parma). Artichoke by-products generated from industrial processing were evaluated for their carbohydrate's profiles and antioxidant potential. Various analytical techniques, such as Fourier-transform infrared spectroscopy (FTIR-ATR), spectrophotometric assays, and high-performance liquid chromatography (HPLC), were used to assess their composition. The study examined how different industrial processing treatments affected the properties of artichoke by-products.

Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC) were assessed after a drying process. Given the seasonal abundance of TBPs, effective storage methods were investigated, with particular attention to the impact of drying temperature and packaging on these parameters. Additionally, carotenoid contents (i.e., β-carotene and lycopene) were monitored by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) throughout the storage period to evaluate their stability and retention under varying conditions.

A visiting period at the CIAL Institute of Madrid in the Foodomics laboratory has also been carried out. The research focused on optimizing green Pressurized Liquid Extraction (PLE) to maximize the recovery of bioactive compounds from artichoke and tomato by-products using Response Surface Methodology (RSM). Analytical characterizations, including spectrophotometric and enzymatic inhibition assays, revealed a high content of bioactive compounds (phenolics and flavonoids) with significant antioxidant potential. Additionally, ABP and TBP extracts, obtained under optimized conditions, were evaluated for their in vitro neuroprotective potential, exhibiting enzymatic inhibition activity against Alzheimer's disease-related enzymes such as Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE), and Lipoxygenase (LOX). An untargeted metabolomic approach using UHPLC-Q-TOF-MS/MS allowed the investigation the chemical profile of these extracts, revealing phenolic and flavonoid compositions having high antioxidant potential and contributing to the neuroprotective activity.

Potential applications for agro-food by-products were also investigated. A first study investigated the nutraceutical potential of pasta enriched with artichoke-based flour. High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) allowed the identification and quantification of carbohydrates with prebiotic properties, such as fructo-oligosaccharides (FOSs). Analytical characterization revealed an enhanced FOS profile in the enriched pasta, showcasing the innovative use of by-products to promote health through diet. A second study focused on evaluating the in vitro permeability of bioactive compounds extracted from artichoke and tomato by-products (ABPs and TBPs) using PLE under optimized conditions. The Parallel Artificial Membrane Permeability Assay for the Blood-Brain Barrier (PAMPA-BBB) demonstrated that several bioactive compounds, including phenolics like protocatechuic acid and ethyl caffeate, and flavonoids such as apigenin and naringenin, could cross an artificial blood-brain barrier,

highlighting their potential neuroprotective applications. Another line of research focused on encapsulation methods to enhance the stability and bioavailability of carotenoid-rich extracts from TBPs for food and nutraceutical applications. Techniques such as spray drying and nano-emulsion improved the sustained release and antioxidant capacity of these compounds compared to untreated extracts. These encapsulation systems are poised for testing in food systems to assess their functionality in enhancing oxidative stability and delivering health benefits. Additional projects developed a bio-based antioxidant spray formulation aimed at extending the shelf life of perishable food products. Enriched with polysaccharides and active principles, the spray demonstrated significant efficacy in reducing lipid oxidation during storage. This natural and sustainable alternative to synthetic preservatives aligns with the growing demand for clean-label solutions. Finally, TBPs powders were also employed as bio-additives in the development of sustainable coatings formulation to improve hydrophobicity and barrier properties of cellulose-based materials. The application of these formulations led to an improvement in terms of water uptake resistance, suggesting a potential use in novel packaging application.

The research carried out demonstrate the huge potential of agro-food by-products as sustainable resources by characterizing them, optimizing green extraction processes, and exploring innovative applications. The outcomes contribute to developing circular agro-food systems, offering practical solutions for reducing waste, enhancing food quality, and promoting sustainability in industrial practices.

RIASSUNTO

La ricerca è stata condotta nell'ambito dei programmi di dottorato di ricerca incentrati su tematiche green nell'ambito dell'Asse IV dell'iniziativa PON Ricerca e Innovazione 2014-2020, “Education and research for recovery - REACT-EU”. Il presente lavoro si è posto come obiettivo la valorizzazione di sottoprodotti agroalimentari utilizzando metodiche di estrazione sostenibili, svolgendo caratterizzazioni analitiche ed esplorando le loro potenziali applicazioni nei settori degli alimenti funzionali, della nutraceutica e del packaging, il tutto in un quadro di economia circolare per migliorare la sostenibilità e ridurre gli impatti ambientali.

Il settore agroalimentare genera grandi volumi di sottoprodotti e rifiuti che sono considerati una fonte a basso costo di composti bioattivi (BC) con un'elevata rilevanza nutrizionale e biotecnologica. Questi BC offrono proprietà antiossidanti, antinfiammatorie e antimicrobiche, che li rendono ideali per il riutilizzo nell'industria alimentare, farmaceutica e cosmetica. A questo proposito, gli studi condotti si sono incentrati sullo studio dei sottoprodotti industriali di carciofo e pomodoro, forniti dal partner aziendale GRECI Industria Alimentare spa, come fonti sostenibili di composti bioattivi.

La prima parte del lavoro si focalizza sulle valutazioni analitiche condotte su sottoprodotti del carciofo generati da differenti processi di trasformazione. In questo senso, sono state impiegate diverse tecniche come spettroscopia infrarossa a trasformata di Fourier (FTIR-ATR), i saggi spettrofotometrici e la cromatografia liquida ad alta prestazione (HPLC), in grado di investigare il loro potere antiossidante e il profilo di carboidrati, i quali possono avere effetti prebiotici

Una seconda parte si incentra sulle caratterizzazioni analitiche condotte sui sottoprodotti di trasformazione del pomodoro. Inizialmente, sono stati valutati il contenuto fenolico totale (TPC) e la capacità antiossidante totale (TAC) subito dopo essiccazione a due differenti temperature (40 e 70 °C). Dati i grandi volumi di sottoprodotti generati dai processi di trasformazione, in questo lavoro si è valutato sia l’effetto delle temperature di essiccazione che l’effetto di diversi tipi di packaging durante la conservazione (plastica, bioplastica e packaging attivo di origine vegetale). Inoltre, i contenuti di carotenoidi (β-carotene e licopene) sono stati monitorati mediante SFE-SFC-QqQ/MS per tutto il periodo di conservazione, per valutarne la stabilità e la ritenzione in condizioni diverse.

Un successivo capitolo riguarda le attività di ricerca svolte durante il periodo svolto all'estero presso il laboratorio di Foodomics all'istituto CIAL di Madrid. Le ricerche si sono focalizzate sull'ottimizzazione dell'estrazione mediante liquidi pressurizzati (PLE) con lo scopo di massimizzate il recupero di composti bioattivi ad alto potere antiossidante, da sottoprodotti di carciofi e pomodori, utilizzando la metodologia delle superfici di risposta (RSM). Le caratterizzazioni analitiche hanno previsto l'utilizzo di saggi spettrofotometrici per valutare il contenuto di composti bioattivi e il potere antiossidante. Una volta ottenuti gli estratti in condizioni ottimizzate, sono stati valutati per il loro potenziale neuroprotettivo mediante saggi di inibizione enzimatica *in vitro* di enzimi correlati alla malattia di Alzheimer, tra cui l'acetilcolinesterasi (AChE), la butirrilcolinesterasi (BChE) e la lipossigenasi (LOX). Infine, analisi di metabolomica mediante UHPLC-Q-TOF-MS/MS ha permesso di esplorare il profilo chimico di questi estratti, portando all'identificazione di composti fenolici e flavonoidi con un elevato potenziale antiossidante e che contribuiscono all'attività neuroprotettiva ed antiinfiammatoria.

L'ultima parte si focalizza sullo sviluppo di applicazioni innovative per la valorizzazione dei sottoprodotti agroalimentari. Il primo studio ha esaminato il potenziale nutraceutico della pasta arricchita con farina di carciofi, identificando e quantificando, attraverso cromatografia anionica con rilevazione amperometrica pulsata (HPAEC-PAD), carboidrati con azione prebiotica, come i frutto-oligosaccaridi (FOS). La caratterizzazione analitica ha evidenziato un miglioramento del profilo di FOS nella pasta arricchita, dimostrando il valore aggiunto dei sottoprodotti nella creazione di prodotti alimentari innovativi e con comprovati benefici per la salute umana.

Un secondo studio ha valutato le performance di permeabilità *in vitro* di composti bioattivi estratti mediante PLE da sottoprodotti del carciofo e del pomodoro (ABP e TBP). Il saggio PAMPA-BBB ha dimostrato che diversi composti bioattivi, come derivati degli acidi caffeoilchinici (acido protocatecuico, etil caffeato) e flavonoidi (apigenina, naringenina), sono in grado di attraversare una barriera emato-encefalica artificiale, evidenziando il loro potenziale come agenti neuroprotettivi.

Un altro studio si è concentrato sullo sviluppo di metodologie di incapsulamento di estratti arricchiti di carotenoidi da TBP per migliorarne stabilità e biodisponibilità. Tecniche come lo spray drying e la nano-emulsione hanno dimostrato miglioramenti in termini di stabilità e di capacità antiossidante rispetto agli estratti non trattati, aprendo la strada a test in sistemi

alimentari per valutarne l'efficacia nell'aumentare la stabilità ossidativa e i benefici per la salute.

È stata inoltre sviluppata una formulazione spray antiossidante completamente vegetale con lo scopo di prolungare la conservazione di alimenti deperibili. Queste formulazioni a base di polisaccaridi e arricchite con principi attivi come Vitamine E o estratti di sottoprodotti di pomodoro, hanno dimostrato un'efficace riduzione dell'ossidazione, rappresentando una soluzione naturale e sostenibile alla crescente richiesta di prodotti "clean label".

Infine, polveri di sottoprodotti di pomodoro sono state impiegate come bio-additivi in rivestimenti sostenibili per migliorare le performance idrofobiche e di barriera dei materiali a base di cellulosa. Queste formulazioni hanno permesso un discreto aumento in termini di resistenza all'assorbimento d'acqua, suggerendo un potenziale utilizzo in applicazioni innovative per il packaging sostenibile.

Questi studi evidenziano il grande potenziale dei sottoprodotti agroalimentari come risorse sostenibili, attraverso la loro caratterizzazione analitica, l'ottimizzazione di processi di estrazione e l'impiego in nuove applicazioni. I risultati ottenuti contribuiscono allo sviluppo di sistemi agroalimentari circolari, fornendo soluzioni concrete per ridurre gli sprechi e migliorare la qualità degli alimenti, promuovendo approcci più sostenibili nelle pratiche industriali.

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Chapter 1

AIM OF THE PROJECT

This project aimed at exploring and demonstrating the potential of agro-food by-products as sustainable resources within a Circular Economy Framework. The research addresses environmental and economic challenges associated with industrial waste management by focusing on their analytical characterization and functional evaluation for further applications. Specifically, the project aims to bridge the gap between waste generation and resource recovery, providing innovative solutions for valorization that align with sustainability principles and emerging industrial needs.

Nowadays, the agro-food sector generates massive quantities of by-products and waste during the industrial processing of fruits, vegetables, grains, and other commodities. These organic residues are typically discarded or reused in low-value applications, such as animal feed or composting. However, they represent a significantly undervalued resource, rich in bioactive compounds, including polyphenols, carotenoids, flavonoids, and dietary fibers, all of which offer a wide range of benefits for human health. The current linear "take-make-dispose" economy prevalent in the agro-food sector contributes to various environmental issues, such as resource depletion, pollution, and greenhouse gas emissions. This research is driven by the urgent need to transition towards a CE model, focusing on reducing, reusing, and recycling waste streams to create sustainable value chains.

The work has been carried out in collaboration with GRECI Industria Alimentare S.p.A, the company partner of this PhD project, which has provided industrial by-products, such as artichoke and tomato by-products. The industrial perspective of this collaboration ensures that the proposed solutions are not only practical but also aligned with real-world needs. By addressing specific challenges - such as the accumulation of large quantities of tomato and artichoke by-products, along with their high disposal costs - the project aims to offer economically and environmentally sustainable alternatives.

Chapter 2

INTRODUCTION

The vast amounts of by-products and wastes generated from industrial processing significantly contribute to the world's environmental issues. For decades, the global economy has been based on a linear model characterized by a "take-make-dispose" approach, leading to unsustainable resource consumption and strongly impacting environmental systems (Edirisinghe et al., 2024). This model prioritizes short-term efficiency over long-term sustainability, resulting in excessive waste generation, resource depletion, and massive greenhouse gas emissions. The global production of industrial waste has steadily increased over the years, reflecting that this issue persists in many countries (Abubakar et al., 2022). According to The World Bank's 2023 report, industrial waste generated approximately 2.02 billion tons of solid waste each year, projected to reach 3.40 billion metric tons by 2050 (Trends in Solid Waste Management, 2023).

This linear system has been considered the leading cause of food waste, pollution, and economic inefficiencies in the agro-food sector. For these reasons, growing consumer awareness and supportive policies are driving a shift towards sustainable models based on reducing waste, reusing resources, and minimizing environmental impact (Cahyadi et al., 2024; Reguengo et al., 2022). Sustainable supply chain management strategies include optimizing logistics to lower emissions, adopting local sourcing to reduce transport distances, and integrating technologies like blockchain for greater transparency. Precision agriculture and eco-design methodologies support efficient resource use, waste reduction, and the development of recyclable or biodegradable products (Chiaraluce et al., 2021; Skalkos, 2023).

2.1. Circular Economy Framework in Europe

The circular economy (CE) framework offers a transformative paradigm shift from the traditional linear “take-make-dispose” model, addressing pressing global concerns around

resource depletion, environmental degradation, and industrial waste (Edirisinghe et al., 2024). The European Union (EU) has embraced CE as a cornerstone of its Green Deal, aiming for carbon neutrality by 2050 through interlinked policies such as the Circular Economy Action Plan (CEAP, 2020), the Farm to Fork Strategy, and the Chemicals Strategy for Sustainability. For instance, Farm to Fork Strategy aims to establish a fair, healthy, and environmentally friendly food system. This strategy targets food loss reduction, the valorisation of by-products, and the support of bio-based innovations. Moreover, the Chemicals Strategy for Sustainability (2020) introduces the "safe and sustainable by design" concept, encouraging the development and use of substances that minimize risk to human and environmental health across their life cycle. These frameworks collectively reflect the One Health approach endorsed by EFSA (2023), which recognizes the interconnectedness of human, animal, and ecosystem health and stresses the importance of integrated risk assessment and sustainable resource use. Together, these initiatives emphasize regenerative growth, sustainable resource management, and the valorisation of by-products into secondary raw materials within agro-industrial systems (Chiaraluce et al., 2021; Reguengo et al., 2022). In the agro-food sector, CE strategies promote the development of value-added compounds such as antioxidants, fibers, and antimicrobials derived from industrial by-products like fruit peels, vegetable stems, and cereal husks, contributing both to economic efficiency and environmental mitigation (Messinese et al., 2023; Berenguer et al., 2022). Indeed, agro-food chains present significant potential for circularity by converting bio-residues for novel application. Such practices align with the CEAP's objective of promoting sustainable supply chains and mitigating the impacts of food production and packaging.

However, a critical regulatory and scientific challenge emerges from the inherent chemical variability of agro-industrial by-products. These materials are often classified as UVCBs (substances of unknown or variable composition, complex reaction products, or biological materials), which complicates their compositional standardization and toxicological risk assessment (EFSA, 2023). As such, the European Food Safety Authority (EFSA) recommends a tiered, evidence-based approach incorporating both whole mixture and component-based assessments to ensure the safety and functional consistency of these materials when used in food, nutraceuticals, or packaging applications (EFSA Scientific Committee, 2019b). Furthermore, the adoption of advanced extraction techniques and green solvents is essential to improve yield, reproducibility, and sustainability.

2.2. Agro-Food By-Products

Agro-food by-products and waste have gained increasing importance. They represent an impressive bioactive compound resource that provides significant health benefits and improves food shelf-life and technological parameters. According to the FAO's State of Food and Agriculture (2022) report, approximately 14% of the world's food, valued at $400 billion annually, is lost at harvesting and retail levels (Zahid & Khedkar, 2024). This amount of lost food could feed 1.26 billion of people each year. Fruit, vegetable, and cereal by-products are notably recognized for their abundance of high-value compounds, such as phenolics, fibers, and essential nutrients, which hold immense potential for reuse in the food, pharmaceutical, and cosmetic industries (Faustino et al., 2019; Messinese et al., 2023; Reguengo et al., 2022). However, conventional disposal methods, including landfilling, animal feed, fertilizer use, and thermo-valorization, contribute significantly (8-10%) to global greenhouse gas emissions, impacting climate instability and leading to extreme weather events like droughts and floods (Agudelo Higuita et al., 2023; Bolan et al., 2024). This growing environmental impact and resource inefficiencies have prompted global efforts to adopt sustainable strategies for agro-food by-products and waste management.

2.2.1. Sources and Compositions

The industrial processing of grains, legumes, fruits, and vegetables generates substantial by-products rich in macronutrients - such as carbohydrates, proteins, and fatty acids - and micronutrients, including vitamins, iron, calcium, and potassium (Faustino et al., 2019). These bioactive compounds (BCs) are phytochemicals produced through the primary and secondary metabolism of plant cells, playing essential roles in plant development and offering various health benefits to humans (Reguengo et al., 2022; Vilas-Boas et al., 2021). Numerous studies have thoroughly characterized these phytochemicals in agro-food by-products, recognizing their health-promoting properties, such as fostering beneficial gut microbiota interactions (Belović et al., 2017; Krumbeck et al., 2016; Pathania & Kaur, 2022), and exhibiting antioxidant (Abd El-Aziz et al., 2021a; Akbari et al., 2022; Baranowska et al., 2021; Jiménez-Moreno et al., 2019; Solaberrieta et al., 2022), antimicrobial (Shallan et al., 2020; Stan et al., 2021; Szabo et al., 2019), anti-inflammatory (Ben Salem et al., 2017; Carpentieri et al., 2022; Giménez-Bastida et al., 2021), cardioprotective (Iglesias-Carres et

al., 2023), and neuroprotective effects (Angeloni et al., 2022; Montenegro et al., 2021; Sanchez-Martinez et al., 2021). These properties make BCs valuable for applications in the food, pharmaceutical, and cosmetic industries.

Fruit and vegetable processing industries produce large quantities of waste - peels, seeds, stems, and pulp - which can account for 10–60% of the raw material, depending on the product and processing methods (Morales-Castro et al., 2021). For example, citrus processing results in over 50% of the biomass becoming by-products, producing around 15 million tonnes of peel waste annually. Similarly, grape processing for winemaking generates significant bagasse waste, including peels, seeds, and stems (Campos et al., 2020; Reguengo et al., 2022). Tomato processing also contributes notably to by-product generation, with about one-third of the tomatoes processed discarded as waste—primarily seeds and skins (Casa et al., 2021; Coelho et al., 2023).

Phenolic and flavonoid compounds, carotenoids, and dietary fibers are prominent phytochemicals in agri-food by-products, valued for their wide-ranging applications. These bioactive compounds demonstrate notable biological activities, such as anti-aging, antiviral, antimicrobial, and anticancer effects (Samtiya et al., 2021). Phenolics and flavonoids—like hydroxycinnamic acids, quercetin, and catechins—are being investigated as functional additives in the food industry (Chadorshabi et al., 2022; Freitas et al., 2021; Grispoldi et al., 2022; Kaderides et al., 2020), biomedical applications (Aluko, 2021; Marino et al., 2022; Tungmunnithum et al., 2018), packaging materials (Grimaldi et al., 2022; Shaik et al., 2022; Visco et al., 2022a), and cosmetics (D'Antuono et al., 2018; Meléndez-Martínez et al., 2019) due to their strong antioxidant and radical-scavenging capacities (Akbari et al., 2022; Mellinas et al., 2022; Samtiya et al., 2021; Teodoro, 2019). Their potent antioxidant, anti-inflammatory, and antimicrobial activities make these compounds ideal for skin health, wound healing, and anti-aging formulations in the cosmetic sector (Abd-El-Aziz et al., 2024; Banwo et al., 2021; Li et al., 2022a; Porro et al., 2024; Turkiewicz et al., 2019a; Zeaiter et al., 2019).

Carotenoids like lycopene, β-carotene, and zeaxanthin are recognized for their antioxidant and anti-inflammatory properties and their role in preventing chronic diseases, including cardiovascular disorders and age-related macular degeneration. Notably, β-cryptoxanthin from citrus fruits inhibits angiogenesis through mechanisms mediated by retinoic acid, highlighting its potential as a functional food supplement ingredient (Belović et al., 2017;

Quesada-Gómez et al., 2018) and a natural additive in pharmaceuticals and cosmetics (Cassani et al., 2022; Lombardelli et al., 2021; Nour et al., 2018; Meléndez-Martínez et al., 2019; Stinco et al., 2016; Szabo et al., 2022).

Dietary fibers, primarily indigestible polysaccharides like inulin from plant cell walls, are beneficial for gut health and probiotic growth (An et al., 2022). Beyond their nutritional roles, dietary fibers derived from industrial processing enhance food product quality. For instance, adding potato peel powder to bread improves dough strength, elasticity, and extensibility, offering valuable applications in baking. Moreover, these fibers serve as bulking agents, emulsifiers, and stabilizers, enhancing food texture and rheology (Difonzo et al., 2022; Jackson et al., 2022). Their water and oil retention, swelling capacity, viscosity, gel formation, and chelating properties make them promising eco-friendly components in biodegradable packaging materials (Berthet et al., 2015; Grimaldi et al., 2022; Pathania & Kaur, 2022). However, the inherent heterogeneity of the agro-food by-products poses challenges not only in compositional standardization but also in ensuring safety and purity, particularly when these by-products are intended for food, cosmetic, or pharmaceutical applications. Besides, it is important to evaluate the stability of the active compounds also during storage, as antioxidant are particularly susceptible to degradation deriving from light and air exposure (Mendonça et al., 2022).

2.2.2. Artichoke By-Products

Artichoke (*Cynara scolymus L.*) by-products (ABP), primarily consisting of external bracts, leaves, and stems, make up as much as 85% of the plant's total biomass following harvest and industrial processing (Ayuso et al., 2024; Colombo et al., 2024). The increasing focus on revalorizing these by-products aligns with global sustainability objectives, addressing environmental concerns related to agricultural waste while fostering new economic opportunities. Notably, the Mediterranean region, which leads global artichoke production, generates over 460,000 tons of artichoke waste annually. These residues are progressively being transformed into high-value ingredients (Feiden et al., 2023). Traditionally regarded as waste, these matrices are now recognized as rich sources of bioactive compounds, including phenolic acids (such as benzoic acid derivatives and mono- and di-caffeoylquinic acids), flavonoids (like apigenin, luteolin, quercetin, and their glucoside complexes), and

dietary fibers (inulin, pectin, cellulose, and hemicellulose) (Ayuso et al., 2024; Colombo et al., 2024; López-Salas et al., 2024).

The concentration of polyphenols—which drive the strong antioxidant capacity of ABPs—varies based on cultivar type, tissue maturity, and extraction methods, reaching up to 12.7 g/kg of dry matter in some bracts. In addition to polyphenols, artichoke by-products are abundant in vitamins (C, E, and B-complex), minerals (potassium, magnesium, iron, and zinc), and prebiotic fibers like inulin. These components support gut health by encouraging the growth of beneficial bacteria such as Lactobacillus and Bifidobacterium species (Difonzo et al., 2022; Jackson et al., 2022; Zeaiter et al., 2019).

Comprehensive studies on the biochemical profile of artichoke by-products have revealed their extensive health benefits, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and lipid-lowering properties (Abd El-Aziz et al., 2021a; Akdogan & Peksel, 2023; Iglesias-Carres et al., 2023; Sokkar et al., 2020). For example, the flavonoids luteolin and apigenin are noted for their neuroprotective, anti-inflammatory, and anti-glycation effects (Maietta et al., 2017). Additionally, cynaropicrin—a sesquiterpene lactone found in artichoke bracts—has been linked to hepatoprotective and digestion-enhancing functions (Elsebai et al., 2016).

From an industrial standpoint, innovative green extraction technologies, such as pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE), have been developed to optimize the recovery of bioactive compounds from ABPs (López-Salas et al., 2024; Mellinas et al., 2022; Pagano et al., 2021; Ruiz-Aceituno et al., 2016). These sustainable techniques utilize eco-friendly solvents - classified as Generally Recognized as Safe (GRAS) - and novel solutions like Natural Deep Eutectic Solvents (NADES). NADES, formed by combining Lewis or Brønsted acids and bases, offer several advantages, including low toxicity, biodegradability, and enhanced solubility of phenolic compounds (López-Salas et al., 2024; Smith et al., 2014). In the food industry, artichoke by-products show considerable promise as functional ingredients for boosting nutritional content and extending product shelf life (Ayuso et al., 2024; Feiden et al., 2023). Research highlights that ABP extracts, rich in bioactive compounds with potent antimicrobial and antioxidant effects, can function as natural food preservatives (Cannas et al., 2024). These properties help slow lipid oxidation and microbial spoilage in products like meat, baked goods, and dairy (Canale et al., 2022; Demir & Ağaoğlu, 2021; Jackson et al.,

2022). Moreover, incorporating ABPs into bakery products has demonstrated prebiotic effects, thanks to inulin and related carbohydrates known for fostering beneficial gut microbiota (Messinese et al., 2023).

Beyond their role in food applications, artichoke by-products are increasingly explored for sustainable packaging solutions. Their abundance of bioactive compounds, particularly polyphenols and fibers, makes them ideal for developing bio-based films and coatings with enhanced functional properties. Active packaging films infused with artichoke-derived extracts have shown improved antioxidant and antimicrobial capacities, effectively prolonging the shelf life of packaged goods (Erguner & Harsa, 2023; Grimaldi et al., 2022).

2.2.3.Tomato By-Products

Tomato (*Solanum lycopersicum*) by-products (TBP), comprising peels, seeds, and residual pulp, are significant biomass generated during the industrial processing of tomatoes for products like juice, paste, and sauces, amounting to approximately 30 - 40% of the initial raw material (Méndez-Carmona et al., 2022; Rajan et al., 2022). According to FAOSTAT's (2022) report, global fresh tomato production was estimated around 186 million tons annually. In California, which produces 95% of US tomatoes, tomato pomace is primarily utilized in cattle feed for dairy farms due to its rich content of BCs including carotenoids (lycopene and β-carotene), polyphenols, dietary fiber, proteins, and essential lipid (Casa et al., 2021; López-Yerena et al., 2024). In contrast, landfilling remains the predominant disposal method employed in other countries such as Italy (Casa et al., 2021).

Bioactive compounds present in TBP are extensively characterized and recognized for their beneficial effects on human health (Elbadrawy & Sello, 2016; López-Yerena et al., 2024; Meléndez-Martínez et al., 2019; Trombino et al., 2021). Lycopene, a carotenoid found in concentrations up to four times higher in tomato peels than in pulp or seeds, is particularly noted for its high antioxidant properties. It has been reported that the consumption of food rich in carotenoid relates with reduced risk of chronic conditions, including cardiovascular diseases, prostate cancer, and age-related disorders (Elbadrawy & Sello, 2016; López-Yerena et al., 2024; Meléndez-Martínez et al., 2019; Trombino et al., 2021). Polyphenols and flavonoids such as caffeic acid and quercetin in TBP also were found to exert potent antimicrobial, anti-inflammatory, neuroprotective effects and may contribute to cognitive

health by reducing neuroinflammation and oxidative damage (Oboh et al., 2015; Rojas-García et al., 2023; Viuda-Martos et al., 2014). The presence of fibers fraction in tomato by-products ranges was also found to contribute to gastrointestinal health and enhances the textural and functional properties of foods (Viuda-Martos et al., 2014). However, ensuring safety requires a thorough assessment of potential pesticide residues or contaminants that may remain on plant surfaces. Additionally, contaminants such as toxins formed during storage, potential occurrence of heavy metals, and other harmful compounds must be closely monitored and controlled to protect human health and ensure compliance with regulatory standards (Pacini et al., 2024).

Advanced extraction techniques, such as supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), and enzymatic methods (EAE), have been optimized over the years to efficiently recover bioactive compounds from TBP in an optic of sustainability (Chada et al., 2022; J. Li et al., 2022b; Mellinas et al., 2022; Méndez-Carmona et al., 2022), allowing minimal environmental impact and high reproducibility, in line with principles of CE. In the food application, tomato by-products have been employed to create enriched functional foods. For example, TBP were employed as bio-based additives in bakery items such as bread and muffins to improve contents of fibres and antioxidant compounds, providing enhancement to shelf-life (Mehta et al., 2018; Silva et al., 2022). Also, in meat-based products, the addition of TBP extracts as colorants provided enhancement of nutritional values, while allowing a reduction of synthetic nitrites content (Cassani et al., 2022; Lombardelli et al., 2021). In other processed foods like ketchup and tomato puree, addition of TBP allowed improvement in terms of rheological properties and nutrients content (Bayod et al., 2008; Torbica et al., 2016).

In addition to their food and health applications, TBP are reported to be suitable as additives in the development of sustainable packaging materials. Extracts obtained from TBP incorporated into bio-based films and coatings were found to enhance their chemical and mechanical properties. For instance, polyvinyl alcohol (PVA)-based films enriched with the extracts reported to enhancing antioxidant and antimicrobial activity, improved physical properties such as thickness and density (Stoll et al., 2019; Szabo et al., 2019, 2020). Carotenoids-rich extracts also demonstrated to improve UV light and oxygen barrier properties when added to the fabrication polylactic acid (PLA) based films (Stoll et al., 2019).

TBP represents a versatile and sustainable resource with several applications across food, health, and packaging industries.

2.3. Safety and Toxicological Aspects Related to the Agro-Food By-Products

A critical aspect in the sustainable repurposing of agro-food by-products lies in achieving adequate purity of these materials, guaranteeing the safety for consumer application. The intrinsic chemical complexity and compositional variability of these matrices pose considerable challenges in isolating compounds of high purity and functional integrity - requirements that are essential for their incorporation into food, nutraceutical, or pharmaceutical formulations. The compositional integrity of the extracted compounds not only influences functional and stability performance but is also important in meeting safety and regulatory requirements (Socas-Rodríguez et al., 2021; Bottex et al., 2023).

Potential contaminations, which may arise during post-harvest handling, storage, transport, or processing can strongly affect the safety of these materials. Residual agrochemicals, heavy metals from soil uptake, microbial proliferation due to moisture retention, or mycotoxin formation under inadequate storage conditions can compromise the safety of the final products. Moreover, mechanical damage and temperature fluctuations during transport can promote microbial proliferation and oxidative degradation, thereby altering the biochemical composition and functional stability of agro-industrial by-products (Socas-Rodriguez et al., 2021; Galanakis, 2021). These degradative processes can lead to the formation of undesirable metabolites, spoilage, or loss of bioactivity, especially in moisture-rich or perishable matrices. Consequently, comprehensive risk assessment protocols - including microbiological screening, chemical contaminant analysis, and moisture and temperature control - must be implemented to safeguard the integrity of these materials (Onyeaka et al., 2024). This aspect is considered particularly critical when high-purity fractions are targeted for use in food, nutraceutical, or biomedical applications, where regulatory compliance, batch reproducibility, and consumer safety are non-negotiable requirements (Bottex et al., 2023).

Although the use of agro-industrial by-products offers considerable economic and environmental benefits, meeting both functional and legal standards demands that purity and safety be assured throughout the entire supply chain, from sourcing and collection to storage and transformation (Socas-Rodríguez et al., 2021). Therefore, implementing analytical quality control protocols - such as contaminant profiling, molecular fingerprinting, and purity quantification - alongside optimized logistics for handling and storage, can represent key practices to ensure safety and scalability for sustainable applications. Currently, European Union food safety regulations state that any novel or modified ingredients derived from agro-food by-products must meet established compositional, toxicological and safety standards (Bottex et al., 2023). These regulations apply equally to materials intended for food and those intended for use in animal feed. Although the scientific community has made substantial progress in extracting high-value nutrients and bioactive molecules from agro-food by-products, safety validation remains a critical step which could affect the industrial scalability (Bottex et al., 2023; Socas-Rodriguez et al., 2021). Comprehensive safety assessments typically include physicochemical and organoleptic traits characterization, microbiological assays targeting key pathogens (e.g., *Salmonella spp.*, *Listeria monocytogenes*, *Escherichia coli*), and contaminant screening for pesticide residues, heavy metals, and naturally occurring toxins (Bottex et al., 2023). Furthermore, toxicological evaluations - such as cytotoxicity and mutagenicity assays - are crucial to establish the potential biological risks associated with the use of these compounds in consumer application (Bottex et al., 2023; Socas-Rodriguez et al., 2021). Finally, migration assessments are required to monitor the release of hazardous compounds from packaging or matrix materials further complement the safety profile requested for regulatory clearance (Bottex et al., 2023; Socas-Rodriguez et al., 2021).

2.4. Valorization Approaches: Extraction Techniques to Recovery Bioactive Compound from Agro-Food I By-Products

The extraction of bioactive compounds from agro-food industrial by-products is crucial for proper valorization into new applications. Effective extraction methodologies must consider both efficiency and sustainability, facing cost, time limitations, and solvent safety. Liquid-solid extraction is the most widely used extraction technique for recovering BCs from natural

matrices. This process relies on diffusion and osmosis principles, involving solvent penetration into a solid matrix to dissolve and transport solutes. The extraction progresses through solvent diffusion, solute dissolution, and solute migration to the bulk solution, then reaches equilibrium between solute concentrations inside and outside the matrix (Gil-Martín et al., 2022; Sagar et al., 2018). Governed by Fick's law, the process is sensitive to prolonged heating or extended times, which can degrade compounds and reduce quality. Once equilibrium is achieved, the solution containing the extracted compounds is mechanically separated from the solid residue and dissolved into the solvent. Conventional techniques, such as maceration, Soxhlet extraction, percolation, and heat reflux extraction, are widely employed in recovering BCs from plant materials (Patra et al., 2022). For instance, maceration, operating at room temperature, is ideal for thermolabile compounds, while Soxhlet extraction is efficient but risks degrading heat-sensitive compounds due to higher temperatures (Patra et al., 2022; Q. Zhang et al., 2018). However, they require long processing times and massive quantities of organic solvents, which can contribute to environmental hazards and degrade heat-sensitive compounds (Patra et al., 2022; Zhang et al., 2018). Consequently, there is an increasing emphasis on mild extraction conditions and adopting greener, safer approaches that maximize yield while preserving compound functionality.

Artichoke and tomato by-products rich in antioxidants, polyphenols, carotenoids, and dietary fibers represent perfect candidates for a valorization approach. By adopting innovative extraction methodologies, industries can transform by-products into valuable resources, reducing environmental impact and supporting the development of sustainable food systems and materials. Novel extraction methodologies involve supercritical fluid extraction (SFE) and enzyme-assisted extraction (EAE) that represent advanced techniques for BCs recovery, offering specific advantages such as mild operating conditions and the preservation of heat-sensitive compounds (Patra et al., 2022). Additionally, using emerging solvent mixtures such as NADES provides high selectivity for classes of compounds and is considered an effective eco-friendly alternative. Although these approaches perfectly align with circular economy principles, the following sections will focus on ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) since they are the object of these investigations. Ultrasound-assisted extraction (UAE) has emerged as an eco-friendly and highly efficient technique for extracting bioactive compounds from plant-based by-products, notably from residues of artichoke and tomato processing. This method utilizes

ultrasonic waves (ranging from 20 to 100 kHz) to generate cavitation bubbles within the extraction medium. The collapse of these bubbles creates localized high-pressure and high-temperature conditions that disrupt plant cell walls, enhance mass transfer, and facilitate the release of bioactive compounds (Gil-Martín et al., 2022; Zhang et al., 2018). Operating under mild conditions, UAE offers significant advantages over conventional extraction methods, including reduced energy and solvent consumption, shorter processing times, and the preservation of heat-sensitive compounds. The cavitation effect—central to UAE's efficiency—improves solvent penetration into plant matrices, increases interfacial contact, and enhances the solubility of target compounds. Research has consistently shown UAE's effectiveness in extracting polyphenols, carotenoids, and other antioxidants, making it highly valuable for applications in the food, pharmaceutical, and nutraceutical sectors (Akdogan & Peksel, 2023; Gómez-Cruz et al., 2021; Mellinas et al., 2022; Rabelo et al., 2016). Specifically, UAE has been widely employed to recover antioxidant-rich polyphenols and dietary fibers from artichoke by-products, significantly reducing extraction times when optimized compared to traditional methods (Colombo et al., 2024; Rabelo et al., 2016). Similarly, in tomato pomace, UAE has proven effective in extracting lycopene and flavonoids, delivering higher yields while preserving their bioactivity (Li et al., 2022a; Solaberrieta et al., 2022). Beyond artichoke and tomato residues, UAE has been successfully applied to various industrial by-products, including grape seeds, pomegranate peels, and citrus residues. In these applications, UAE consistently outperforms traditional techniques, achieving superior recovery rates of phenolics, flavonoids, and anthocyanins (Solaberrieta et al., 2022).

Pressurized liquid extraction (PLE), also referred to as accelerated solvent extraction (ASE), is a highly efficient and advanced method used to extract bioactive compounds (BCs) from agro-food by-products (Bragagnolo et al., 2022; Plaza et al., 2013; Ruiz-Aceituno et al., 2016; Tripodo et al., 2018). This technique utilizes moderate pressures ranging from 10 to 20 MPa and elevated temperatures between 50 °C and 200 °C to significantly enhance the mass transfer of phytochemicals (Alvarez-Rivera et al., 2020). The application of these conditions not only reduces solvent usage and shortens extraction time but also results in high recovery yields of essential compounds such as phenolics, flavonoids, and carotenoids. A key advantage of PLE lies in its ability to operate under subcritical conditions, which alters solvent properties—including viscosity and polarity—to improve the solubilization and extraction efficiency of target compounds (Alvarez-Rivera et al., 2020). Additionally, PLE's capacity to precisely control temperature and pressure safeguards heat-sensitive bioactive

compounds, making it particularly suitable for extracting delicate phytochemicals in the food, pharmaceutical, and nutraceutical industries (Perra et al., 2023). Applications of PLE extend to various agro-food by-products, such as grape pomace, citrus peels, and pomegranate residues, where it has consistently demonstrated higher efficiency in recovering phenolics, flavonoids, and anthocyanins compared to conventional techniques (Alvarez-Rivera et al., 2020; Perra et al., 2023; Sánchez-Martínez, Valdés, et al., 2022)Alvarez-Rivera et al., 2020; Perra et al., 2023; Sánchez-Martínez, Valdés, et al., 2022). Previous research on artichoke and tomato by-products has demonstrated that PLE is particularly effective in extracting bioactive compounds like polyphenols and lycopene (Pagano et al., 2021; Strati et al., 2015). Compared to other technologies, PLE demonstrated higher recovery rates of phenolics and anthocyanins, phytochemicals presenting high antioxidant activities, than supercritical fluid extraction, suggesting its potential use in the context of industrial-scale valorization (Alvarez-Rivera et al., 2020; Bragagnolo et al., 2022). This combination of efficiency, sustainability, and versatility makes PLE a powerful tool for valuing agro-food by-products. It offers significant potential for large-scale industrial applications in developing new products through friendly workflow.

2.5. Analytical methods for agro-food by-products characterization

The analytical characterization of agro-food by-products has emerged as a critical area of study, addressing the growing need for sustainable and innovative resource utilization. These by-products, often rich in bioactive compounds, present valuable opportunities for applications in functional foods, nutraceuticals, and pharmaceuticals. Several analytical techniques such as spectroscopy, spectrophotometry, chromatography and mass spectrometry have been developed to meet the challenges of characterizing the complex matrices such as food or its industrial by-products, offering insights into their chemical composition, functional properties, and potential applications.

This section will focus on discussing the primary analytical techniques employed throughout this project, highlighting their role and relevance in the comprehensive characterization and valorization of agro-food by-products.

2.5.1. Oxidative Stability Techniques

Oxidation is a significant challenge in maintaining the quality and shelf life of food products, particularly those rich in lipids, such as oils and processed foods. Lipid oxidation leads to the deterioration of sensory properties, nutritional value, and safety of food products, resulting in rancidity, off-flavors, and the formation of potentially harmful compounds. These changes not only compromise product quality but can also negatively impact consumer health (Wang et al., 2023). The addition of antioxidants, particularly natural ones, has gained considerable attention as a strategy to mitigate oxidation. However, their efficacy varies depending on oxidation conditions and the analytical methods used to evaluate stability (Kumar et al., 2015). The evaluation of oxidative stability is crucial for ensuring product quality and predicting shelf life. Methods such as the Oxitest and Rancimat are widely employed for this purpose, each offering unique advantages. The Oxitest reactor accelerates oxidation by applying high oxidative stress, enabling rapid and reliable assessment of oxidative stability. Moreover, it allows for the direct analysis of whole samples without the need for preliminary fat extraction, making it particularly effective for complex food matrices (VELP Scientifica), including agro-food by-products. Studies have demonstrated the efficacy of the Oxitest in monitoring oxidative stability across various food products. For example, research on bakery items with high fat content utilized the Oxitest to predict shelf life and assess the impact of lipid oxidation over time (Caruso et al., 2017). Similarly, studies on the addition of chili pepper extracts to edible oils have shown improved oxidative stability, demonstrating the potential of natural antioxidants in preserving food quality (Cavazza et al., 2015). The instrument's ability to test samples in their entirety streamlines the analysis process, providing a more accurate representation of real-world conditions. In the context of agro-food by-products, the Oxitest can be employed to evaluate the oxidative stability of these materials when incorporated as ingredients or additives. For example, the oxidative stability of oils and food products enriched with by-product-derived bioactive compounds has been shown to correlate strongly with their phenolic content, underscoring the importance of these antioxidants (Cavazza et al., 2015). By providing insights into how by-products influence the oxidative stability of final products, manufacturers can make informed decisions regarding formulation and storage to maintain quality and extend shelf life.

2.5.2. Spectroscopic Techniques

Non-separative analytical techniques offer high throughput and low resource consumption, while separative methods provide greater selectivity (Mandrioli et al., 2022). These techniques are easy, quick and affordable for the evaluation antioxidant properties, and other bioactive compounds content in food matrices. For example, spectrophotometric assays can operate on redox or radical reactions, where bioactive compounds act as reducing or scavenging agents, generating spectral changes in terms of absorbance (Mir-Cerdà et al., 2023). These methods include the Folin-Ciocalteu (FC) assay, ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and oxygen radical absorbance capacity (ORAC) assays. These assays are categorized based on their reaction mechanisms into hydrogen atom transfer (HAT) and single electron transfer (SET) methods (Apak et al., 2016; Munteanu & Apetrei, 2021). For instance, FC, FRAP, and DPPH rely on SET mechanisms, ORAC employs a HAT mechanism, while ABTS involves both (Apak et al., 2016; Munteanu & Apetrei, 2021). Results are typically expressed relative to calibration standards, such as gallic acid for total phenolic content (TPC) in FC assays or Trolox for antioxidant capacity like DPPH.

Beyond general assays for evaluating antioxidant potential and bioactive compound content, spectroscopic techniques such as infrared spectroscopy have gained prominence for analytical characterization. Fourier-Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) is a powerful non-separative method used to measure molecular vibrations, providing detailed information about functional groups and chemical bonds in complex food matrices (Krähmer et al., 2021; Subramanian & Rodriguez-Saona, 2009). This technique requires minimal sample preparation and offers rapid, non-destructive analysis. FTIR-ATR has been successfully applied in the study of agro-food by-products to determine compositional characteristics, such as phenolic content, lipids, and carbohydrates, contributing to a comprehensive understanding of their bioactive profiles.

2.5.3. Chromatographic Techniques

Chromatographic techniques are separative methods widely employed to characterize agro-food by-products, enabling bioactive compounds' separation, identification, and quantification from complex matrices. Among these techniques, gas chromatography (GC),

high-performance liquid chromatography (HPLC) and its advanced variant, ultra-high-performance liquid chromatography (UHPLC), are particularly relevant for their versatility and precision. These techniques are based on the interaction of analytes with the stationary and mobile phases to achieve separation, enabling the quantification and structural elucidation of target compounds. The broad range of separation modes and the large selection of commercially available columns allow wide adaptability of these methods to different analytical requirements (Mir-Cerdà et al., 2023). Gas chromatography is especially suitable for the analysis of volatile and semi-volatile compounds, such as essential oils, aroma constituents, and low-molecular-weight fatty acids, often found in agro-food by-products (Louw et al., 2021). GC typically uses a capillary column with a liquid stationary phase and requires analyte volatility or prior derivatization to improve thermal stability and detectability. Coupled with detectors such as flame ionization (FID) or mass spectrometry (GC-MS), it offers excellent resolution, sensitivity, and reproducibility for complex sample matrices (Louw et al., 2021). GC has been successfully applied in studies related to the valorization of by-products like citrus peels, grape pomace, and tomato skins for profiling volatile aroma compounds, and identifying antioxidant or antimicrobial components (El Kady et al., 2024).

Concerning liquid chromatography, reversed-phase is one of the main using mode for analyzing bioactive compounds such as phenolic and flavonoid compounds in food-derived matrices. It often involves stationary phases with chemically bonded aliphatic groups, such as octadecyl (C18), and other functional moieties like butyl or phenyl, to achieve selective separations (Cavaliere et al., 2018; Mir-Cerdà et al., 2023). Mobile phases typically consist of hydro-organic mixtures, such as methanol or acetonitrile, often supplemented with small amounts of organic acids like formic or acetic acid (Mir-Cerdà et al., 2023). Gradient elution programs are tailored to handle the complexity of phenolic and polyphenolic compositions, ensuring efficient resolution of diverse compounds within a single run. Indeed, such techniques have been widely employed to characterize phytochemicals in agro-industrial residues, including polyphenols, carotenoids, and volatile organic compounds (Barth et al., 2019; Ben Salem et al., 2017; Donno et al., 2020; Otify et al., 2023).

Another separative method involves anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) offers a valuable alternative to mass spectrometry for the analysis of specific classes of bioactive compounds. HPAEC-PAD is particularly effective in the detection of highly polar and ionic analytes, such as carbohydrates,

oligosaccharides, and certain phenolic acids, which may be challenging to analyze using conventional HPLC or GC-MS methods (Borromei et al., 2009; Jumaah et al., 2016; Mechelke et al., 2017; Rohrer et al., 2013). This technique provides high sensitivity and specificity without requiring derivatization, making it a cost-effective and straightforward option for laboratories without access to mass spectrometry. Its ability to directly measure electroactive compounds offers a reliable approach for targeted analyses in agro-food by-product characterization.

More recently, emerging approaches such as supercritical fluid chromatography (SFC) and ultra-high-performance SFC (UHPSFC) have gained importance, particularly for analyzing non-polar bioactive compounds like carotenoids (Giuffrida et al., 2020; Jumaah et al., 2016; Zoccali et al., 2021). These methods combine rapid analysis with reduced solvent usage, aligning with the principles of green chemistry while offering high efficiency in compound separation.

The integration of chromatographic methods with mass spectrometry has revolutionized the analysis of food matrices. By coupling separation techniques with MS, is possible to gain sensitivity, specificity, and structural elucidation capabilities, enabling a deeper understanding of the bioactive compounds within these matrices (Mir-Cerdà et al., 2023).

2.5.4. Mass Spectrometry

Mass spectrometry (MS) is widely employed among the analytical techniques to obtain extensive characterization of complex biological matrices. Its high sensitivity allows accurate discrimination and quantification of chemical compounds present in samples, which are crucial for determining their potential applications in functional foods, nutraceuticals, and pharmaceuticals.

Both liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are commonly used in the analysis of agro-food by-products and waste, offering complementary coverage depending on the volatility and polarity of target analytes. GC-MS is particularly effective for volatile and thermally stable compounds, such as aroma compounds and short-chain fatty acids, while LC-MS is suited for non-volatile and thermally labile compounds, including polyphenols, flavonoids, and glycosylated metabolites (Louw et al., 2021).

The analytical characterization of food matrices exploiting MS is predominantly performed using targeted and non-targeted approaches. Targeted approaches rely on the quantification

of pre-established compounds or compound families, based on the availability of commercial standards for accurate quantification and confirmatory analysis (Mir-Cerdà et al., 2023). Over the years, agro-food by-products and waste have been extensively characterized with this methodology, mainly adopting tandem mass spectrometry (MS/MS) by means of mass analyzers such as triple-quadrupole (QqQ) working in multiple reaction monitoring (MRM) modes, which can enhance selectivity and sensitivity of the analytes of interest (Di Lena et al., 2021; Och et al., 2023). On the other hand, non-targeted analysis, performed in full-scan mode, are particularly suited for comprehensively identifying known and unknown compounds, aiming to explore the full chemical composition of samples . These approaches are typically implemented using high-resolution mass spectrometry (HR-MS) platforms such as Orbitrap and time-of-flight (TOF) analyzers, coupled with either LC or GC depending on the nature of analytes. These instruments operate in full-scan acquisition mode, allowing exact mass measurements, structural elucidation through isotopic pattern analysis, elemental composition determination, and tandem MS/MS fragmentation (Mir-Cerdà et al., 2023). The combination of HRMS with MS/MS fragmentation is particularly powerful for elucidating structures of novel or unexpected bioactive molecules occurring in agro-food by-products extracts. Moreover, innovative software algorithms further enhance the annotation and interpretation of mass spectral data, making this strategy essential for discovering new compounds of interest (Mir-Cerdà et al., 2023). When coupled with chemometric tools, non-targeted analysis can support comparative profiling, biomarker discovery, and compositional mapping (Valdes et al., 2021). MS versatility is also evident in the range of ionization methods available (e.g., electrospray ionization, ESI, and electron impact, EI), which accommodate diverse compound classes and sample types. The availability of hybrid systems such as QTOF, Orbitrap, and Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) ensures ultra-high resolution and accurate mass capabilities essential for untargeted workflows and structural elucidation (González-Domínguez et al., 2020).

A significant application of MS-based techniques in the analytical evaluation of food and its by-products is their integration within the Foodomics approach - a pioneering research field where the synergy of analytical chemistry, food science, and biotechnology comes together to provide a comprehensive understanding of food matrices (Cifuentes, 2009; García-Cañas et al., 2012; Herrero et al., 2012; Valdés et al., 2021). Foodomics adopts a systems biology perspective, utilizing advanced MS technologies to investigate food's chemical

composition, functionality, biological impact, and related matrices (Valdés et al., 2021). This multidisciplinary approach enables identifying and quantifying bioactive compounds, their metabolic pathways, and their interactions within complex food systems.

2.6. Agro-food By-Products for Nutraceuticals and Functional Foods Enrichment

The concept of circularity in agro-food systems highlights the importance of "closing the loop" within production and consumption cycles by reintegrating waste materials into the value chain. Agro-food by-products, which are rich in bioactive compounds, have potential applications as nutraceuticals or bio-additives that can enhance the technological properties and shelf life of food products (Messinese et al., 2023). By transforming these by-products into valuable ingredients, industries can reduce their environmental impact while creating new economic opportunities. For instance, incorporating apple and orange pomaces into bakery products has been shown to improve dough hydration, increase fiber content, and extend freshness by enhancing water retention capacity (Gasparre et al., 2024; Usman et al., 2020). Similarly, artichoke by-products generated from industrial processing have been utilized to produce bread with higher levels of phenolic compounds and dietary fiber (Canale et al., 2022). In meat processing, by-products like pomegranate peel extracts and avocado seed powders help reduce lipid oxidation, extend shelf life, and improve sensory attributes, providing a natural alternative to synthetic preservatives (Gullón et al., 2020; Rodríguez-Carpena et al., 2011). Additionally, artichoke extracts have shown antioxidant benefits by lowering oxidative stress markers, such as metmyoglobin, in freeze-stored minced meat (Demir & Ağaoğlu, 2021). Dairy products have also benefited from the addition of carrot peel powder, which enhances color, antioxidant capacity, and antimicrobial properties while increasing dietary fiber content (Kamel et al., 2023). These enriched food items often qualify as functional foods due to their health-promoting properties. For example, yogurts supplemented with pomegranate peel powder, which is a rich source of dietary fiber and phenolic compounds, exhibit enhanced antioxidant activity and gut health benefits (Lai & Tang, 2024). Likewise, natural pigments derived from tomato by-products offer a sustainable alternative to synthetic dyes in sauces and dressings while maintaining product appeal (López-García et al., 2021). Moreover, the antioxidant properties

of tomato by-products can extend the shelf life of lipid-rich foods by reducing oxidative spoilage. For instance, tomato seed powder has been shown to delay rancidity and preserve sensory qualities in meat products (Putnik et al., 2021). Advancements in encapsulation techniques further enhance the potential of bioactive compounds found in agro-food by-products. For example, nano-emulsions using lupin protein isolate (LPI) protect phenolics like chlorogenic acid and luteolin during gastrointestinal transit, preserving their antioxidant activity and enabling targeted delivery to the colon, where they exhibit antiproliferative effects against cancer cells (Siles-Sánchez et al., 2024). Biocompatible encapsulation systems that adhere to the INFOGEST protocol ensure the retention of antioxidant efficacy during digestion, making them ideal for incorporating bioactive compounds into functional foods (Kondrashina et al., 2024). Liposomal systems, especially those enriched with inulin, have also been effective in stabilizing carotenoids and terpenoids in tomato by-products, preserving their antioxidant potential and bioavailability over time (Amador-Luna, 2024). These applications highlight the dual role of agro-food by-products in improving both the functional and health-related attributes of food products. Their strategic utilization not only contributes to human health but also aligns with sustainability and circular economy principles, adding value to food systems while reducing waste.

2.7. Agro-Food By-Products as Components for Novel Materials Manufacturing

Agro-food by-products are increasingly recognized as valuable resources for developing novel materials, particularly in the packaging industry. These by-products, derived from plant and animal sources, offer sustainable alternatives to traditional plastics (Messinese et al., 2023). Notable examples of by-products used in biopolymer production include starchy biomass, such as corn and potato residues, lignocellulosic waste, like wheat straw and wood fibers, as well as processing waste from fruits, vegetables, and nuts (Cinelli et al., 2020; Visco et al., 2022a). These materials are transformed into biodegradable polymers such as polylactic acid (PLA), polyhydroxyalkanoates (PHA), and polybutylene succinate (PBS), which are increasingly employed in packaging applications (De Luca et al., 2023; Mohanty et al., 2018). The European Union (EU) has adopted a comprehensive policy

framework to support the circular economy, emphasizing resource efficiency, waste reduction, and sustainable industrial practices (Visco et al., 2022b). Within this context, agro-food by-products can be recycled and reused, contributing to the EU's goals of minimizing waste generation and reducing dependency on fossil-based resources.

Agro-food by-products are well known for being reach in bioactive compounds, such as phenolic compounds, flavonoids and carotenoids, which revealed to improve bioplastics functionalities. These compounds confer antimicrobial, antioxidant, and UV-protective properties, making bioplastics suitable for active food packaging applications and extending product shelf life (Kumar Gupta et al., 2024; Mariño-Cortegoso et al., 2022; Visco et al., 2022a). Recent studies have further expanded the potential applications of agro-food by-products by demonstrating their ability to improve the mechanical properties of biopolymers, thereby reducing reliance on costly biopolymers. For instance, in a study involving PBAT (polybutylene adipate-co-terephthalate) blended with zein (a protein derived from corn) and TiO_2, it was shown that the by-product component enhanced the material's mechanical strength and flexibility (Togliatti et al., 2022), making it suitable for packaging applications. Blend of hemp fibers used as reinforcement agent in PHBV-PBAT (poly(butylene adipate-co-terephthalate) composites demonstrated that by-product component can enhance material's mechanical strength and flexibility (Pereira et al., 2024), making it suitable for packaging applications. Specifically, Hemp cellulose-based cryogels, derived from hemp stems, offer eco-friendly alternatives to conventional meat packaging pads. With high water sorption capacity (2.20 mL/g), structural stability, minimal shrinkage, and antioxidant activity from rice straw bioactive extracts, they effectively protect against lipid oxidation during refrigeration, making them sustainable and functional materials for food preservation (Cabrera-Villamizar et al., 2025).

Other research demonstrated the potential of using agro-industrial by-products, including fruit peels, cereal husks, and dairy by-products, to produce biodegradable films, coatings, and other packaging solutions (Messinese et al., 2023). For example, PLA films enriched with citrus extracts have demonstrated improved preservation qualities, thereby contributing to food waste reduction (Kumar Gupta et al., 2024; S. Zhang et al., 2024). Edible alginate-film enriched with agro-food by-products (obtained from onion, artichoke, thristle) demonstrated to enhance food shelf-life thanks to the improved antioxidant properties (Grimaldi et al., 2022). Dairy by-products, such as whey protein, have also been transformed into bio-based films and coatings with excellent barrier properties for food packaging

applications (Cinelli et al., 2014; Song et al., 2022). Additionally, the biomasses generated from thermo-valorization processes that treat exhausted materials collected from secondary treatment of waste and by-products from industrial processing have also shown promise.

Biochar, a carbon-rich material derived from pyrolysis of organic waste, has demonstrated excellent potential as a functional filler in biopolymer composites. By integrating biochar into biopolymers, such as PLA or PBAT, researchers have reported significant improvements in mechanical strength, thermal stability, and water vapor barrier properties, making the resulting materials highly suitable for food packaging applications (Sirico et al., 2021).

The innovative use of agro-food by-products align with global sustainability goals by reducing reliance on petrochemical-based resources and promoting resource efficiency (Cinelli et al., 2020). These developments underline the dual advantage of valorizing waste streams while addressing the environmental concerns associated with conventional plastic use.

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Chapter 3

ANALYTICAL CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM ARTICHOKE BY- PRODUCTS EXTRACTS

This chapter investigates the unexploited potential of artichoke by-products (ABPs) generated from industrial transformation as valuable resources of bioactive compounds, serving as ingredients for sustainable and functional food development. Analytical characterizations of water extracts of ABPs revealed a high content of inulin, dietary fibers, and phenolic compounds, providing high antioxidant potential. These compounds are well-known correlated to antioxidant activities and anti-inflammatory properties, contributing to health benefits and enhancing food stability (Ayuso et al., 2024; Colombo et al., 2024). n the circular economy framework, green extraction methodologies involving ultrasound-assisted extraction (UAE) were performed to maximize bioactive recovery. High-performance liquid chromatography confirmed the chemical profiles, while oxidative stability tests of enriched oils demonstrated enhanced shelf-life and functionality.

Furthermore, innovative applications of ABPs in nutraceutical formulations, such as functional pastas, demonstrated their potential to deliver bioactive-rich diets while reducing environmental waste. The findings highlight artichoke by-products as a dual-purpose resource for waste valorization and the development of health-promoting food products. This dual approach supports environmental sustainability and addresses growing consumer demand for functional and nutraceutical foods, underscoring the broader applicability of ABPs in food science.

3.1. Materials and methods

3.1.1. Chemicals

Water (Milli-Q), sodium acetate, 50% (w/w) sodium hydroxide, xylitol, sorbitol, mannitol, rhamnose, glucose, galactose, fructose, sucrose, lactose, raffinose, maltose, 1-ketose, and 1-nystose analytical standards were purchased from Sigma Aldrich (Steinheim, Germany)

with the highest purity degree (>99%). Beneo (Mannheim, Germany) purchased inulin from chicory roots.

3.1.2. Samples

Artichoke by-products (ABP) samples were supplied by Greci Industria Alimentare S.p.A (Ravadese, Parma, Italy) during 2023 harvesting. ABP samples involved untreated artichoke by-products (U-ABP), treated artichoke by-products (T-ABP), and artichokes' blanching water (BW) obtained from the industrial transformation to produce artichoke preserves (Table 3.1).

Table 3.1: Samples of artichoke by-products (ABP) collected from GRECI Alimentare spa

Sample	Description
U-ABP	Outer bracts and stems collected before blanching step
T-ABP	Outer bracts and stems collected after blanching step
BW	Water used for the blanching treatment

U-ABP and T-ABP were dried at 40 °C for 48 h and minced to obtain powders. BW was used for analysis without any treatment.

3.1.3. Oxitest

The oxidative stability of vegetable oil, used as a food model, was evaluated with the Oxitest reactor (Velp Scientifica, Usmate, MB) after supplementation with artichoke by-product (ABP) powders. This assessment involved accelerating the oxidation process of 10 g of each sample under controlled conditions—maintaining a constant temperature of 90 °C and an oxygen pressure of 6 bar. Sunflower oil served as the reference sample for comparison.

To achieve a uniform mixture, the ABP powders were infused into the model oil for 48 hours prior to analysis. The Oxitest software enabled real-time monitoring of the oxidation process across both reaction chambers, generating graphical oxidation curves and calculating the

induction period (IP), which reflects the oxidative stability of the samples. All tests were performed in duplicate, utilizing both chambers to ensure consistent and reliable results.

3.1.4. Extraction of carbohydrates

Carbohydrates were extracted from artichoke by-products (ABP) following a previously established protocol by Grimaldi et al. (2022). Briefly, 3 g of ABP were mixed with 50 mL of Milli-Q water and heated at 80 °C for one hour. This was followed by ultrasound-assisted extraction (UAE) for 30 minutes to enhance the extraction process. Post-extraction, the samples were centrifuged at 6000 rpm for 15 minutes at 4 °C. The supernatant was collected, and the remaining residue underwent a second extraction with fresh solvent. Both supernatants were combined to a final volume of 100 mL, then filtered through a 0.45 µm membrane to remove solid residues. To eliminate polyphenols that could interfere with subsequent analysis, the solution was further purified using On-Guard IIP pre-packed cartridges. The purified samples were then analyzed using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

3.1.5. Extraction of antioxidant compounds

The extraction of bioactive compounds with antioxidant properties from artichoke by-product (ABP) powders was carried out following a validated protocol from previous research (J. Li et al., 2022). Specifically, 1 g of each sample was extracted with 35 mL of 96% (v/v) ethanol at 65 °C for 15 minutes. This was followed by a 15-minute ultrasonic bath treatment to enhance extraction efficiency. The mixture was then filtered, and the supernatant was collected. The remaining residue underwent a second extraction under identical conditions. Both supernatants were combined, resulting in a total volume of 70 mL. The solvent was subsequently evaporated using a rotary evaporator, and the remaining extract was dissolved in 7 mL of ethanol. The final solutions were filtered through a 0.45 µm PTFE membrane filter and analyzed using colorimetric assays—specifically, the Folin-Ciocalteu method for total phenolic content and the DPPH assay for antioxidant activity.

3.1.6. Fourier transform infrared spectroscopy – Attenuated Total Reflectance (FTIR–ATR)

A Perkin Elmer Spectrum Two spectrophotometer was employed for the analysis of artichoke by-product (ABP) samples. The samples, along with standard compounds—D-glucose, D-fructose, and commercial inulin—were analyzed using attenuated total reflectance Fourier-transform infrared spectroscopy (FTIR-ATR). This analytical method requires no pre-treatment; therefore, both samples and standards were directly analyzed after recording the air background spectrum. For each sample, spectra were recorded by averaging 16 scans over the wavenumber range 4000-400 cm^{-1}.

3.1.7. Size Exclusion Chromatography (SEC)

Artichoke extract samples were prepared at a concentration of 1 mg/L in Milli-Q water. The molecular weight distribution and the corresponding degree of polymerization (DP) of fructans-derived saccharides occurring in the ABP extract were analyzed by Agilent 1100 HPLC equipped with an Agilent Refraction Index Detector. A PolySep-GFC-P 2000 Phenomenex column (7.8 mm × 300 mm) was used with a mobile phase composed of ultrapure degassed water with an addition of sodium azide (NaN3) at 0.02% w/v to prevent microbial contamination at a flow rate of 0.5 mL/min. The column temperature was 25 °C, and the injection volume was 100 µL. Five sugar standards were selected to build a calibration curve based on their specific molecular weights: dextran 5000 Da, dextran 1000 Da, maltopentaose (828 Da), maltotetraose (666 Da), glucose (182 Da).

3.1.8. High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The separation of fructo-oligosaccharides (FOS) and inulin with varying degrees of polymerization was conducted using a Dionex DX500 series liquid chromatography system (Sunnyvale, CA, USA). This system was equipped with a Dionex AS50 autosampler featuring a 25 µL injection loop and a model ED50 pulsed amperometric detector (PAD) with a gold working electrode against an Ag/AgCl reference electrode. To maintain stability and prevent eluent degassing, a continuous helium flow was applied throughout the analysis. The mobile

phases consisted of degassed Milli-Q water (Eluent A), 600 mM NaOH (Eluent B), and 500 mM sodium acetate (Eluent C) (Corradini et al., 2004).

Chromatographic separation was carried out using a CarboPac PA100 analytical column (4 × 250 mm) coupled with a CarboPac PA100 guard column (4 × 50 mm). The system operated at room temperature with a flow rate of 0.5 mL/min. A linear gradient of sodium acetate (2.5–60 mM) was employed to achieve optimal elution of oligosaccharide fractions from both commercial inulin and ABP samples. This was followed by a column washing step and re-equilibration. The specific elution gradient program is detailed in Table 3.2.

Table 3.2: Elution gradient employed for oligosaccharides separation in CarboPac PA100

Time (min)	Milli-Q water (%A)	NaOH 0.6 M (%B)	CH_3COONa 0.5 M (%C)
0	72.5	25	2.5
50	15	25	60
60	10	25	60
70	35	25	30
75	72.5	25	2.5
90	72.5	25	2.5

For compound identification, commercially available standards—sorbitol, *D*-glucose, *D*-fructose, *D*-sucrose, 1-kestose, and 1-nystose—were used by comparing their chromatographic retention times. A calibration curve for these standards was prepared using five concentration levels ranging from 0.25 to 10 µg/mL. All analytical procedures were performed in triplicate to ensure accuracy and reproducibility. Chromeleon software (Dionex) was employed to manage and analyze the chromatographic data.

3.1.9. Total Phenolic Content (TPC)

The total polyphenol content (TPC) was determined using UV-Vis spectrophotometry with a Thermo Scientific™ Evolution™ 201/220 spectrophotometer (Milan, Italy), following the Folin-Ciocalteu (FC) method. This method involves the addition of an oxidizing reagent that reacts with phenolic groups, causing a measurable change in absorbance and the development of

a characteristic color. Specifically, the reaction utilizes phosphotungstate and phosphomolybdate compounds, which, under basic conditions, oxidize hydroxyl (-OH) groups to carbonyl groups, resulting in the reduction of the reagent and the formation of a blue-colored complex (Ammor & Jennan, 2019). In brief, 50 µL of the sample was combined with 1160 µL of Milli-Q water, 300 µL of 20% (w/w) sodium carbonate solution, and 100 µL of Folin-Ciocalteu reagent. The mixture was incubated at 40 °C for 30 minutes to allow the reaction to proceed. Absorbance was then measured at 760 nm. TPC results were expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g dry extract). A calibration curve was constructed using gallic acid standards within the concentration range of 1–30 µg/mL. All measurements were performed in triplicate to ensure data accuracy and reproducibility.

3.1.10. Total Antioxidant Capacity (%TAC)

The antioxidant power (measured as %TAC, percentage of Total Antioxidant Capacity) of the samples was determined by UV-vis spectrophotometry (Thermo Scientific™ Evolution™ 201/220, Milan, Italy) using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay. The assay is based on the neutralization of the stable DPPH- radical by chemical compounds present in the sample that can function as radical scavengers. The method used is the same as that reported by Pasqualone (2014) with some slight modifications (Pasqualone et al., 2014): 500 µL of phenolic extract obtained from the ABP samples was added to 1500 µL 60 µM solution of DPPH- in methanol. After 16 min of reaction (turning yellow), absorbance was recorded at 517 nm using a UV-Vis spectrophotometer (Thermo Scientific™ Evolution™ 201/220). At the same wavelength but at t=0 min, the absorbance of the DPPH 60 µM methanolic solution (reference) was measured. The absorbance reading at 517 nm of methanol as it was used to set the instrument to zero (as absorbance value). Antioxidant radical scavenging activity was calculated as follows (Kaderides et al., 2020):

$$\%\,TAC = \frac{A_R - A_S}{A_R} \times 100$$

A_R refers to absorbance of reference (517 nm); A_S refers to the absorbance of sample (517 nm). The analysis was carried out on all the conditions assessed for all analysis. The analysis was performed in duplicate.

3.1.11. Statistical Analysis

Microsoft Excel was used to calculate means and standard deviations (SD), as well as to assess significant differences between samples using one-way analysis of variance (ANOVA). This was followed by Tukey's post-hoc test at a significance level of $p \leq 0.05$ to determine differences among the groups. Additionally, Pearson's correlation was performed to evaluate the linear relationships between oxidative stability (Induction Period, IP), total phenolic content (TPC), and total antioxidant capacity (TAC). Correlation analyses were performed by means of Pearson's correlation test, and results were considered preliminary due to the limited size of dataset (n = 6).

3.2. Results and discussion

This project was carried out in collaboration with Greci Industria Alimentare spa, company partner of the PhD project, which provided ABP from the transformation chain. Three types of by-products were analysed:

- **Untreated Artichoke By-Products (U-ABP):** outer bracts and stems collected before blanching step.
- **Treated Artichoke By-Products (T-ABP):** outer bracts and stems collected after blanching step.
- **Blanching water (BW):** water used for the blanching treatment.

The artichoke samples used in this study were obtained from two different industrial processing chains that are depicted in Figure 3.1.

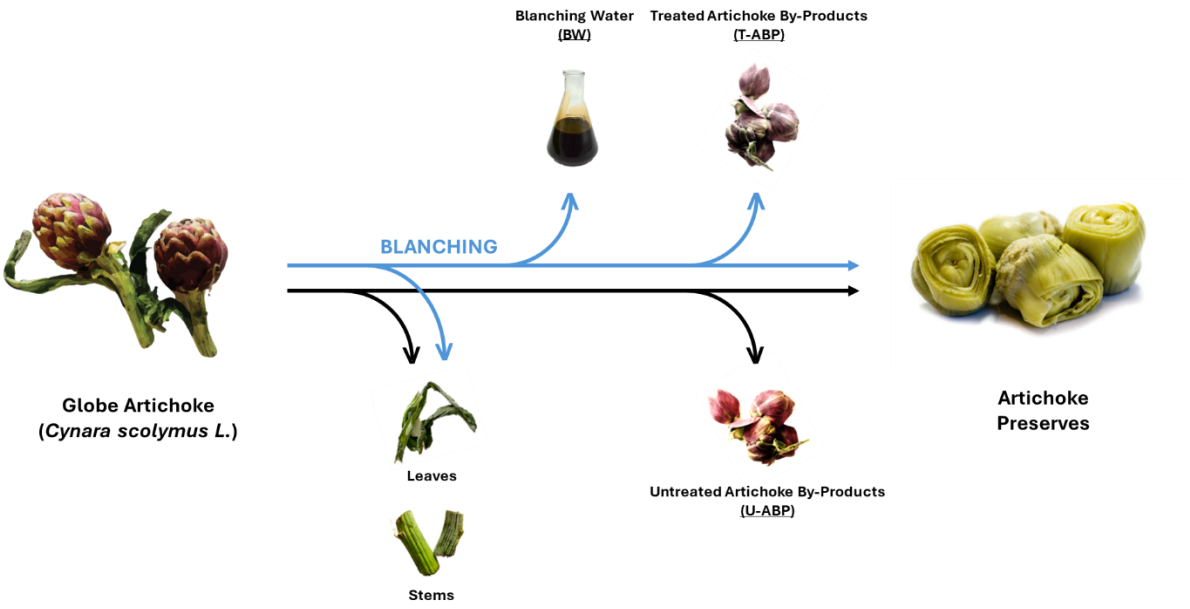

Figure 3.1: industrial processing flowchart of artichokes and generation of ABPs samples (BW, U-ABP, and T-ABP).

During industrial transformation, artichokes are normally processed by thermal treatment in hot water, producing U-ABP samples. Alternatively, a blanching step may be performed during industrial processing for some preparations, thus producing T-ABP sample. The resulting solution from the blanching step (BW) was analyzed directly without any additional treatment, as it originates from the industrial artichoke transformation process. However, due to the nature of this process, the correct ratio of the artichoke-to-water used was unknown.

This section focuses on the characterization of the carbohydrate patterns and the evaluation of the antioxidant capacity of ABPs extracts.

3.2.1. Preliminary investigations on ABP powders and extracts

ABP powders were obtained after drying and grinding to ensure suitability for analysis and to prevent potential contamination or microbial fermentation. Such treatments are critical in stabilizing samples, as microbial activity can alter biochemical properties (Bourdoux et al., 2016), leading to inaccurate results. As reported in Table 3.3, a mild condition (40 °C, 48 h) was selected for ABP drying based on conditions used in previous work (Grimaldi et al., 2022).

Table 3.3: Drying conditions and yields (% on dry basis) of the two drying treatments for artichoke. g d.w. = grams of dry weight; g f.w. = grams of fresh weight.

Sample	Temperature	Drying time	% Yield
	(°C)	(h)	(g d.w./g f.w)
U-ABP	40	48	18.8
T-ABP	40	48	21.4

Drying treatment allowed to remove all the water kept inside the matrices, leading to a loss of weight of about 80%.

***Oxitest reactor*.** Preliminary experiments were performed to evaluate the oxidative stability of the vegetable oils enriched with ABP powders using an Oxitest reactor. This equipment is designed for accelerated shelf-life evaluation of food matrices having a minimum fat content of 25 %. Since ABP does not present a consistent lipids fraction, samples were suspended in sunflower oil (also used as positive control) for two days and then analyzed by Oxitest reactor, which gives information about the induction period (IP). The Induction Period corresponds to the time required to achieve a marked pressure reduction within the oxidation chamber, resulting in the onset of lipid oxidation. Figure 3.2 displays the IP values, expressed in minutes, for each condition tested. The results showed that ABP powders significantly enhanced ($p < 0.05$) the oxidative stability of the vegetable oil in terms of IP, highlighting their potential to mitigate lipid oxidation during storage. A significant increase ($p < 0.05$) in IP was observed for each condition compared to the control, indicating that the addition of by-products by-product powders (1 %, w/v) effectively enhanced the antioxidant properties of sunflower oil. The results related to both samples highlighted a significant

improvement in IP (849.5 ± 4 and 854.5 ± 5 minutes for U-ABP and T-ABP, respectively) than the control (810 ± 9 minutes).

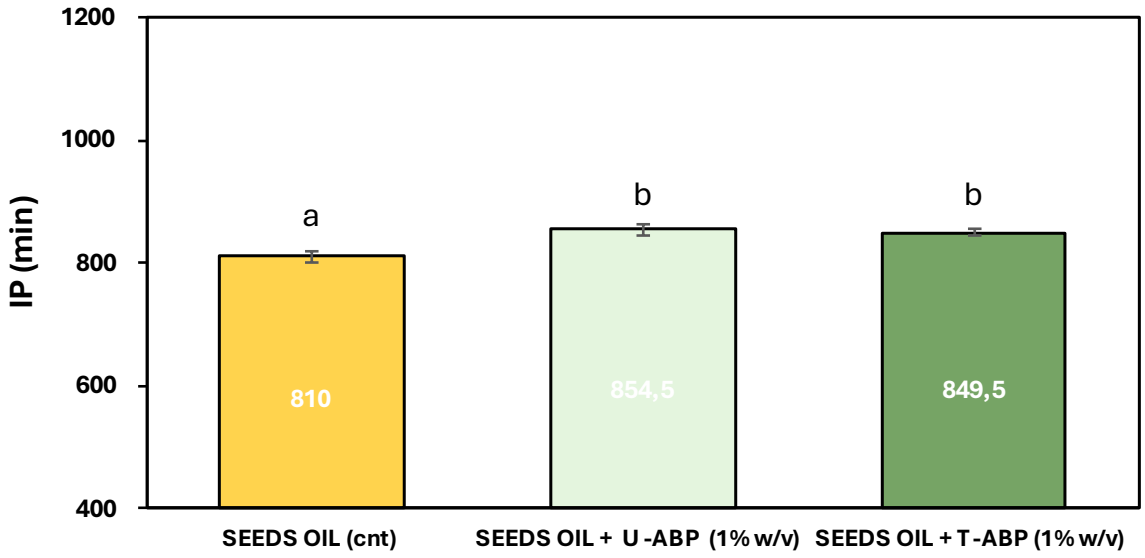

Figure 3.2: Comparison of Induction Periods (IP) values of the control (yellow bar), seeds oil enriched with U-ABP (light green), seeds oil enriched with T-ABP (dark green).

Conversely, there were no significant differences found between the various types of technological treatments (U-ABP vs T-ABP). Our findings align with earlier studies utilizing the same methodologies, showing that incorporating agro-food industrial by-products can impede the oxidation processes in vegetable oils due to the antioxidant compounds present in these matrices (Cannas et al., 2024; Cavazza et al., 2015; Grimaldi et al., 2022). The evaluation of oxidative stability indicated that adding by-product powders to vegetable oils helps to reduce the rate of decline in IP, which is typically accelerated by oxidation-related stress.

FTIR-ATR. Literature studies reported that artichoke are vegetables rich of fibers like fructo-oligosaccharides (FOS) and inulin (Ayuso et al., 2024). The Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR–ATR) is an effective technique for rapid qualitative analyses of solid or liquid samples, avoiding further preparation (Subramanian & Rodriguez-Saona, 2009).

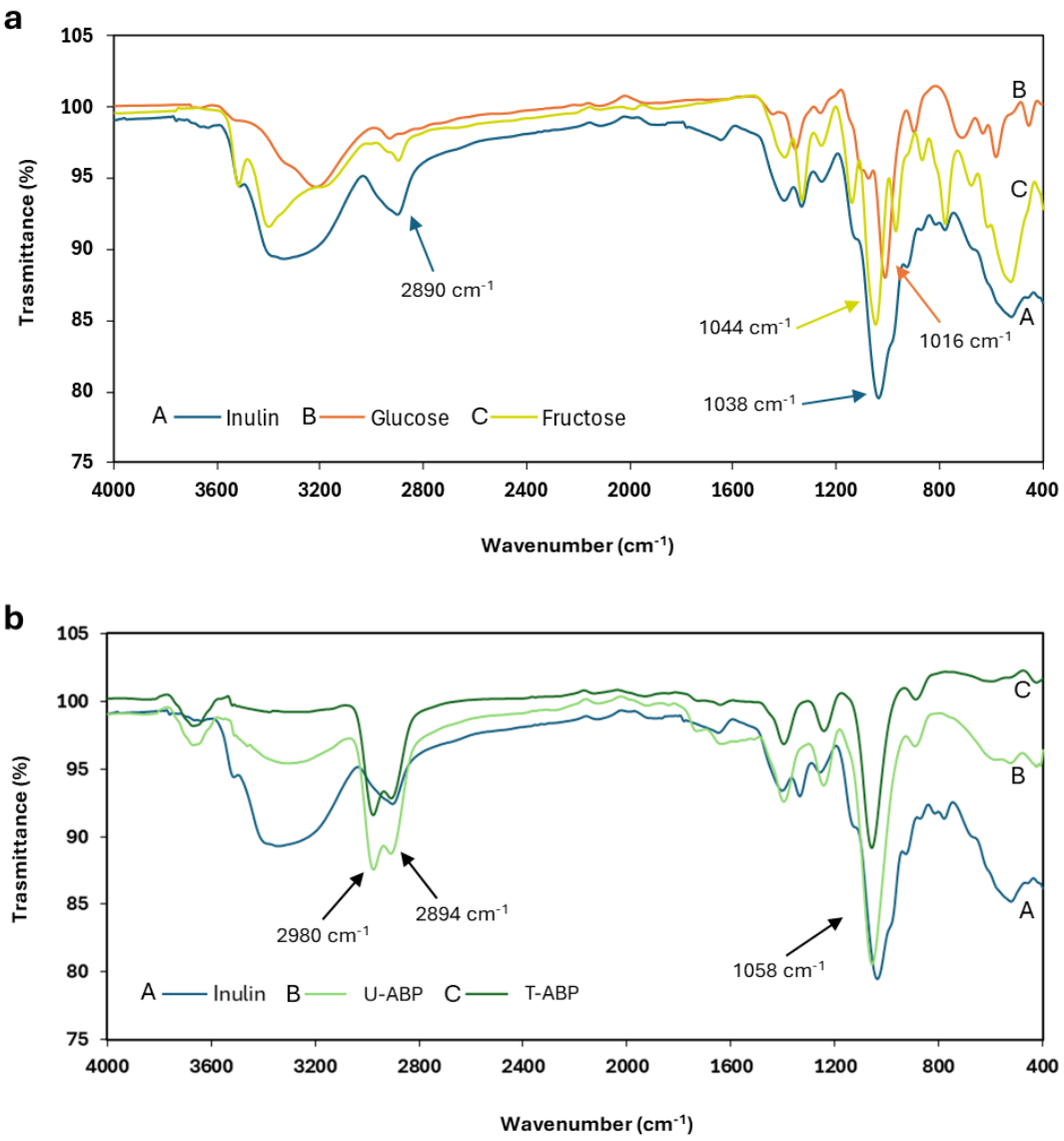

Figure 3.3: (a) Comparison of FTIR-ATR spectra of the standard selected: inulin (blue), glucose (orange), fructose (yellow); (b) Comparison of FTIR-ATR spectra of the ABP powder: inulin (blue), untreated artichoke by-products (light green), treated artichoke by-products (dark green).

FTIR-ATR provides information regarding the presence or absence of specific functional groups of the occurring compounds in the samples and the chemical structure of polymers. FTIR-ATR analyzed the U-ABP and T-ABP samples to assess the presence of bands related to FOS/inulin compounds. The spectra of samples were compared with those obtained with glucose, fructose, and commercially available inulin powders, which were used as standards. The results are reported in Figure 3.3. Inulin showed the characteristic band of the β(2-1) glycosidic bond of FOSs at 1038 cm^{-1}, while glucose and fructose showed bands at 1016 cm^{-1} and 1044 cm^{-1}, which were attributed to the stretching of the only hemiacetal group and the ring's hemichetal, respectively (figure 3.3a). Moreover, another characteristic

band at 2890 cm-1 of the CH stretching of the furanoside ring was seen by comparing both the inulin and fructose spectra. Figure 3.3b shows the spectra of the U-ABP and T-ABP compared to the inulin spectrum. The profiles were comparable, presenting a characteristic band related to the glycosidic bond stretching band typical of FOSs (1058 cm^{-1}) and CH stretching of the furanosidic ring (2894 - 2980 cm^{-1}). Moreover, the band in the 3000 - 3400 cm^{-1} range was attributed to hydroxyl group stretching. Our results aligned with previous research investigating the presence of inulin in artichoke by-product samples by FTIR-ATR approach (Castellino et al., 2020; Redondo-Cuenca et al., 2021). Preliminary experiments on ABP powders highlighted their high content of bioactive compounds such as antioxidants and dietary fibers, making them viable candidates for recycling and reuse in the context of the circular economy. Further characterization of these compounds will be discussed in the following paragraphs.

HPLC-SEC. The water extracts of ABP powders were subjected to size exclusion chromatography (SEC) analysis to determine the M_W of oligo- and polysaccharides present in the samples. The extraction procedure used was a combined extraction approach which involved conventional heating extraction at 80 °C followed by a short UAE treatment. The elution profile of standards was used to create a calibration curve correlating retention time (RT) to the log M_W of each standard; the coefficient of linearity was determined and the equation calculated as followed: $y = -0.2759x + 7.1821$; $R2 = 0.9845$, highlighting a correspondence between the two variables (figure 3.4).

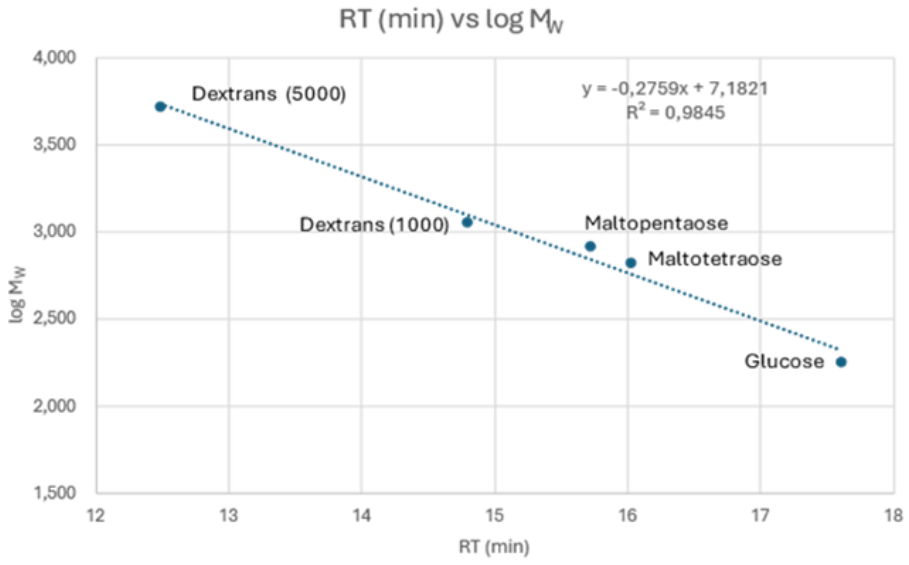

Figure 3.4: Calibration curve of five sugar standards with different molecular weight. 1: Glucose (182 Da); 2: Maltotetraose (666 Da); 3: Maltopentaose (828.72 Da); 4: Dextrans (≈1000 Da); 5: Dextrans (≈5000 Da).

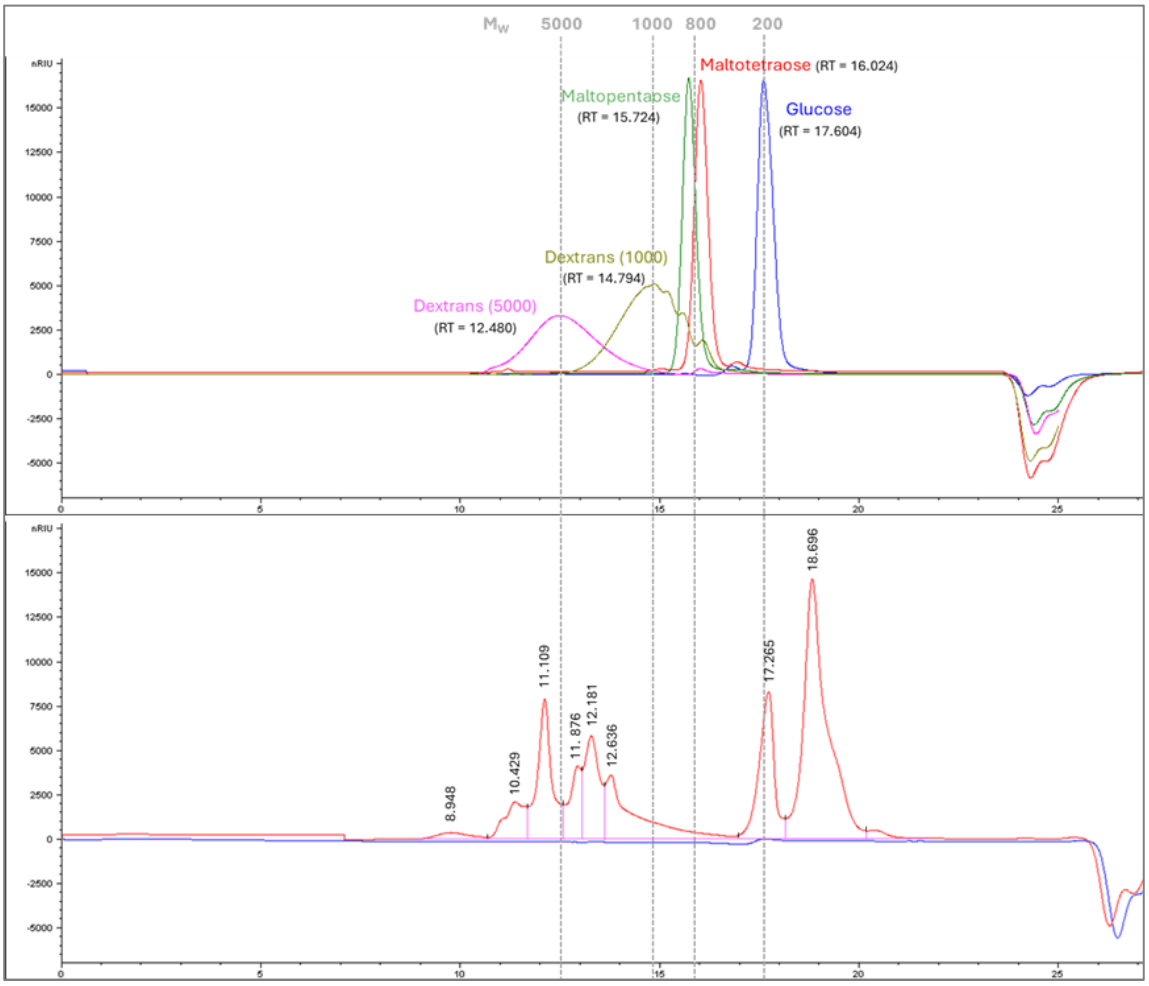

Figure 3.5: Comparison of SEC profile between standards and U-ABP water extract.

In Figure 3.5, the comparison between the standards' SEC-HPLC profile and the one related to the U-ABP is reported. The highest peak distribution inside the selected range of 182 - 666 Da, referring to simple sugars elution, was recorded at RT = 17.265 min, resulting in an estimated M_W of 262 Da. Other peaks with high signal overlapped the same region where dextran compounds were separated, recording retention times at RT = 12.181 min and RT = 11.109 min with an estimated M_W of 6628 and 13096, respectively. As shown in Figure 3.5, the comparison between two chromatographic profiles confirmed the presence of compounds with high DP, probably associated to inulin-type polymers occurring in the extracts of globe artichoke and their industrial by-products. The presence of components related to FOS/inulin compounds with comparable M_W distribution was also confirmed in other studies (Zeaiter et al., 2019). However, other non-carbohydrate compounds potentially occurring in aqueous extracts of ABP - such as proteins, phenolics, or pigments

- may limit the reliability of the method. This limitation arises from the use of a universal refractive index (RI) detector in the HPLC-SEC system, which lacks compound specificity. The RI detector responds to any solute differing in refractive index from the mobile phase, without providing structural or chemical information. As a result, co-eluting compounds of similar hydrodynamic size, such as proteins or small polyphenols, can produce overlapping signals with carbohydrates, making it difficult to assign peaks identity unambiguously based on molecular weight alone (Knol et al., 2021; Swartz, 2010). In this regard, High-Performance Anion Exchange Chromatography with Pulsed Electrochemical Detection (HPAEC-PED) was also used, as it can accurately identify sugar fractions.

3.2.2. Analysis by HPAEC-PAD and Method Validation

HPAEC-PAD technology allows the separation of carbohydrates, and in this section the method validation is described. Qualitative and quantitative identification of sugars was performed using a CarboPac PA100 column. Calibration curves were built using the following standards: sorbitol, glucose, fructose, sucrose, 1-kestose and 1-nystose, in a concentration range of 0.25 - 10 µg/mL. For the quantification of oligosaccharides with DP > 4, the calibration curve of 1-nystose was used (Pitirollo et al., 2023) since no commercially available standards with higher DP were available. All the analyses were performed in triplicate. Limit of detection (LOD), limit of quantification (LOQ),precision of retention time, precision of peak area intraday and interday, and linearity were calculated according EURACHEM 2014 guidelines (The Fitness for Purpose of Analytical Methods, 2014). Results about the mentioned parameters are reported in Table 3.4.

Table 3.4: Method validation

Sugar standard	RT (%RSD) n = 9	LOD/LOQ (mg/L)	Concentration level (mg/L)	Area Intra-day (%RSD) n = 3	Area Inter-day (%RSD) n = 6	Linearity R^2
Sorbitol	0.8	0.02/0.04	0.25	0.35	3.41	0.9986
			0.5	0.91	3.63	
			1.0	0.21	3.19	
			2.5	0.24	2.93	
			5.0	0.22	2.88	
			7.5	0.18	3.05	
			10.0	0.15	2.96	
Glucose	0.4	0.02/0.08	0.25	0.16	1.77	0.9919
			0.5	1.05	1.85	

			1.0	1.06	1.82	
			2.5	0.24	1.79	
			5.0	0.35	2.04	
			7.5	0.28	1.84	
			10.0	0.46	1.74	
Fructose	0.2	0.07/0.22	0.25	1.88	1.82	0.9832
			0.5	1.16	1.87	
			1.0	0.81	1.56	
			2.5	1.01	1.95	
			5.0	2.03	2.50	
			7.5	1.75	2.66	
			10.0	1.33	1.89	
Sucrose	2.1	0.06/0.18	0.25	0.29	3.25	0.9854
			0.5	2.86	3.10	
			1.0	0.62	2.13	
			2.5	1.75	2.78	
			5.0	1.75	3.02	
			7.5	1.24	2.56	
			10.0	0.62	2.87	
1-kestose	2.4	0.05/0.10	0.25	0.91	4.03	0.9849
			0.5	3.40	3.56	
			1.0	1.78	1.71	
			2.5	2.62	1.79	
			5.0	2.53	1.75	
			7.5	1.79	1.68	
			10.0	1.17	1.50	
1-nystose	2.2	0.06/0.09	0.25	1.50	3.09	0.9971
			0.5	5.87	2.25	
			1.0	4.42	1.74	
			2.5	5.10	1.76	
			5.0	4.17	1.56	
			7.5	3.37	1.90	
			10.0	1.90	1.86	

3.2.3. Qualitative and quantitative analysis of sugar profile by HPAEC-PAD

HPAEC-PAD was used to obtain a chromatographic profile of carbohydrates in ABP, and qualitative and quantitative analyses were performed using commercially available inulin as standard. CarboPac PA100 column was employed for its high selectivity for oligosaccharides separation. First, the commercially available inulin (Farmalabor) was injected at a 500 µg/mL concentration. In Figure 3.6, the chromatogram of inulin was reported. It is possible to divide the chromatogram into two regions: from 0 to 16 min of

analysis were related to the elution of polyols, mono-, di-saccharides, 1-ketose, and 1-nystose, which are the DP3 and DP4 of FOS, respectively.

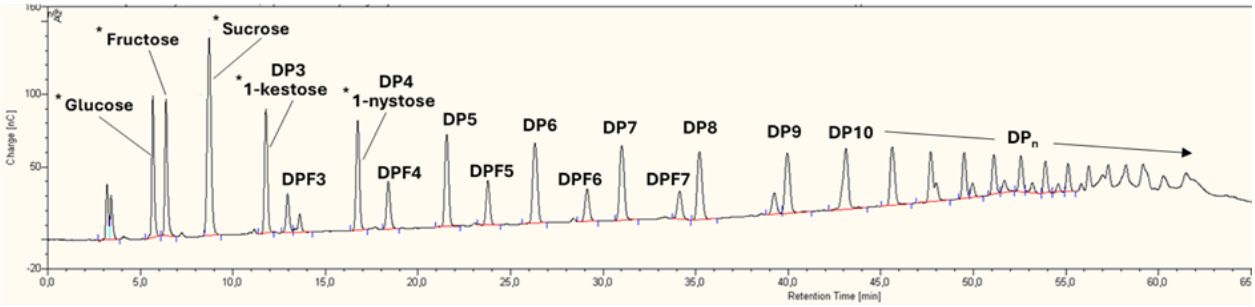

Figure 3.6: Oligosaccharides separation by HPAEC-PAD of commercial inulin (Farmalabor). DP_n refers to FOS peak series; DPF_n refers to IOS peak series. Asterisks (*) mark the standards available used for the analysis.

The identification of simple sugars and 1-ketose and 1-nystose was carried out by comparison with commercially available standards.

The highest signal intensities were recorded for simple such as D-glucose, D-fructose, and sucrose eluted at 6.075, 6.867, and 9.336 min, respectively; oligosaccharides such as 1-ketose and 1-nystose eluted at 12.942 and 17.933 min, respectively. Regarding the evaluation of FOS with DP higher than 4, tentative identification was based on their consecutive increase in retention time per fructose unit since standards of FOS having DP higher than 5 are not commercially available. However, in previous research FOSs compounds with higher DP were identified by comparison with standards (up to DP9) (Pöhnl et al., 2017).

It is already reported that the oligosaccharides with DP > 4 elute with increasing polymerization when a linear gradient of sodium acetate is used (Borromei et al., 2009). In addition, a similar distance ratio (in terms of RT) between the peaks is attributed to FOS with DP_n and the corresponding DPn+1. Comparable behavior was observed for DPF_n (where F_n refers to a unit of Fru) corresponding to the inulin-oligosaccharides (IOS), which consist of linear chains of fructose (Borromei et al., 2009).

To identify the peak corresponding to FOS with degree of polymerization greater than 4, we plotted a calibration curve relating DP and RT (Figure 3.7).

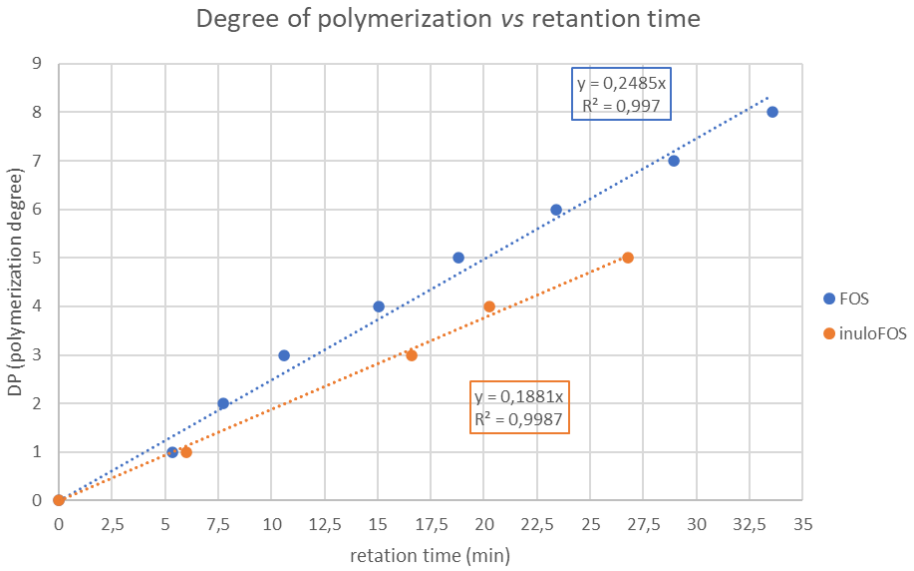

Figure 3.7: Trends of oligosaccharides elution for FOSs compounds (blue linen) and IOSs (orange linen).

The graph in Figure 3.7 illustrates the correlation between DP and RT for two families of oligosaccharides: fructo-oligosaccharides (FOS) (blue line) and inulo-oligosaccharides (IOS) (orange line), analyzed by HPAEC-PAD under a linear sodium acetate gradient.

Regarding FOS series, glucose and sucrose appear as the first two data points near the origin of the blue line; while for IOS series, which consists of a linear backbone of repeated fructosyl units, includes only fructose as the first point after the origin. Sucrose is not detected in the IOS series because it contains a terminal glucose unit, which is not occurring in this class of compounds. Both FOS and IOS datasets were evaluated using linear and second-order polynomial regression models. The linear model showed a better fit, yielding the highest coefficients of determination: $R^2 = 0.9913$ for FOS and $R^2 = 0.9954$ for IOS. In both cases, the second-order polynomial model reduced the R^2 value ($R^2 = 0.9955$ for FOS and $R^2 = 0.9965$ for IOS), indicating no significant improvement in fit. Thus, linear fit relationship better described the correlation between DP and RT under these chromatographic conditions. The regression lines allowed the discrimination between Glu-Fru_n units of FOS and Fru_n units of IOS, and to predict the DP of oligosaccharides within the analyzed range. No statistically significant differences ($p < 0.05$) were observed between the RTs of peaks corresponding to DP_n and DP_{n+1} up to DP9. Indeed, average RT difference was 5.506 ± 1.032 minutes. Differences in slope and concentration points between FOS and IOS relies on their structural differences. Notably, each IOS point is shifted relative to its FOS counterpart because it presents a glucosyl unit less. That is, for a given concentration point on the FOS

curve, the corresponding point on the IOS curve (at the same retention time) represents a compound with DP_{n-1}. This shift reflects the absence of the terminal glucose unit in IOS, meaning the same compound appears in both series but with a one-unit difference in assigned DP. The apparent offset is therefore not primarily due to measurement error or variability in elution but to this systematic structural difference between the two families. Consequently, DP values were estimated based on RT progression and known elution behavior of FOS and IOS homologs. Consequently, adopting this approach, degrees of polymerization for the two classes were estimated based on RT progression and the known elution behavior of FOS and IOS homologs. For this reason, we assigned specific DPs to each peak after 1-nystose, identifying oligosaccharides up to DP9. The same method was applied to identify IOSs with DPs between 3 and 5. Identification of FOS and IOS compounds with DP > 4 was only achievable by employing a linear sodium acetate gradient. This gradient increased the system's ionic strength, allowing the effective separation and identification of oligosaccharides differing by a single glucose unit (Borromei et al., 2009).In Figure 3.8, representative chromatograms of inulin standard and ABP extracts are compared. For each condition, peaks related to FOSs compounds at different degrees of DP were qualitatively observed. Similar qualitative profiles comparable to those of inulin were observed, suggesting the presence of FOSs in all the ABP extracts and BW sample, which was also assessed by means of FTIR-ATR (section 3.2.1).

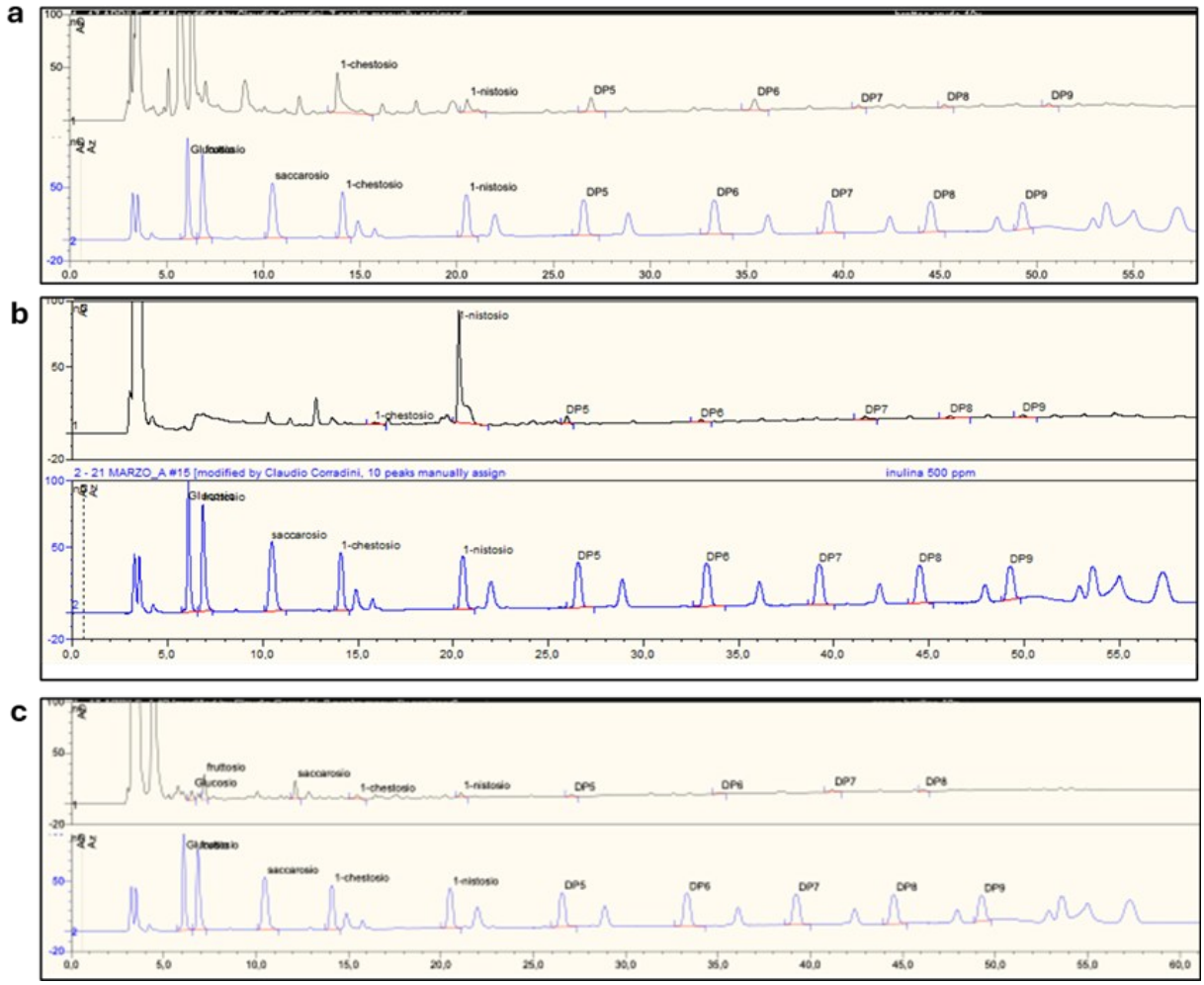

Figure 3.8: Comparison of representative chromatograms of (a) U-ABP and commercial inulin, (b) T-ABP extract and commercial inulin; (c) BW and commercial inulin.

Quantitative results for U-ABP and T-ABP extracts, expressed as mg/100 g dry sample are reported in Figure 3.9. A great variation of sugars among the two industrial treatments on the same matrix was observed. This effect could be due to the cooking step that involved T-ABP since no simple sugars were observed in T-ABP. Indeed, the evaluation of the qualitative profile of the blanching water (BW) highlighted the presence of simple sugars (Figure 3.8c). The amount of sorbitol remained high in both samples but showed significant differences ($p < 0.05$) among the two treatments. Moreover, U-ABP provided a content of 1-kestose (37.87 mg/100 g dry weight) significantly higher than T-ABP (1.12 mg/100 g d.w.), suggesting that the industrial treatment had a considerable significant impact on oligosaccharides content. On the other hand, the average content of 1-nystose in T-ABP was higher values (72.54 mg/100 g d.w.) than in the untreated samples (12.41 mg/100 g d.w.). Also, in this case, this variation could be related to the cooking process employed during industrial treatment, which might

have affected the FOS pattern occurring in the samples by hydrolysing larger polymers into shorter chains, thus affecting the amount of 1-ketose and 1-nystose.

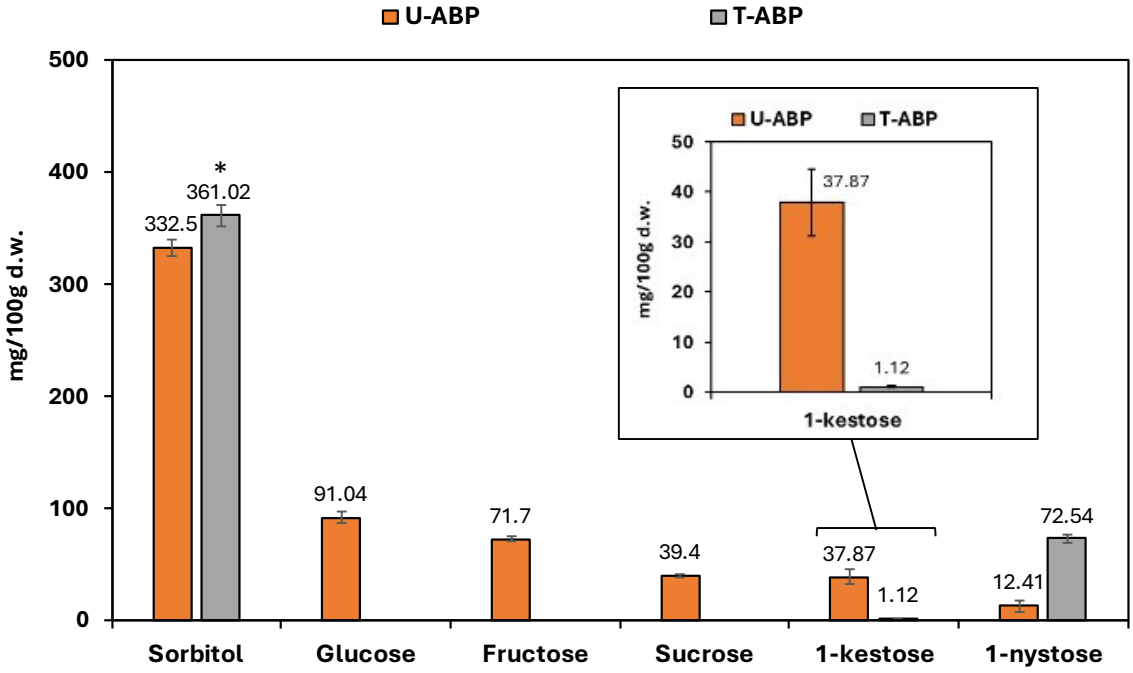

Figure 3.9: Quantification (express as mean ± sd) of polyols, monosaccharides and oligosaccharides in U-ABP and T-ABP samples. Asterisk (*) indicates statistically significant differences between the samples (U-APB vs T-APB).

Considering BW used during the industrial treatment, it was not possible to quantify the content of sugars since both the amount of raw artichoke and the volume of water derived from the technological process were unknown. However, the presence of potential compounds related to FOS was qualitatively confirmed. Quantification analyses were also conducted on ABP samples, highlighting the notable amounts of sugars, especially involving 1-ketose and 1-nystose, which are well-known for their prebiotic properties for gut microbiota.

3.2.4. Total phenol content (TPC) and total antioxidant capacity (%TAC) of ABP extracts

Artichoke by-products (ABP), which include bracts, leaves, and stalks, are well-documented source of phenolic compounds that contribute to their antioxidant properties (Gibson et al., 2017; Krumbeck et al., 2016; Varney et al., 2017). To quantify their bioactive potential, the Total Phenolic Content (TPC) and Total Antioxidant Capacity (%TAC) of ABP extracts were

evaluated using the Folin-Ciocalteu and DPPH radical scavenging assays, respectively. Both water and ethanolic extractions were conducted to investigate solvent efficacy in isolating antioxidant constituents. TPC values were expressed in mg of gallic acid equivalents (GAE) per gram of dry sample (Figure 3.10a), while %TAC values measured the inhibition rate of DPPH radicals (Figure 3.10b).

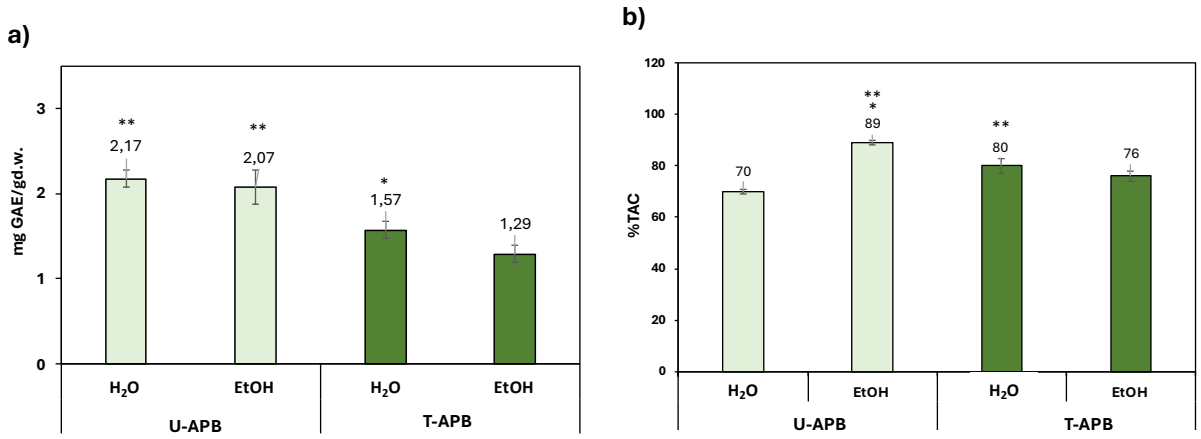

Figure 3.10: (a) TPC of BW and U-ABP, T-ABP extracts in EtOH and water; (b) %TAC BW and U-ABP, T-ABP extracts in EtOH and water. Asterisk (*) indicates statistically significant differences between the samples (U-APB vs T-APB); ** indicates statistically significant differences between the solvents (H_2O vs EtOH)

The analysis revealed that U-ABP exhibited significantly higher TPC values than T-ABP in both solvents ($p < 0.05$), highlighting the impact of thermal processing on phenolic compound content. Across all samples, aqueous extracts consistently yielded higher TPC than ethanolic ones. This suggests that water is more effective in extracting a wider spectrum of polar bioactive molecules, such as phenolic acids and flavonoid glycosides, commonly found in plant-based materials (Xu et al., 2017; Galanakis, 2020). Interestingly, no significant difference in TPC between solvents was observed for U-ABP, indicating that both ethanol and water are equally efficient at extracting phenolics when heat degradation is absent. A similar trend was observed in antioxidant activity (%TAC), with aqueous extracts exhibiting higher radical-scavenging capacities in both untreated (U-ABP) and thermally treated (T-ABP) agro-industrial by-products. However, this difference was statistically significant ($p < 0.05$) only for U-ABP. In the case of T-ABP, thermal treatment likely induced a reduction or structural alteration in antioxidant molecules, thereby limiting solvent-related differences. This reduction can be attributed to the leaching and denaturation of critical antioxidant compounds during blanching, as demonstrated by the remarkably high antioxidant activity (>90% TAC) detected in the blanching water (data not shown). Furthermore, thermal

processing likely reduced the range of extractable antioxidant compounds in T-ABP, making it less sensitive to differences in solvent polarity. In general, these results highlight the importance of pre-treatment and solvent selection in maximising the recovery of bioactive compounds from agro-industrial by-products. The combined use of Folin-Ciocalteu and DPPH assays allowed a simple, rapid and economical evaluation for profiling the antioxidant potential of plant-derived matrices.

3.2.5. Correlation analysis between Oxidative Stability, Phenolic Content, and Antioxidant Capacity

To better elucidate the relationship between oxidative stability (Induction Period, IP), total phenolic content (TPC), and antioxidant activity (TAC) in artichoke by-product extracts, a Pearson correlation analysis was conducted. The results revealed generally positive correlations among the investigated parameters, with some differences depending on the technological treatment and extraction solvent applied to the samples.

Regarding U-ABP water extract, positive but weak correlations were observed between IP and TPC ($r = 0.3083$) and between IP and TAC ($r = 0.2876$), whereas a moderate positive correlation was found between TPC and TAC ($r = 0.6485$). Similarly, in treated ABP (T-ABP) extracted with water, positive correlations were observed for all pairs: IP vs TPC ($r = 0.2638$), IP vs TAC ($r = 0.6171$), and TPC vs TAC ($r = 0.6379$). Ethanolic extracts exhibited consistent trends. For U-ABP ethanolic extracts, a moderate positive correlation was observed between IP and TPC ($r = 0.4310$), whereas weaker positive relationships were seen between IP and TAC ($r = 0.2880$) and TPC and TAC ($r = 0.5394$). In the case of T-ABP ethanolic extracts, a moderate positive correlation was maintained between IP and TPC ($r = 0.3471$), with additional moderate positive correlations for IP and TAC ($r = 0.4487$) and TPC and TAC ($r = 0.5269$).

In general, these results indicate that higher total phenolic content (TPC) and greater antioxidant activity (TAC) tend to be associated with improved oxidative stability (higher IP values) in artichoke by-product extracts. Although the strength of the correlations varies, especially depending on the solvent used for extraction, the positive trends suggest that phenolic compounds and antioxidant capacity synergistically contribute to enhancing the oxidative resistance of lipid matrices supplemented with ABP extracts. Nevertheless, due to the limited sample size ($n = 6$), these findings should be considered as preliminary results.

Further research with larger samples is recommended to verify the observed relations and understand the interplay between phenolic compounds, antioxidant activity and oxidative stability.

3.2.6. Conclusions

The chemical characterization of artichoke by-products (ABPs), presented in this chapter, demonstrates their considerable potential as functional ingredients in the context of circular bioeconomy and sustainable food systems. Through an integrative approach combining advanced chromatographic techniques (HPAEC-PAD, SEC-HPLC), spectroscopic analysis (FTIR-ATR) and spectrophotometric assays (Folin-Ciocalteu and DPPH), this study demonstrated that ABP, particularly the untreated fraction (U-ABP), contain high levels of inulin-type fructans, mono- and oligosaccharides, and polyphenolic compounds. The extract obtained from ABP also exhibits high in vitro antioxidant activities. Notably, TPC and TAC evaluations demonstrated that aqueous extracts from untreated ABPs (U-ABP) consistently yielded the highest values, underscoring water's superior efficacy in solubilizing hydrophilic antioxidant constituents such as phenolic acids and flavonoid glycosides. In contrast, thermally treated ABPs (T-ABP) showed significant reductions in both TPC and TAC, likely due to compound degradation and migration into blanching water. Indeed, thermal processing, although advantageous from a technological point of view, led to a marked decrease in mono- and oligo-saccharides sugar pattern, phenolic compounds integrity, and antioxidant potential. Nevertheless, even thermally treated by-products (T-ABP) contributed to enhance oxidative stability when incorporated into lipid matrices, confirming the presence of compounds presenting discrete antioxidant potential. This work underscored the critical influence of pre-treatments and solvent polarity on extractability, highlighting the need for tailored sustainable extraction strategies for bioactive compounds recovery.

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Chapter 4

CHEMICAL CHARACTERIZATION OF TOMATO BY-PRODUCTS AND EVALUATION OF THE EFFECT OF DRYING AND STORAGE CONDITIONS ON BIOACTIVE COMPOUNDS STABILITY

The valorization of tomato by-products (TBP), such as peels and seeds derived from industrial processing, represents a sustainable strategy to recover high-value bioactive compounds for food and nutraceutical applications. In this context, the present study aimed to investigate how post-harvest drying temperatures and packaging materials affect the chemical stability of key antioxidant constituents in TBP powders during storage. These matrices are well-documented for their rich phenolic and carotenoid content, as well as their high antioxidant capacity.

This study systematically evaluated the impact of two controlled drying treatments (40 °C and 70 °C), which involved different drying times but were designed to deliver an identical thermal load (Cook Value), on the oxidative stability, total phenolic content (TPC), total antioxidant capacity (%TAC), and carotenoid retention (β-carotene and lycopene) of TBP powders. Subsequently, aliquots of TBP were stored in four different packaging materials - plastic, bioplastic, active film, and antioxidant-enriched active film - and key parameters such as TPC and %TAC were monitored throughout the storage period. Initial analyses revealed that a higher drying temperature (70°C) significantly increased TPC, antioxidant capacity and carotenoid content compared to milder drying (40°C). However, these parameters were affected by storage condition, particularly in conventional plastic and bioplastic packaging, highlighting the critical balance between thermal treatment and compound preservation. Furthermore, innovative active film packaging enriched with antioxidant agents was evaluated alongside conventional materials. Results demonstrated that active films mitigated bioactive losses over prolonged storage, maintaining comparable TPC and %TAC values to bioplastic and plastic packaging. Correlation analyses confirmed strong positive relationships between oxidative stability (Induction Period), TPC, and %TAC for samples subjected to high-temperature drying, highlighting the functional relevance of phenolic preservation for shelf-life extension.

In parallel, LC/MS analyses allowed quantification of carotenoids over time, showing that although initial β-carotene and lycopene concentrations were similar between drying treatments, significant degradation occurred during storage, especially under oxygen-permeable packaging. While bioplastic showed some protective effect at early stages, conventional plastic offered better carotenoid retention in the long term.

In general, the integrated findings of this study emphasize that the optimization of drying temperature combined with tailored packaging solutions is essential for maximizing the retention of antioxidant and functional compounds in tomato by-products. This supports the broader framework of sustainable resource utilization and the development of high-value ingredients from agro-industrial waste streams.

4.1. Materials and Methods

4.1.1. Chemicals and Reagents

Water (MilliQ), 96% ethanol (EtOH), 99% methanol (MeOH), lab-grade ethyl acetate, lab-grade methyl tert-butyl ether, Folin-Ciocalteu reagent, sodium carbonate, sodium alginate, calcium carbonate, glycerol, gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), nylon filters (0.2 µm x 25 mm), and PTFE (Polytetrafluoroethylene) filters (0.2 µm x 25 mm) were all obtained from Agilent Technologies in Milan, Italy. Additionally, lycopene and β-carotene standards were purchased from Sigma Aldrich (Steinheim, Germany). Tomato by-products (TBP), made of peels and seeds, were provided by GRECI Industria Alimentare s.p.a.

4.1.2. Samples

Tomato by-product samples, consisting of peels and seeds from industrial processing, were supplied by GRECI Industria Alimentare S.p.A (Ravadese, Parma, Italy) and were collected during the 2023 tomato season. To prevent fermentation, the samples were dried, and two different conditions selected to achieve the same cook value (Cook value = 30.9) were explored:

- TBP-70: Dried at 70°C for 4 hours

- TBP-40: Dried at 40°C for 48 hours

The dried matrices were minced and packaged in various materials, including plastic bags, bioplastic bags, films, and active films. The storage duration of TBP in these packaging materials was monitored over six months using colorimetric assays (e.g., Total Phenolic Content and Total Antioxidant Capacity) and for 11 months through LC/MS analyses.

4.1.3. Extraction Procedure

The extraction procedure was adapted from (J. Li et al., 2022) with some modifications. Specifically, 1 gram of TBP was extracted with 35 mL of ethanol under reflux conditions for 30 minutes. The samples were then subjected to ultrasound-assisted extraction for 15 minutes. After the first extraction, the supernatant was collected and separated from the solid residue. The remaining matrix was re-extracted using 35 mL of fresh ethanol under the same conditions (30 minutes reflux followed by 15 minutes UAE). The supernatants from both extraction steps were combined and evaporated under a vacuum using a rotary evaporator. Before analysis, the extract was centrifuged at 6000 rpm for 15 minutes, filtered through a 0.45 µm membrane filter, and stored at –20°C. Oxitest analysis, Folin-Ciocolteau assay, and DPPH assay were performed following the procedure reported in Chapter 3.

4.1.4. Film Preparation

The bio-based films were prepared by solvent casting (Grimaldi et al., 2022). Briefly, glycerol (0.300 g/g of sodium alginate) was added to a 1% w/v water solution of sodium alginate at 70 °C under vigorous stirring. In another beaker, a suspension of CaCO3 (0.04g/g alginate) and GDL (5.4g/g $CaCO_3$) was prepared in 100ml distilled water. The two solutions were mixed, and the temperature was maintained at 55 °C for 30 min. To formulate the active film enriched with antioxidant compounds, vitamin E (2% w/v) was added to produce active films. The mixture was poured and dried in an oven at 60 °C for 16 h.

4.1.5. Total Phenolic Content (TPC)

The protocol used to evaluate total phenolic content is reported in Chapter 3, section 3.1.9.

4.1.6. Total Antioxidant Capacity (%TAC)

The protocol used to evaluate total phenolic content is reported in Chapter 3, section 3.1.10.

4.1.7. LC/MS analysis

Carotenoid analyses were carried out by Prof. Luigi Mondello and Prof. Daniele Giuffrida at the University of Messina. A 1 g aliquot of lyophilised sample was first extracted with 10 mL of hexane, followed by a further extraction with 10 mL of ethyl acetate (EtOAc). To improve extraction efficiency, the mixture was sonicated for 15 minutes and then centrifuged at 3000 rpm for 15 minutes. The supernatant obtained was filtered through a 0.45 µm syringe filter to remove any particulate matter and then collected. This filtered supernatant was evaporated to dryness under vacuum and reconstituted in a 1 mL mixture of methyl tert-butyl ether (MTBE) and methanol in a 1:1 (v/v) ratio. Chromatographic conditions were based on a previously described method (Giuffrida et al., 2011), with minor modifications. A C30 column was employed for the separation. The column used was C30 (150 mm × 4.6 mm, 2.7 µm); mobile phases: Solvent A was methanol/MTBE/water (90:8:2, v/v/v). Solvent B was methanol/MTBE/water (8:90:2, v/v/v). The gradient program ranged from 0 to 35 minutes. Solvent B was increased from 10% to 95%. The flow rate was set at 1.0 mL/min with an injection volume of 2 µL. The PDA detection was performed in the range of 200 - 700 nm with a sampling frequency of 12.5 Hz and a time constant of 0.160 s. The chromatograms were extracted at λ 450 nm. APCI-MS acquisition was performed in both positive and negative modes over a mass range of 200-1200 m/z with an event time of 0.6 s, scan speed of 1875 µ/s, nebulized gas (N_2) flow rate of 4 L/min, detector voltage of 0.5 kV, interface temperature of 300°C, desolvation line (DL) temperature of 250°C, heat block temperature of 300°C and drying gas flow rate of 5 L/min. Data acquisition was performed using LabSolution ver. 5.91 (Shimadzu Corporation). This method allows accurate extraction and separation of the compounds of interest for further analysis.

4.1.8.Statistical analysis

All quantitative data were statistically analyzed using two-way ANOVA to assess the effects of both storage time and packaging type on TPC, %TAC and carotenoid contents (β-carotene and lycopene). Post hoc tests (Tukey's HSD and Dunnett's test) were applied to determine significant differences between packaging groups at each time point ($\alpha = 0.05$) and with reference sample (plastic bag). Data were reported as mean ± standard deviation ($n = 3$).

Statistical analysis was performed using Microsoft Excel. In addition, Pearson correlation coefficients (r) were calculated (n = 6) to evaluate the linear relationships between oxidative stability (Induction Period, IP), total phenolic content (TPC), and total antioxidant capacity (TAC). Correlation strength was interpreted according to standard thresholds, and results were considered preliminary due to the limited sample size.

4.2. Results and Discussion

4.2.1. Preliminary experiments

The selection of two different drying conditions this study was based on the temperatures typically used for processing tomatoes to ensure microbiological safety: 40 °C and 70 °C (Lazzarini et al., 2022). This step ensures that variations in antioxidant stability and compound retention can be directly attributed to drying temperature rather than differences in thermal exposure. Given that antioxidant compounds are thermolabile, applying a consistent and low Cook Value (C = 30.9) helps prevent excessive thermal degradation during drying. The C value was calculated with the following equation:

$$C = \int_0^t 10^{\left(\frac{T - T\,rif}{z}\right)} \cdot dt$$

Where: T, refers to thermal treatment temperature; Tref, refers to cooking treatment temperature (100 °C); z, refers to thermal resistance constant for the most thermolabile components in the tomato (33.1 °C); t, refers to the duration of thermal treatment.

With the same C value = 30.9, the drying treatments of tomato byproducts (TBP) were performed at 40 °C for 48 hours and at 70 °C for 4 hours. Then, TBP was minced to obtain a powder. Preliminary experiments evaluating the oxidtive stability of sunflower oil enriched with TBP powders were performed by an Oxitest reactor (see section 3.2.1). Figure 4.1 shows the IP values for the two drying conditions assessed. The results showed that TBP powders had a significant ($p < 0.05$) effect on the oxidative stability of the vegetable oil, as observed for ABP. The IP values for enriched TBP samples ranged from 979.5 ± 38 minutes (TBP-40) to 938 ± 37 minutes (TBP-70), improving oil oxidative stability of up to 40 %, which was more evident at higher drying temperatures.

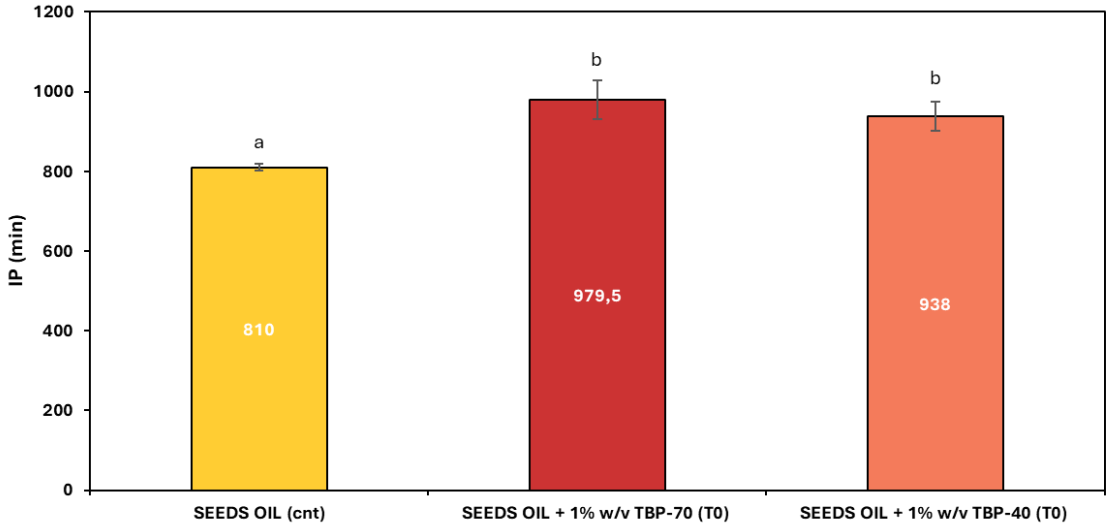

Figure 4.1: Comparison of Induction Periods (IP) of seeds oil (used as positive control) enriched with TBP (1 w/v).

No significant differences in terms of IP values were observed between the two drying treatments. These results agreed with the literature results: indeed, it has been reported the efficacy of higher temperatures in enhancing antioxidant capacity, carotenoid retention, and oxidative stability (Al-Hilphy et al., 2021; Mendelová et al., 2013, 2020; Yusufe et al., 2017). Tan et al. (2021) reported that air-drying at elevated temperatures (up to 80 °C) increased lycopene content and antioxidant activity while reducing drying time and speed. However, in contrast with our findings, some studies reported a decrease in total phenol content with increased drying temperature, leading to a reduction in terms of carotenoid content (Moreno G. & Díaz-Moreno, 2017).

4.2.2. Total Phenolic Content (TPC) and Total Antioxidant Capacity (%TAC) of TBP After Drying Treatments

The Phenolic content (TPC) and antioxidant capacity (%TAC) were evaluated using spectrophotometric assay immediately following oven drying. Figure 4.2 shows the TPC and %TAC values of the extract obtained from TBP matrices subjected to two different drying treatments. As shown in Figure 4.1a, TBP-70, dried at 70°C for 4 hours, had a significantly higher TPC (15.02 ± 0.12 mg GAE/g dry weight) compared to TBP-40, dried at 40°C for 48 hours, which had a TPC of 5.87 ± 0.11 mg GAE/g dry weight). These differences could be explained by the higher temperature used during drying, which promotes the formation and

stabilisation of antioxidant compounds, including phenolics. Furthermore, mechanisms such as enzyme inactivation and reduction of water activity promoted by higher drying temperatures may prevent further oxidative degradation of components occurring in the matrix (Kim & Chin, 2016).

In addition, the higher levels observed for TBP-70 may be partly due to the formation of compounds such as melanoidins during high temperature drying. Melanoidins, Maillard reaction end products, are known to enhance antioxidant activity and TPC through their intrinsic free radical scavenging ability and phenolic compound interaction (S. Yang et al., 2022; Yang et al., 2023). The Maillard reaction during drying processes above 55°C has been shown to yield melanoidins, which contribute to the browning of food matrices and significantly enhance antioxidant properties (Kitchen & Williamson, 2024; S. Yang et al., 2022).

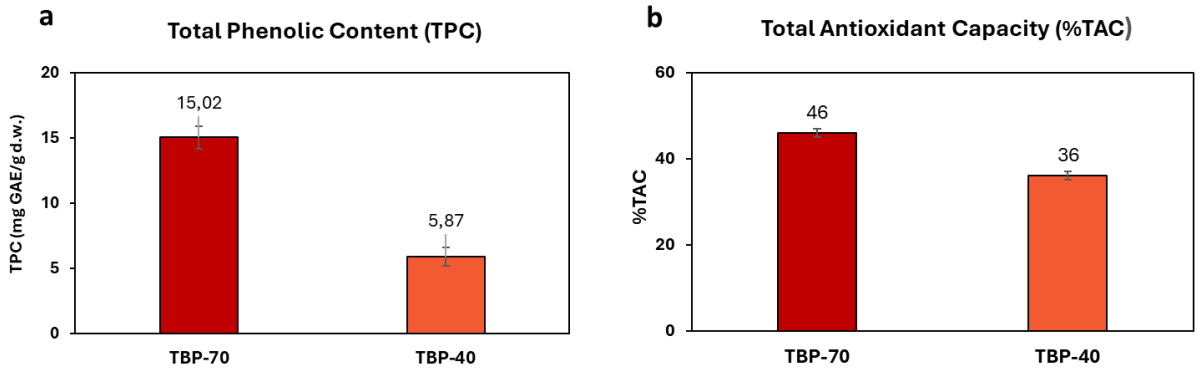

Figure 4.2: (a) Total Phenolic Content (TPC) of TBP-70 and TBP-40 after drying treatment (T_0); (b) Total Antioxidant Capacity (%TAC) of TBP-70 and TBP-40 after drying treatment.

Similarly, as shown in Figure 4.2b, TBP-70 demonstrated a higher %TAC (46 ± 1%) than TBP-40 (36 ± 1%). The elevated antioxidant capacity in TBP-70 is consistent with the retention of higher phenolic content, as phenolic compounds are key contributors to antioxidant activity. These results suggested that higher drying temperatures can stabilize and enhance the bioactive compounds' content and TBP extract's antioxidant properties.

4.2.3.TPC and %TAC Evaluation of TBP During Storage in Different Packaging

Total phenolic content (TPC) was evaluated on extracts obtained from TBP powders stored in the four distinct packaging over time. Figure 4.3 displays the results of decay trends in

terms of TPC across all storage conditions for highly dried samples (TBP-70, 70 °C drying temperature).

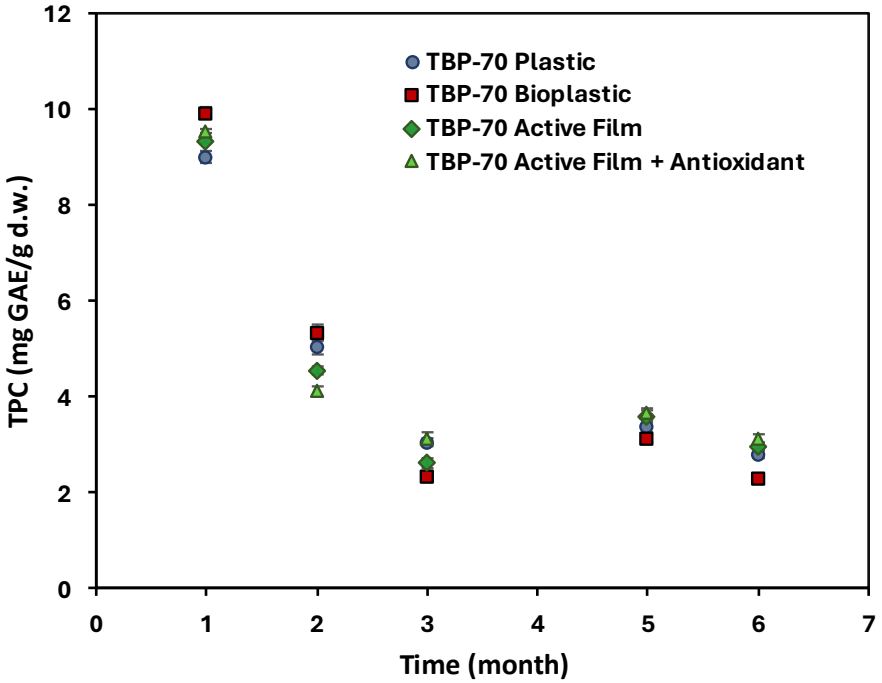

Figure 4.3: TPC values (mean ± standard deviation) of TBP-70 during 6 months of storage in different packaging types at room temperature and light exposure.

As shown in Figure 4.3, different trends in terms of TPC for TBP-70 were observed over time for each condition assessed.

TPC of TBP-70 during storage exhibits a non-linear trend characterized by an initial decline in the first three months, followed by a slight increase at month five, and a final reduction approaching a plateau by month six. The initial decrease in TPC can be attributed to the oxidative degradation of free phenolic compounds, a well-documented phenomenon during storage, especially under light and oxygen exposure (Lombardelli et al., 2021). The slight increase observed in month five may be due to matrix restructuring or hydrolysis of bound phenolics, which can release additional phenolic fractions previously unavailable to the extraction solvent. This phenomenon has been reported in dried plant matrices during aging or mild degradation, leading to a transient rise in extractable antioxidants (Verzelloni et al., 2010). The subsequent decline and apparent plateau by month six likely reflect a balance between continued degradation and the exhaustion of releasable bound phenolics. Despite the higher oxygen permeability than plastic, bioplastic packaging showed good barrier

performance against oxidative degradation compared to conventional plastic, in the initial monitoring stages. Previous studies demonstrated that poly(lactic acid) (PLA) composites, biodegradable polyesters, provided intermediate barrier performances to gas compared to poly(ethylene terephthalate (PET) with better suitability for extending the shelf-life of vegetables (Patanè et al., 2019; Rocca-Smith et al., 2019). In the later stages of storage, active films outperformed conventional packaging, demonstrating improved TPC preservation. Among these, active films incorporating antioxidant substances showed the most significant improvement by the sixth month, highlighting the potential of antioxidants to enhance preservation by releasing protective compounds from the active formulation.

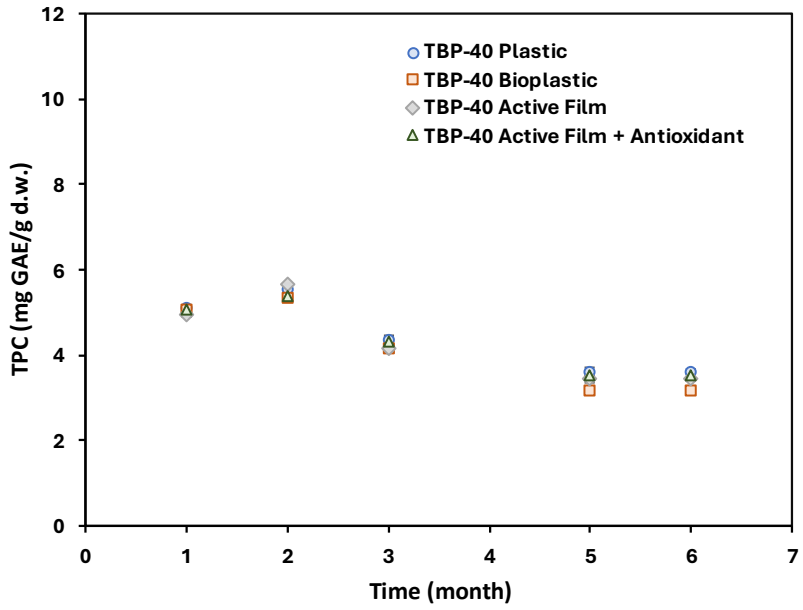

Figure 4.4: TPC values (mean ± s.d.) of TBP-40 during 6 months of storage in different packaging types at room temperature and light exposure.

In contrast, samples dried at lower temperatures (TBP-40) exhibited a slower and more stable degradation trend across all assessed conditions (Figure 4.4), suggesting that mild drying temperatures may contribute to prolonged TPC stability over time. Slight differences in TPC values among packaging types became evident during the later stages of storage (T_5 and T_6) confirming that bioplastic was the worse effective one in preserving TPC.

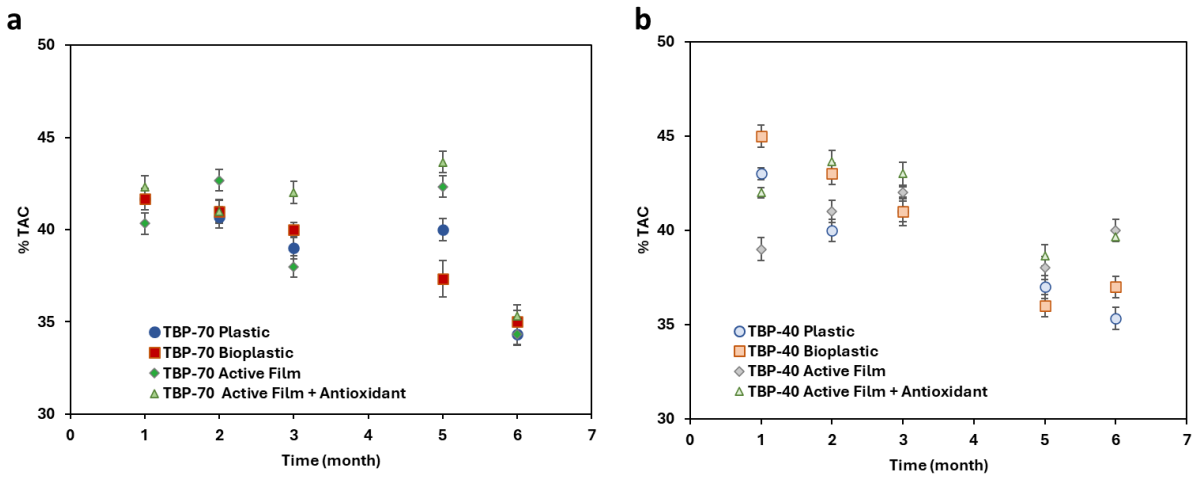

Figure 4.5: (a) %TAC values (mean ± s.d.) of TBP-70 during 6-months of storage under different packaging types at room temperature and light exposure; (b) %TAC values of TBP-40 during 6-months of storage under the same conditions.

Regarding the evaluation of Total Antioxidant Capacity (%TAC) in extracts obtained from TBP dried and stored under various conditions, the results are presented in Figure 4.5. Distinct trends in %TAC were observed over time for TBP-70 and TBP-40 under different packaging conditions. Figure 4.5a displays the overall %TAC values for TBP-70, which ranged from approximately 42% to 34% over six months of storage. A sharp decline in %TAC was observed in the early stages of storage (T_1-T_3), particularly for plastic and bioplastic. TBP-70 stored in active films, particularly those with incorporated antioxidants, demonstrated improved %TAC preservation during early and mid-storage periods, with values remaining consistently higher than others despite showing a non-linear trend over time. This behaviour could be related to the effect of biobased packaging in releasing active compounds over time (Grimaldi et al., 2022). Regarding TBP-40, as illustrated in Figure 4.5b, the %TAC values also showed distinct trends over the six months of storage under different packaging conditions. The overall %TAC values for TBP-40 ranged from approximately 40% to 32%, indicating a similar decline in antioxidant capacity as observed with TBP-70, but at a slightly lower rate. The sharpest reduction in terms of %TAC for TBP-40 occurred in plastic and bioplastic packaging during the initial three months (T_1 -T_3). In contrast, active films, particularly those enriched with antioxidants, consistently outperformed other materials, maintaining higher %TAC levels throughout storage.

To further investigate TPC, %TAC and oxidative stability (measured as Induction Period) a Pearson correlation analysis was performed. Despite the limited sample size (n = 6), strong

correlations were observed in TBP-70 samples. Specifically, the Pearson correlation coefficients were 0.908 for IP vs. TAC, 0.896 for IP vs. TPC, and 0.868 for TAC vs. TPC. These results suggest that, in samples dried at higher temperatures (70 °C), phenolic content and antioxidant capacity are closely associated with oxidative stability. In contrast, TBP-40 samples (dried at 40 °C) showed weaker and even negative correlations for some parameters: the correlation coefficient between IP and TAC was -0.511, and between IP and TPC was -0.891, whereas TAC vs. TPC showed a moderate positive correlation of 0.576. These inverse trends in TBP-40 could indicate complex interactions under milder drying conditions, possibly reflecting different oxidative degradation kinetics or variations in the matrix structure. It should be noted, however, that due to the small sample size, these findings must be considered preliminary. Although suggestive, further studies with larger datasets are needed to confirm statistical robustness and fully elucidate the relationships between oxidative stability, phenolic content and antioxidant capacity.

4.2.4. LC/MS Analysis of β-carotene and Lycopene Contents During TBP Storage

This part of the research was carried out in collaboration with the University of Messina (Prof. Mondello and Giuffrida). LC/MS analyses were performed to evaluate the carotenoids content (β-carotene and lycopene) present in the extracts obtained from TBP, after drying and then during long-term storage in different packaging. In general, also in the case of carotenoids, their stability is strongly influenced by drying temperatures, storage conditions and packaging materials. In Figure 4.6 are displayed the initial content of carotenoids measured in TBP-70 and TBP-40 after drying treatment.

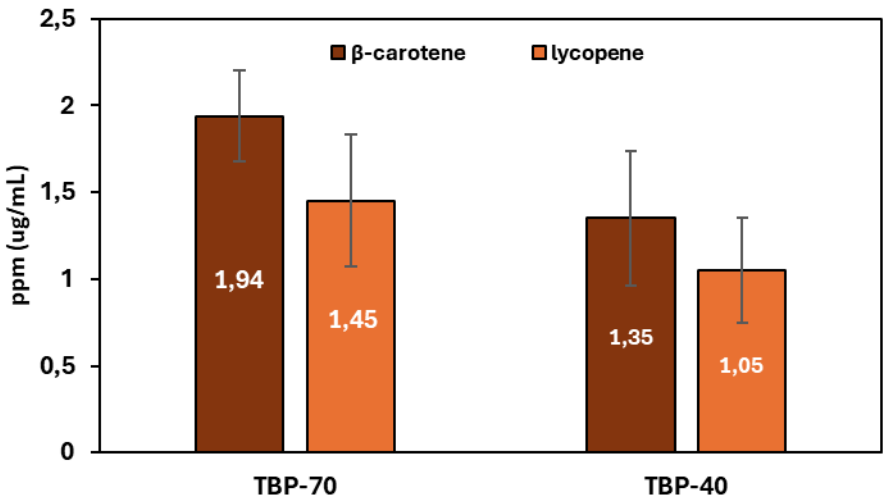

Figure 4.6: β-carotene and lycopene contents (mean ± s.d.) in ppm of TBP-70 and TBP-40 samples. Error bars represent the standard deviation of the measurements.

As visible in Figure 4.6, although the different values recorded, no significant differences ($p > 0.05$) were observed in the β-carotene (1.94 ppm) and lycopene (1.45 ppm) contents between the two samples (TBP-70 vs TBP-40). Therefore, the observed variations cannot be attributed directly to the drying temperature effect, and a further analysis was focused on additional antioxidant parameters. The degradation of both carotenoids is marked for all samples during storage as shown in Figure 4.7.

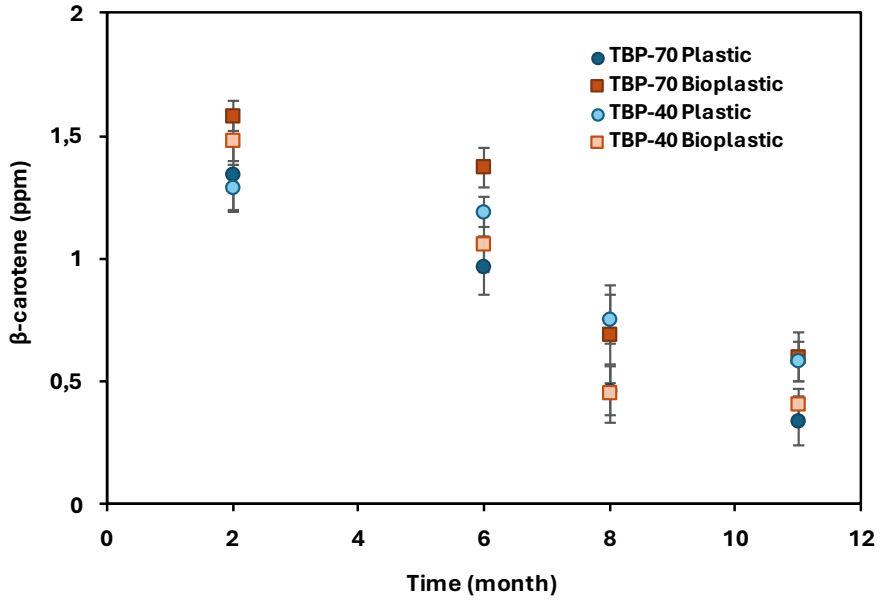

Figure 4.7: Comparison of β-carotene (mean ± s.d.) degradation trends in TBP-70 and TBP-40 over time during storage in Plastic and Bioplastic at room temperature.

For instance, a pronounced degradation was observed for β-carotene by the end of the monitoring period when stored in plastic. The reduction, exceeding 80% over the storage period, may be attributed to multiple interacting factors, primarily involving oxidative and thermal stresses. β-carotene, as a highly unsaturated compound, is known for its susceptibility to oxidation, especially in the presence of oxygen, light, and elevated temperature conditions (Lombardelli et al., 2021; Yang et al., 2022). Indeed, packaging materials used in this study, particularly plastic and bioplastic, differ in their oxygen permeability, which likely influenced the extent of carotenoid degradation. Poor oxygen barrier properties, especially over prolonged storage, can accelerate oxidative reactions leading to the degradation of bioactive compounds Patanè et al., 2019; Rocca-Smith et al., 2019). Moreover, the absence of UV-absorbing additives in these materials may have further contributed to carotenoid instability, as UV-protective agents are commonly employed in commercial packaging to mitigate photo-degradation and preserve bioactive compounds. As also observed for TPC and TAC, bioplastic packaging exhibited a lower reduction in the initial monitoring phases for samples dried at higher temperatures. However, during prolonged storage periods, a significant degradation rate was observed.

Analyses carried out on TBP dried at lower temperatures (Figure 4.8) showed improved stability over time, as was observed for TPC and %TAC.

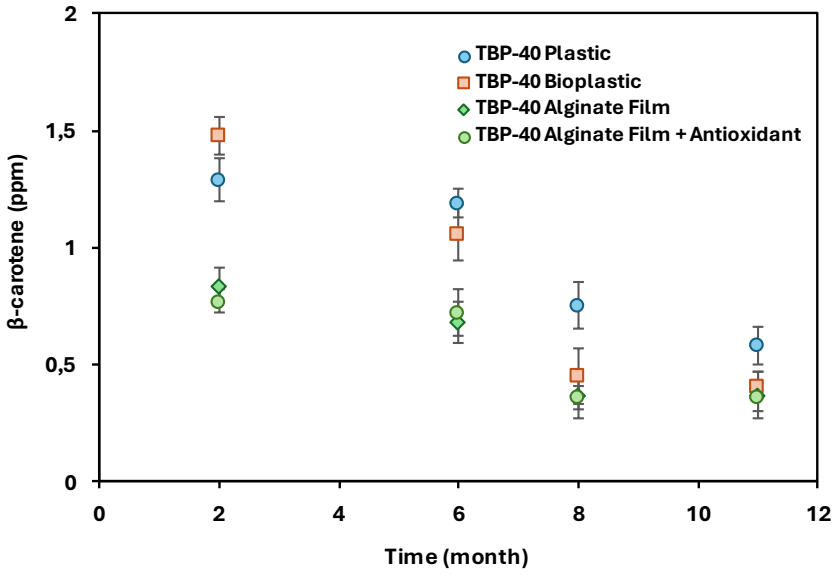

Figure 4.8: Comparison of β-carotene (mean ± s.d.) degradation trends in TBP-40 over time during storage in the four different packaging types.

Although β-carotene content detected in TBP-40 at the beginning of storage was lower than TBP-70 (see Figure 4.6), the results related to the storage provided less reduction of approximately 70%. In general, plastic packaging provided the best performances, overcoming active films. As for lycopene, Figure 4.9 displays its degradation in TBP dried at the two temperatures during storage in plastic and bioplastic.

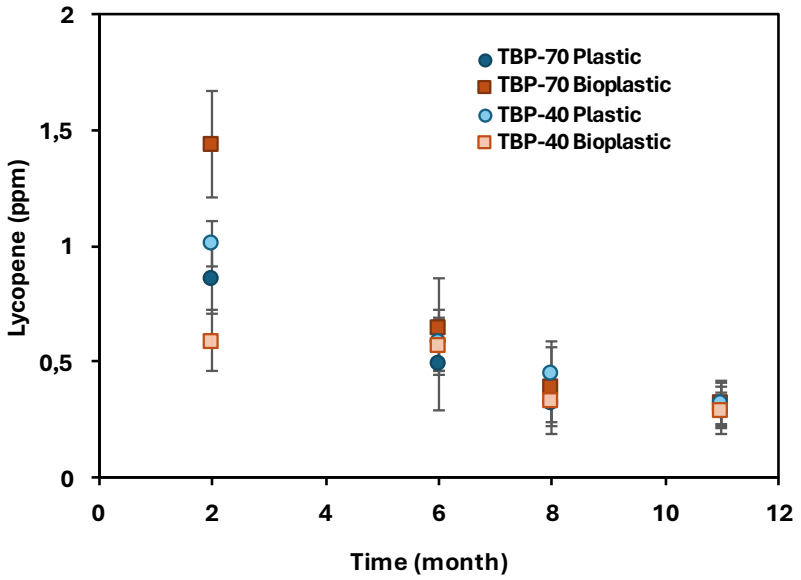

Figure 4.9: Comparison of lycopene degradation trends in TBP-70 and TBP-40 over time during storage in Plastic and Bioplastic at room temperature.

Higher drying temperatures yielded initially higher lycopene concentrations in TBP-70 (1.45 ppm) compared to TBP-40 (1.05 ppm) (see Figure 4.6). High temperature also promoted faster degradation rates during subsequent storage. Several studies have reported that high drying temperatures or prolonged exposure to oxygen and light can induce structural changes in carotenoids, leading to accelerated degradation and reduced stability during storage (Lombardelli et al., 2021; S. Yang et al., 2022; Kitchen W & Williamson, 2024). As for packaging, bioplastic provided superior short-term protection for high-temperature-dried powders whereas an opposite effect was recorded for low-temperature dried sample.

Stability performances of active films in preserving lycopene were also monitored, as shown in Figure 4.10.

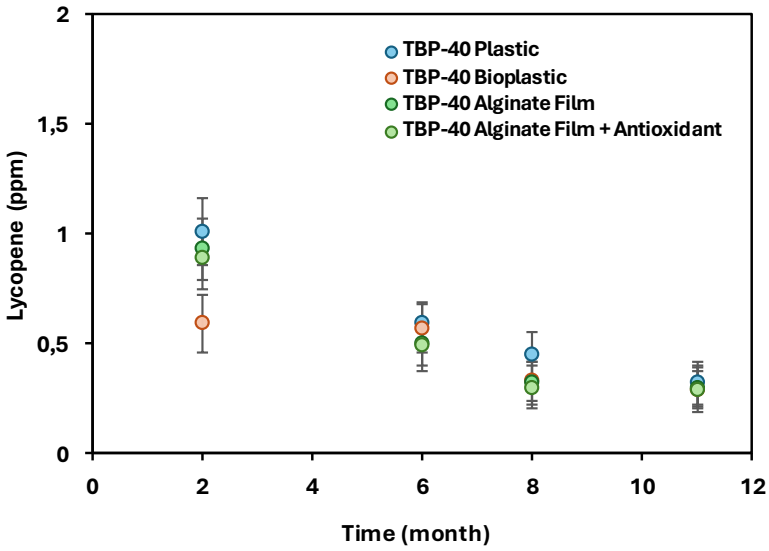

Figure 4.10: Comparison of lycopene degradation trends in TBP-40 over time during storage in Plastic and Bioplastic, and Active Films at room temperature.

The TBP-40 sample in plastic showed a 70% of decline in lycopene content, dropping from about 1 to 0.3 ppm by the end of the monitoring period, as shown in Figure 4.10. Active films did not demonstrate improved stability. In general, conventional packaging showed its effectiveness in preserving carotenoids during storage.

These findings underscored the need for tailored storage solutions, combining optimal drying temperatures and the choice of suitable packaging technologies to ensure a longer preservation of bioactive compounds in TBP.

4.3. Conclusions

This study demonstrated both drying temperature and packaging material significantly influence the stability of bioactive compounds in tomato by-product powders after drying treatment and during storage. Higher drying temperature (70 °C) initially enhanced the total phenolic content (TPC), antioxidant capacity (%TAC). However, these benefits were followed by accelerated degradation across all packaging types during storage. Notably, bioplastic packaging preserved higher levels of TPC, %TAC, and carotenoids (β-carotene and lycopene) during the early stages of storage. In contrast, active packaging formulations performed comparably to conventional plastic by the end of the monitoring period.

On the other hand, milder drying at 40 °C preserved antioxidant stability more effectively over time, despite lower initial values in terms of TPC and %TAC. Among the packaging systems tested, active film formulations provided comparable performances to the plastic in terms of TPC, %TAC and carotenoids retention, thereby mitigating oxidative and light-induced degradation of phenolic and carotenoid compounds.

These results emphasize the importance of integrating optimized thermal treatment with functional packaging to preserve the nutritional and functional properties of agro-industrial by-products, thereby supporting sustainable valorisation framework in food, nutraceutical, and pharmaceutical applications.

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Chapter 5

OPTIMIZATION OF A *GREEN* APPROACH FOR AGRO-FOOD BY-PRODUCTS VALORIZATION: EXPLORING IN VITRO NEUROPROTECTIVE POTENTIAL OF ARTICHOKE AND TOMATO BY-PRODUCTS

As part of my Ph.D. project, a visiting period of about 10 months was spent in the Foodomics Laboratory at the CIAL Institute (Madrid), supervised by Prof. Alejandro Cifuentes and Dr. Alberto Valdés. The research group was the first to define the new field of *Foodomics* in an SCI journal (Cifuentes, 2009). Introduced in 2009, *Foodomics* is defined as "a discipline that studies the food and nutrition domains through the application and integration of advanced -omics technologies to improve consumer well-being, health, and knowledge" (García-Cañas et al., 2012; Herrero et al., 2012; Valdés et al., 2021). This interdisciplinary field combines food chemistry, biological sciences, and data analysis. It focuses mainly on transcriptomics, proteomics, and metabolomics approaches to gain a deeper understanding of foods, their functional components, and their interactions with the human genome. Additionally, advanced MS-based omics techniques have been applied to investigate issues related to food quality and safety.

This study optimized a pressurized liquid extraction (PLE) technique to isolate phenolic and flavonoid-rich extracts from artichoke (ABP) and tomato (TBP) industrial by-products. By means of response surface methodology (RSM) by central composite design (CCD), the optimal conditions in terms of solvent mixture compositions and extraction temperatures were evaluated, aiming to maximize bioactive compounds recovery. For ABP, a mixture of GRAS solvents such as ethyl acetate (EtOAc) and ethanol (EtOH) at 180 °C was found to be most effective in enhancing the extraction of antioxidant compounds. In the case of TBP, PLE with cyclopentyl methyl ether (CPME) at 180 °C yielded the best results. An untargeted metabolomic approach using UHPLC-Q-TOF-MS/MS was adopted for the identification of phenolic and flavonoid profiles present in artichoke and tomato by-products pressurized liquid extracts (obtained under optimized conditions), contributing to the anti-inflammatory

and neuroprotective potential. The parallel artificial membrane permeability assay for the blood-brain barrier (PAMPA-BBB) assay demonstrated the potential of several extracted compounds to diffuse across an artificial blood-brain barrier. In silico molecular docking, analysis revealed characteristic interactions between Acetylcholinesterase (AchE), Butyrylcholinesterase (BChE), and Lipoxygenase (LOX) active sites and the most abundant flavonoids occurring in PLE extracts. These findings highlighted the potential of green extraction techniques to valorise food waste to isolate neuroprotective agents.

5.1. Materials and methods

5.1.1. Solvents, reagents, and standards

Milli-Q water was collected from the Millipore system (Billerica, MA, USA). Absolute ethanol (EtOH), ethyl acetate (EtOAc), HPLC-grade acetonitrile (ACN), and HPLC-grade methanol were supplied by VWR Chemicals (Barcelona, Spain). For conducting in vitro biological activity assays, sodium chloride, sodium acetate, sodium hydroxide, sodium dihydrogen phosphate, potassium phosphate, and monopotassium phosphate were obtained from Panreac Quimica SLU (Barcelona, Spain). The Folin-Ciocalteu reagent was acquired from Merck (Darmstadt, Germany). Trizma hydrochloride (Tris-HCl), acetylcholinesterase (AChE) Type VI-S derived from *Electrophorus electricus*, Butyrylcholinesterase from equine serum (BChE), linoleic acid (LA), acetylthiocholine iodide (AChI), aluminum chloride ($AlCl_3$), phosphoric acid, sodium carbonate, potassium phosphate, monopotassium phosphate, fluorescein, gallic acid, quercetin, galantamine hydrobromide, and a 96-well acceptor plate were sourced from Sigma-Aldrich (Madrid, Spain). Additionally, lipoxygenase from *Glycine max* (soybean) (LOX), 4-(amino-359-sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F), and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from TCI Chemicals (Tokyo, Japan).

5.1.2. Agro-food by-products samples

Artichoke and tomato by-products (ABP and TBP) were provided by Greci Industria Alimentare S.p.A (Ravadese, Parma, Italy) and dried and minced as reported in chapter 3. Powders were finely ground using a laboratory-grade knife mill Grindomix GM 200 (Retsch GmbH, Haan, Germany), and then sieved obtaining powders with a homogeneous particle

size distribution (300 – 600 µm). The resulting powder was vacuum-packed (C400 Multivac Wolfertschwenden, Germany) and stored at -18 °C.

5.1.3. Pressurized liquid extraction (PLE) method

Extraction of bioactive compounds from ABP and TBP were performed by means of Pressurized Liquid Extraction (PLE) in an ASE 200 (Dionex, Sunnyvale, CA, USA) instrument, equipped with a solvent controller. In this work, Milli-Q H_2O, EtOH, EtOAc and CPME were the solvents used and the PLE parameters – such as pressure, temperature, number of cycles and time– were set at 10.34 MPa, 115 °C, and 1 cycle of extraction for 20 min.

To perform the extraction, 1 g of ABP or TBP powder was placed into a 5 mL extraction cell and mixed with 2 g of sand which was used as supporting material. The cell was filled with the selected solvent and then two cellulose filters were placed on the top and bottom of the cell (Restek, Bellefonte, PA, USA). The extracts were collected in a glass vial (final volume of 17 mL), dried using nitrogen solvent evaporator at 30 °C (TurboVap LV, Caliper LifeSciences, MA, USA) and the total yield was calculated as Eq. 1. Sample extracts were stored at -18 °C until futher use.

5.1.4. Total Phenolic Content and Total Flavonoid Content

The total phenolic content (TPC) and total flavonoid content (TFC) of the ABP and TBP extracts were determined using the protocol described by Tripodo et al. (Tripodo et al., 2018). For TPC measurement, 10 µL of each extract, resuspended in ethanol (at a concentration of 5 mg/mL), were added to 50 µL of Folin reagent. After 1 minute, 150 µL of a 20% (w/v) aqueous sodium carbonate solution was included, and the total volume was adjusted to 1 mL with Milli-Q water. The mixture was incubated at room temperature in the dark for 2 hours. After incubation, 300 µL of the solution was transferred into a microplate, and the absorbance was measured at 760 nm using a Synergy HT microplate reader from Bio-Tek Instruments (Winooski, VT, USA). A calibration curve was prepared using gallic acid as the standard compound, within a range of 0.031 to 2 µg/mL in Milli-Q water. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract (mg GAE/g dry extract). All analyses were performed in triplicate.

For TFC measurement, 100 µL of each extract, also resuspended in ethanol (at 5 mg/mL), were added to 140 µL of methanol and 60 µL of an 8 mM aqueous solution of AlCl3. The mixture was incubated at room temperature in the dark for 30 minutes, after which the absorbance was measured at 425 nm. TFC was calculated from a calibration curve using quercetin as the standard compound, within a range of 1 to 14 µg/mL. The results were expressed as milligrams of quercetin equivalents (QE) per gram of dry extract (mg QE/g dry extract). All analyses were performed in triplicate.

5.1.5. DPPH radical scavenging activity

The radical scavenging capacity was assessed using the DPPH radical, following a modified protocol based on Abderrezag et al. (2021). Specifically, each well was filled with varying volumes of the sample, ranging from 10 to 100 µL at a concentration of 1.5 mg/mL. Next, 150 µL of DPPH solution (6×10^{-5} M in ethanol) was added to each sample. After incubating in the dark at room temperature for 30 minutes, absorbance was measured at 517 nm using a microplate reader. The results were expressed as IC_{50} in µg/mL and calculated using linear regression, with concentration as a function of free radical scavenging activity. A solvent mixed with the sample extract served as the blank, while the DPPH solution (150 µL; 6×10^{-5} M) was the negative control. All measurements were conducted in triplicate.

5.1.6. Optimization PLE extraction by experimental design

A response surface methodology (RSM) by central composite design (CCD) approach was employed to evaluate the best PLE conditions for bioactive compounds extraction from ABP and TBP. Two factors were considered at three levels: X_1: solvent composition (%, v/v) and X_2: extraction temperature (°C), including four factorial, four axial and three replicates at the central point.

For ABP different EtOH volumes were added in EtOAc according to the experimental design (Table 5.1).

Table 5.1: Central composite design (CCD) for two independent variables: ethanol volume in ethyl acetate (%, v/v) and temperature (°C) used for the extraction of bioactive compounds from ABP.

Variables	Factors	Levels		
		-1	0	+1

EtOH in EtOAc (%, v/v)	X_1	0	50	100
Temperature (°C)	X_2	50	115	180

For TBP different CPME volumes were added in EtOAc according to the experimental design (Table 5.2).

Table 5.2: Central composite design (CCD) for two independent variables: ethanol volume in ethyl acetate (%, v/v) and temperature (°C) used for the extraction of bioactive compounds from ABP.

Variables	**Factors**	**Levels**		
		-1	0	+1
CPME in EtOAc (%, v/v)	X_1	0	50	100
Temperature (°C)	X_2	50	115	180

Statgraphics Centurion XVI software (v.16.1.11) (StatPoint Technologies, Inc., Warrenton, VA, USA) was employed to design experiments assessing the selected response variables (Y_i): Extraction yield, TPC (mg GAE/g dry extract), TFC (mg QE/g dry extract), ORAC and DPPH assays (both expressed as IC_{50}, µg/mL). The proposed quadratic model (Eq. 1) for each response variable was:

$$Y_i = a + X_1 + X_2 + X_1 X_2 + X_1^2 + X_2^2 + \varepsilon \qquad (1)$$

In table 5.3 are reported the 11 extractions performed for ABP and TBP.

Table 5.3: Central composite design (CCD) for two independent variables: solvent* volume in EtOAc (%, v/v) and temperature (°C) used for the extraction of bioactive compounds from ABp and TBP.

Run	**X_1: solvent* volume (% v/v)**	**X_2: temperature (°C)**
1	0	180
2	0	115
3	50	50
4	50	115
5	50	115
6	50	115
7	50	180

8	100	115
9	100	50
10	0	50
11	100	180

*Solvent is EtOH for ABP and CPME for TBP

5.1.7. Oxygen Radical Absorbance Capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) method was employed to assess antioxidant activity by evaluating fluorescence degradation, as previously reported by Sánchez-Martínez et al. (2022). A 96-well microplate reader (BioTek Instruments Inc., Winooski, USA) was utilized for the analysis. Briefly, 100 µL of AAPH (590 mM) in 30 mM phosphate-buffered saline (PBS) at pH 7.5 was added to 100 µL of each extract sample in ethanol, with concentrations ranging from 500 µg to 5000 µg/mL (six levels of dilution) to generate peroxyl radicals. Subsequently, 25 µL of fluorescein (11 µM) was added. Fluorescence measurements were taken under the following conditions: excitation wavelength (λ) at 485 nm and emission wavelength (λ) at 530 nm. Absorbance readings were recorded every 5 minutes at 37 °C for a duration of 1 hour. The calibration curve was constructed using Trolox at 15 µM as the standard. The results were expressed as IC50 (µg/mL), with all measurements performed in triplicate.

5.1.8. Total Chlorophyll Content (TTC)

For the determination of chlorophyll pigments, the method described by Clesceri, Greenberg, and Eaton (1988), modified for micro-concentrations, was employed. Absorbance measurements were conducted using a UV-VIS spectrophotometer (Spectronic 200E, version 4.07i, Thermo Fisher Scientific, Mundelein, Illinois, USA) at wavelengths of 664 nm, 647 nm, and 630 nm for chlorophyll a (Equation 2), chlorophyll b (Eq. 3), and chlorophyll c (Eq. 4), respectively. Total chlorophyll content was calculated by summing each chlorophyll content (Eq. 5). Turbidity correction was performed by measuring absorbance at 750 nm. Chlorophyll concentrations were calculated using the formulae established by Arnon (1949).

$$Chl\ a\ \left(\frac{mg}{L}\right) = 12.7\ \times A_{664} - 2.69\ \times A_{647} \qquad (2)$$

$$Chl\ b\ \left(\frac{mg}{L}\right) = 22.9\ \times A_{647} - 4.68\ \times A_{664} \quad (3)$$

$$Chl\ c\ \left(\frac{mg}{L}\right) = 24.36\ \times A_{630} - 3.85\ \times A_{647} \quad (4)$$

$$Chl\ Total\ = Chl\ a + Chl\ b + Chl\ c \quad (5)$$

5.1.9. Total Carotenoids Content (TCC)

Total carotenoids contents (TCC) were measured by colorimetric assays following the protocol proposed by Amador-Luna et al. (2024). The extract (0.05 mg/mL) was dissolved in cold acetone/ Milli-Q water (90:10, v/v) after an overnight incubation in dark and 4 ºC, the mixture was centrifuge at 1500 rpm for 10 min at 4 ºC. The supernatant was collected and diluted with 5 mL acetone/Milli-Q water (90:10, v/v) and the absorbance were measured at 480 and 750 nm. The value of TCC was obtained with Britton (1985) formula (6).

$$\text{TCC}\ (\%) = \text{A} \cdot \frac{10^6}{A_{1cm}^{1\%} \cdot 0.03 \cdot [M^{ext}]} \cdot 100\% \quad (2)$$

Where: A, refers to the sample absorbance corrected for turbidity (A_{480}-A_{750}) (UA); $A_{1\text{cm}}^{1\%}$, refers to the molar absorptivity coefficient of β-carotene (2500 - 100 mL g^{-1} cm^{-1}); $[M^{ext}]$, refers to sample concentration (g/mL).

5.1.10. HPLC-Q-TOF-MS/MS analysis

The extracts of ABP and TBP were dissolved in EtOH reaching a final concentration of 2 mg/mL. The samples were then vortexed for 30 seconds and centrifuged at 14800 rpm for 5 minutes at 4 °C. The supernatant was collected and stored at -80°C. A volume of 2 µL was then injected into the UHPLC system model 1290 (Agilent Technologies) coupled to the Q-TOF series 6540 using an Agilent Jet Stream thermal orthogonal ESI source (Agilent Technologies, Waldbronn, Germany). Compound separation was performed using a ZORBAX Eclipse Plus C18 analytical column (100 mm x 2.1 mm, 1.8 µm particle size) and a C18 guard column (0.5 cm x 2.1 mm, 1.8 µm particle size) supplied by Agilent Technologies (Waldbronn, Germany). Chromatographic analysis was performed using a gradient elution with Milli-Q water (mobile phase A) modified with 0.1% formic acid and acetonitrile (ACN,

mobile phase B): the gradient consisted of 0-30% B over 7 min, 30-80% B over 2 min, 80%-100% B over 2 min, held at 100% B for 2 min, and a post time of 3 min to return to initial conditions. The column temperature was maintained at 40 °C with a flow rate of 0.5 mL/min. The mass spectrometer was operated in ESI-positive and ESI-negative modes using the following parameters: capillary voltage of 3000 V for ESI-positive and -3000 V for ESI-negative; mass range from 25 to 1100 m/z; nebuliser pressure of 40 psig; and drying gas flow rate of 8 L/min and 300◦C. Sheath gas flow was 11 L/min at 350 °C. MS/MS analyses were performed using the Auto MS/MS mode with five precursors per cycle, dynamic exclusion after two spectra (released after 0.5 min) and collision energies of 20 and 40 V. To ensure accurate mass measurements, the spectra were calibrated using ions m/z 121.0509 ($C_5H_4N_4$) and 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) in ESI positive mode and m/z 119.0363 ($C_5H_4N_4$) and 980.0164 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + acetate) in ESI negative mode. These ions were injected simultaneously into the ionisation source. A blank sample of EtOH was included for blank subtraction. The chromatograms were processed using MS-DIAL v4.9 software for tentative identification of compounds, supplemented by MS/MS spectral comparisons with the NIST, LipidBLAST and MoNA databases. Additionally, Agilent MassHunter Qualitative Analysis software (v.10.0) was utilized to gain further qualitative insights into the tentatively identified compounds, employing filtering tools based on diagnostic product ions and/or neutral losses of interest to enhance data interpretation.

5.1.11. Molecular docking

A docking simulation was performed to predict the binding affinity of the most abundant bioactive compound specifically extracted by PLE for the active site of AchE (PDB: 5HFA), BChE (PDB: 6EQP) and LOX (PDB: 1JNQ) enzymes. A single crystal structure of each enzyme was downloaded from the Protein Data Bank (PDB) (http://www.rscb.org/pdb). Apigenin was the most abundant flavonoid in artichoke by-products PLE extracts, and its structure was obtained from PubChem in SDV format (https://pubchem.ncbi.nlm.nih.gov/). Prior to docking, AChE, BChE and LOX were pre-processed (dehydrated, hydrogenated and polarised) and then defined as the receptors. The molecule apigenin (CAS: 520-36-5) was defined as the ligand. Both proteins and the ligand were prepared for docking using Chimera software (version 1.14). In the active site of each enzyme, the following XYZ coordinates were used to construct the grid box: X: -2.686, Y: -49.014, Z: 30.162 for AChE; X: 132.836733, Y:

116.056133, Z: 41.709600, for BChE; X: 27.383, Y: 4.270, Z: 15.298 for LOX. Using Discovery Studio 4.5 (COMPASS, COMPASS-II, Forcite, Discover and Materials Studio software), the highest free binding energy of the ligand with each enzyme active site was selected and analysed. BIOVIA2015, San Diego, CA) using the Lamarckian algorithm. Finally, the results of the docking simulation for the positive control group (galantamine) were compared with those for apigenin, which showed the best statistical correlation with the enzyme inhibition activity.

5.1.12. Statistical analysis

Statistics were calculated using GraphPad Prism (v. 6.0; GraphPad Software). Results are expressed as mean ± standard error of the mean (SEM). Statistical comparison between groups was estimated using the one-way analysis of variance (ANOVA), followed by the Tukey's post-hoc test. In all cases, p-values lower than 0.05 were considered as statistically significant. The type of inhibition parameters was all calculated with GraphPad Software.

5.2. Results and discussion

5.2.1. Preliminary investigations

Artichoke and tomato by-products (ABP and TBP) were subjected to preliminary extraction experiments with PLE for evaluating the extraction performances of 5 output variables selected for the development of Design of Experiment (DoE), such as extraction yield, Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and DPPH assay values. Four distinct solvents were employed for the extractions - Milli-Q water, ethanol (EtOH), ethyl acetate (EtOAc), and cyclopentyl methyl ether (CPME). The selection of these solvents was based on their wide range of polarities, allowing for the assessment of solubility and extraction efficacy of various phytochemicals. All solvents are classified as green and are Generally Recognized as Safe (GRAS) for food applications, ensuring their suitability for sustainable and food-grade extraction processes. The results of the extractions are reported in Figure 5.1.

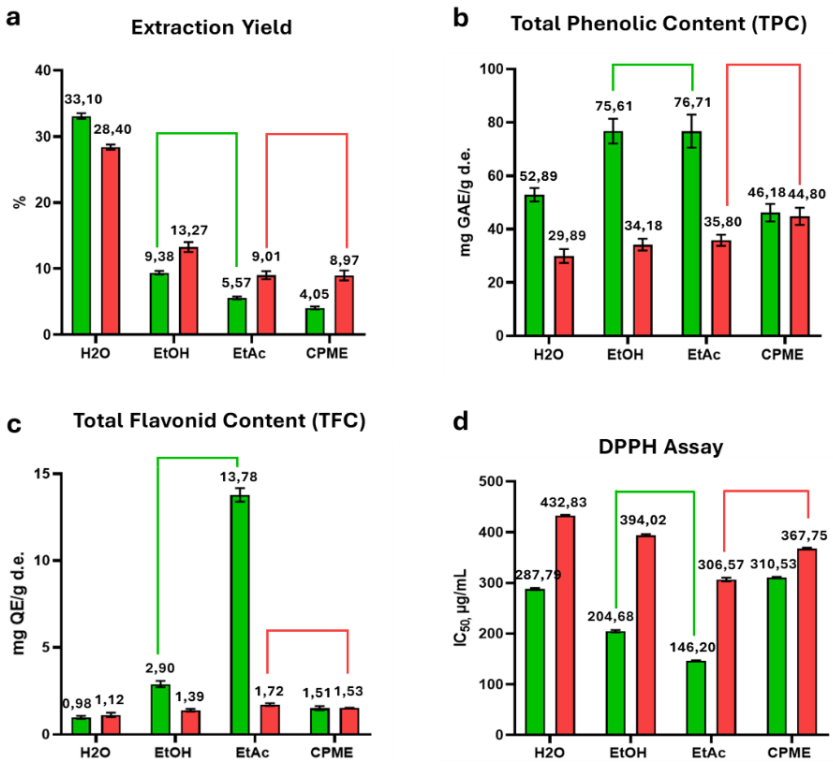


Figure 5.1: Preliminary results of (a) Extraction Yield, (b) Total Phenolic Content, (c) Total Flavonoid Content and (d) DPPH assay of four independent extraction on ABP (green bars) and TBP (red bars). Green lines refer to best results for ABP, red lines for TBP.

While the extraction yield obtained using water achieved approximately 30% (mg/g dry extract) for both ABP and TBP (Figure 5.1a), which can be considered satisfactory given its strong affinity for hydrophilic compounds, the overall performance of water as a solvent was limited when considering the other output variables. Specifically, water extracts exhibited significantly lower values in TPC, TFC, and antioxidant activity (DPPH assay) compared to the other solvents tested (Figure 5.1b–d).

However, PLE water extracts provided lower results in terms of TPC, TFC, and DPPH values when compared to the other solvents (Fig. 1b, c, d). EtOH and EtOAc enhanced extraction of phenolic and flavonoid compounds as well as DPPH values providing the lowest IC_{50} values for both samples ABP and TBP (Fig. 1b, c, d). In addition, the ability of CPME in extracting nonpolar compounds from both matrices was investigated but resulted in effective performances only in the case of TBP. Based on these results, both the effects of the temperature and the different nature of the solvents mixture were further investigated in DoE. Specifically, solvent compositions were set at 0, 50 %, and 100 % of EtOH in EtOAc for ABP and CPME in EtOAc for ABP.

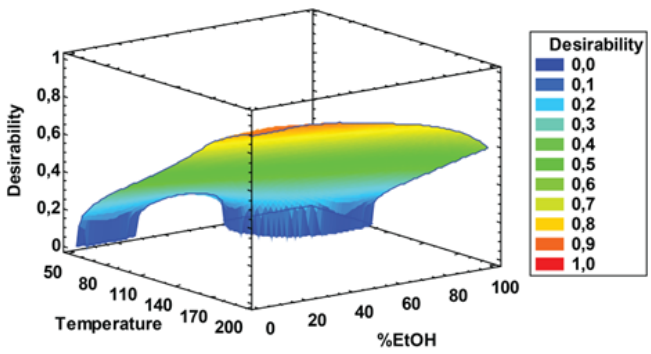
5.2.2. Design of Experiment (DoE)

In this study, a Central Composite Design (CCD) approach of Response Surface Methodology (RSM) was applied to optimize the pressurized liquid extraction (PLE) parameters in order to improve the extraction of bioactive compounds from ABP and TBP. The experimental setup involved a three-level factorial design, testing two independent variables (temperature and % EtOH or % CPME in EtOAc). Specifically, 3 levels of temperature were selected (50, 115, 180 °C) and 3 levels of solvent mixture composition were set by using 0 %, 50 %, and 100 % of the solvent in EtOAc. In this way, the effect on extraction performances was investigated by maximizing the extraction yield, TPC, TFC, DPPH and by minimizing the Oxygen Radical Absorbance Capacity (ORAC) values (i.e., better antioxidant capacity expressed as IC_{50}). The ABP results of the DoE are reported in Table 5.4.

Table 5.4: Experimental design and results of response variables Recovery Yield, Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Oxygen Radical Absorbance Capacity (ORAC), Antioxidant capacity (DPPH assay) of artichoke by-products extracts (ABP) obtained by pressurized liquid extraction (PLE). Asterisk (*) refers to the standard used for antioxidant capacity evaluation.

Run	T	EtOH	Extraction Yield	TPC	TFC	DPPH	ORAC
	°C	%solvent* in EtOAc	% mg/g d.e.	mg GAE/g d.e.	mg QE/g d.e.	IC_{50} (µg/mL extr.)	
1	180	0	7.1	177.3 ± 2.3	10.3 ± 0.4	119.2 ± 2.3	1.5 ± 0.1
2	115	0	5.8	79.2 ± 1.8	8.9 ± 0.2	146.2 ± 3.5	2.1 ± 0.3
3	50	50	3.7	72.1 ± 1.2	4.8 ± 0.1	217.2 ± 1.2	3.6 ± 0.3
4	115	50	5.7	98.4 ± 1.1	7.8 ± 0.6	220.3 ± 2.8	4.2 ± 0.4
5	115	50	7.1	123.4 ± 0.3	6.3 ± 0.5	215.5 ± 3.2	5.3 ± 0.6
6	115	50	6.4	103.6 ± 1.1	7.1 ± 0.6	209.4 ± 3.2	4.6 ± 0.3
7	180	50	9.4	146.3 ± 1.2	9.4 ± 0.4	128.3 ± 1.3	2.0 ± 0.2
8	115	100	9.5	85.9 ± 0.6	5.3 ± 0.4	204.7 ± 1.4	10.7 ± 0.8
9	50	100	7.6	38.1 ± 2.3	6.5 ± 0.3	220.6 ± 2.8	11.7 ± 0.7
10	50	0	3.9	35.3 ± 1.2	7.8 ± 0.1	165.4 ± 1.1	2.5 ± 0.2
11	180	100	12.0	137.3 ± 2.0	4.5 ± 0.3	174.8 ± 1.4	3.3 ± 0.2
*Trolox**						4.82 ± 0.24	0.3 ± 0.04

Temperature and solvent composition significantly influenced the extraction efficiency of ABP samples. As shown in Table 5.4, higher recovery yields were achieved at elevated temperatures (run 1, 7, 8, and 11), whereas the lowest recovery yield occurred at 50 °C. The solvents employed also had a notable impact: increasing percentage of EtOH allowed a significant enhancement of the recovery yield. For TPC and TFC, higher values were observed when high temperatures were applied, regardless of the solvent used. However, extraction with EtOAc yielded the highest TPC and TFC results (run 1). The combination of EtOAc and high temperatures proved to be particularly effective for extracting slightly polar phenolic and flavonoid compounds with high antioxidant potential, aligning with findings from (Mejri et al., 2020; Yang et al., 2020). The impact of temperature and solvent mixture composition was also observed in terms of IC_{50} values from ORAC and DPPH assays. Higher extraction temperatures were related to lower IC_{50} values, with the best results observed at 180 °C. In the ORAC assay, the highest values were acquired as the percentage of EtOAc increased, with an IC_{50} of 1.5 µg/mL. Similarly, the DPPH assay showed optimal radical scavenging activity at 180 °C with increasing volume of EtOAc (run 1 and 7). Figure 5.2 displays the graphical output of RSM for PLE optimization.



Optimize Desirability
Optimum value = 0,843671

Factor	*Low*	*High*	*Optimum*
Temperature	50,0	180,0	180,0
%EtOH	0,0	100,0	10,4563

Response	*Optimum*
Yield	7,55923
TPC	166,886
TFC	10,3623
ORAC	1,08877
DPPH	119,21

Figure 5.2: Experimental Design and Optimization Results Using Response Surface Methodology for PLE of ABP

Regarding optimization of PLE of TBP samples, the DoE results are displayed in Table 5.5.

Table 5.5: Experimental design and results of response variables Recovery Yield, Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Oxygen Radical Absorbance Capacity (ORAC), Antioxidant capacity (DPPH assay) of tomato by-products extracts (TBP) obtained by pressurized liquid extraction (PLE). Words marked with asterisk (*) refers to the standard used for antioxidant capacity evaluation.

Run	T	CPME	Recovery Yield	TPC	TFC	DPPH	ORAC
	°C	%solvent* in EtOAc	% mg/g d.e	mg GAE/g d.e.	mg QE/g d.e.	IC_{50} (µg/mL)	
1	180	0	8.3	54.5 ± 1.1	4.3 ± 0.3	306.6 ± 3.3	1.1 ± 0.0
2	115	0	6.9	28.5 ± 0.9	3.5 ± 0.2	396.4 ± 1.4	10.1 ± 0.9
3	50	50	4.6	14.7 ± 1.2	2.4 ± 0.1	506.0 ± 3.9	23.9 ± 1.6
4	115	50	6.8	20.9 ± 0.8	3.0 ± 0.1	411.8 ± 1.8	19.7 ± 1.7
5	115	50	7.0	24.1 ± 0.3	2.9 ± 0.1	404.5 ± 2.0	16.7 ± 2.5
6	115	50	6.2	28.5 ± 0.7	3.7 ± 0.3	406.4 ± 1.8	15.8 ± 1.8
7	180	50	7.3	42.8 ± 1.2	4.8 ± 0.3	342.8 ± 1.6	1.4 ± 0.1
8	115	100	6.9	25.8 ± 0.5	4.1 ± 0.3	464.9 ± 3.4	9.6 ± 1.8
9	50	100	4.1	11.8 ± 0.6	2.2 ± 0.1	535.5 ± 3.4	24.4 ± 1.0
10	50	0	4.1	11.4 ± 0.7	3.0 ± 0.2	447.1 ± 1.4	21.2 ± 1.8
11	180	100	8.8	66.1 ± 1.6	5.5 ± 0.4	367.7 ± 2.6	5.7 ± 0.5
*Trolox**						*4.8 ± 0.2*	*0.3 ± 0.0*

The highest recovery yield was achieved at elevated temperatures with only CPME (run 11), while lower yields were observed at milder temperatures and lower percentage of CPME in solvent mixture composition. Similarly, TPC and TFC provided higher result at high temperatures and high volume of CPME; milder temperatures and CPME percentages resulted in significantly lower values. Antioxidant capacity, assessed by ORAC and DPPH assays, showed that improved significantly with higher temperatures and CPME concentrations, demonstrating strong radical scavenging activity under these conditions. Figure 5.3 illustrates the graphical output of RSM global optimization for TBP.

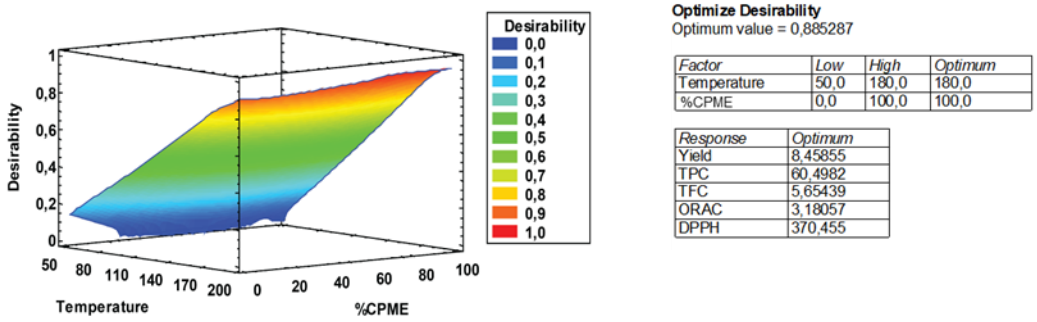


Figure 5.3: Experimental Design and Optimization Results Using Response Surface Methodology for PLE of ABP

Theoretical predictions for the optimal pressurized liquid extraction conditions were calculated based on the previously discussed outcomes. Table 5.6 shows the theoretical and experimental results for optimizing PLE on agro-food by-products.

Table 5.6: Comparison between theoretical and experimental results of the optimal ABP and TBP extracts obtained under optimized PLE conditions.

				Bioactive Compounds Content		Antioxidant Activities	
Sample	**T**	**Solvent mixture**	**Extr. Yield**	**TPC**	**TFC**	**ORAC**	**DPPH**
	°C	%solvent* in EtOAc	%	mg GAE/g d.e.	mg QE/g d.e.	IC_{50}. (µg/mL)	
Predicted Opt ABP	180	10%	7.5	166.88	10.4	1.0	119.2
Experimental Opt ABP	180	10%	7.7	164.8 ± 1.2	11.2 ± 0.7	1.2 ± 0.04	123.2 ± 1.5
Predicted Opt TBP	180	100%	8.5	60.5	5.6	3.2	370.4
Experimental Opt BP	180	100%	8.1	58.3 ± 0.9	6.2 ± 1.1	3.4 ± 0.1	365.9 ± 2.7

*solvent is EtOH for ABP and CPME for ATP

For ABP samples, the predicted optimal conditions were 180 °C with a solvent mixture of EtOAc/EtOH (90:10 v/v), yielding a high desirability score of 84.4%. For TBP samples, the optimal conditions were predicted to be 180 °C with 100% CPME, presenting desirability

score of ...%. Predictions were experimentally confirmed ($p > 0.05$) by performing extraction under the proposed conditions and then assessing same response variables (TPC, TFC, ORAC, and DPPH assays). The experimental results were consistent with the predicted values for both ABP and TBP confirming the accuracy of the regression models in predicting response outcomes.

The spectrophotometric evaluation of chlorophyll and carotenoid contents was conducted on the optimal extracts to extend the analysis of bioactive compounds. For ABP, the total chlorophyll content was approximately 15.25 μg/g, while the total carotenoid content was around 1.62 ± 0.16 μg/g. These results indicate a higher pigment concentration compared to PLE obtained through UAE, as previously reported by Akdogan and Peksel (2023). Furthermore, compounds such as chlorophylls and carotenoids have been shown to positively correlate with antioxidant capacity, as highlighted in the study by Turkiewicz et al. (2019). In the case of TBP, the results exhibited an opposite trend. Carotenoid content was significantly higher, at 15.14 ± 0.4 μg/mL, while the total chlorophyll content was lower, at 2.51 μg/mL. This outcome aligns with the characteristic red coloration of tomatoes, which is strongly associated with the presence of carotenoid compounds. These findings underscore the variability of pigment composition based on the specific agro-food by-products analyzed.PLE demonstrated to be highly efficient in extracting bioactive compounds with strong antioxidant potential from industrial artichoke and tomato by-products.

These findings proved the efficacy of this technology by modifying pressure on the solvent to modify its properties, thus improving extraction performance (Alvarez-Rivera et al., 2020). The selection of solvent mixtures also plays a crucial role in enhancing the extraction performances of biologically active compounds, as solvent polarity influences hydrogen bond interactions, impacting on recovery rates.

5.2.3. Chemical Characterization of Artichoke By-Products PLE Optimal Extract by UHPLC-C18-Q-TOF MS/MS

UHPLC-Q-TOF MS/MS analyses were performed to investigate phenolic and flavonoids profiles of artichoke by-products pressurized liquid extracts obtained under optimal conditions. Analyses were performed in negative and positive mode for both matrices allowing the identification of 43 compounds in ABP and 13 in TBP. The tentative assignment

for each metabolite was possible considering the retention times, MS2 spectra, and comparing with databases and data reported in literature. In Table 5.7 are reported the tentative metabolite identifications for APB, their retention times, experimental mass, proposed formula, corresponding chemical family, and main MS/MS fragments is reported.

Table 5.7: Chemical profile of ABP PLE extracts obtained by UHPLC-C18-Q-TOF MS/MS ESI (−/+) analyses.

No	RT (min)	Tentative Metabolite name	$[M-H]^-$ (m/z)	$[M-H]^+$ (m/z)	Monoisotopic Mass	Δ ppm	Molecular Formula	Family	MS/MS (*m/z*, *relative abundance*)	Ref
1	0.742	Methylmalonic acid	117.0209		118.02817	4.8	C4H6O4	Carboxylic acids	99.92 (100); 73.03 (73); 55.02 (8)	database
2	1.672	4-(Methylamino)phenol		124.0756	123.06831	-4.3	C7H9NO	Benzoic acids	108.04 (100); 80.04 (65); 96.04 (60)	database
3	2.218	Protocatehuic acid	153.0204		154.02776	3.9	C7H6O4	Benzoic acids	109.03 (100); 78.97 (52)	(Chen et al., 2015; Masala et al., 2024b)
4	2.690	5-O-Caffeoylquinic acid (neochlorogenic acid)	353.0890		354.09635	2.0	C16H18O9	Hydroxycinnamic acids	179,03 (69), 191,05 (48), 135,04 (8)	(Pagano et al., 2016)
5	3.532	3-O-Caffeoylquinic acid (chlorogenic acid)	353.0896		354.09687	3.5	C16H18O9	Hydroxycinnamic acids	191,05 (100), 179,03 (3), 135.04 (2)	(Pagano et al., 2016)
6	3.841	3-O-Caffeoylquinic acid methyl ester	367.1049		368.11219	2.5	C17H20O9	Hydroxycinnamic acids	133.03 (100); 161.02 (58);	(Zhao et al., 2014); Jaiswal & Kuhnert, 2011)
7	3.846	2-Hydroxy-5-methoxybenzaldehyde	151.0410		152.04836	3.1	C8H8O3	Hydroxyphenols	55.02 (100); 62.01 (28)	database
8	4.060	1-O-Caffeoylquinic acid ethyl ester	381.1208		382.12812	3.1	C18H22O9	Hydroxycinnamic acids	161.02 (100); 133.03 (40); 191.05 (7); 135.04 (7)	-
9	4.441	4-O-Caffeoylquinic acid methyl ester	367.1052		368.11246	3.2	C17H20O9	Hydroxycinnamic acids	161.02 (100); 135.04 (17); 133.03 (6)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
10	4.446	3-O-Caffeoylquinic acid lactone	335.0791		336.085066	4.0	C16H16O8	Hydroxycinnamic acids	133.03 (100); 161.02 (73); 135.14 (14)	(Jaiswal et al., 2011, 2014)

11	4.613	Methyl 4-methoxycinnamate		193.0861	192.07882	-1.9	C11H12O3	Hydroxycinnamic acids	90.04 (100); 77.03 (50); 55.05 (39); 131.04 (26)	(Ancillotti et al., 2019)
12	4.616	4-O-Caffeoylquinic acid lactone	335.0786		336.08643	2.6	C16H16O8	Hydroxycinnamic acids	133.03 (100); 161.02 (88); 135.04 (13)	(Jaiswal et al., 2011, 2014)
13	4.737	3-O-Caffeoylquinic acid ethyl ester	381.1208		382.126931	2.9	C18H22O9	Hydroxycinnamic acids	161.02 (100); 133.03 (37)	-
14	4.860	5-O-Caffeoylquinic acid methyl ester	367.1054	369.1180	368.111281	-1.5	C17H20O9	Hydroxycinnamic acids	135.04 (100); 161.02 (23); 133.03 (21); 179.03 (10)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
15	5.098	1-O-Caffeoylquinic acid lactone	335.0788		336.08613	3.2	C16H16O8	Hydroxycinnamic acids	161.02 (100); 133.02 (79); 135.04 (17)	(Jaiswal et al., 2011, 2014)
16	5.262	4-O-Caffeoylquinic acid ethyl ester	381.1206		382.12794	2.6	C18H22O9	Hydroxycinnamic acids	161.02 (100); 133.03 (82); 135.04 (22)	-
17	5.416	Kaempferol 3-rungioside	593.1523		594.15958	0.9	C27H30O15	Flavonoids-O-glycosides	285.04 (100); 61.98 (16)	(Man et al., 2022)
18	5.506	Luteolin-7-O-glucoside (cynaroside)	447.0943		448.10157	1.0	C21H20O11	Flavonoid-O-glycosides	285.04 (100)	(Luca et al., 2022; Masala et al., 2024b)
19	5.625	5-O-Caffeoylquinic acid ethyl ester	381.1208		382.12815	3.2	C18H22O9	Hydroxycinnamic acids	135.04 (100); 161.02 (19); 133.03 (13); 179.03 (13)	-
20	5.745	(+)-Pinoresinol hexoside	519.1883		520.195352	1.1	C26H32O11	Lignan derivates	357.13 (100); 151.04 (91); 179.03 (20)	(Abu-Reidah et al., 2013a; Boldizsár et al., 2010; Masala et al., 2024b)
21	5.762	3,5-Dicaffeoylquinic acid	515.1213	517.1343	516.12858	2.4	C25H24O12	Hydroxycinnamic acids	161.02 (100); 135.04 (50); 179.03 (43); 133.03 (28); 363.11 (7)	(Pagano et al., 2016; Gouveia et al., 2012)
22	5.921	Spherobioside	577.1565	579.1713	578.16379	-0.5	C27H30O14	Flavonoids-O-glycosides	269.04 (100)	database
23	6.072	Apigenin 7-O-glucoside	431.0996		432.10694	1.7	C21H20O10	Flavonoid-O-glycosides	269.05 (100)	(Pagano et al., 2016)

24	6.091	1,4- Dicaffeoylquinic acid methyl ester	529.1364		530.14371	1.4	C26H26O12	Hydroxycinnamic acids	161.02 (100); 135.04 (53); 133.03 (22)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
25	6.133	1,4-Dicaffeoylquinic acid	515.1208		516.12815	1.6	C25H24O12	Hydroxycinnamic acids	191.05 (100); 179.03 (62); 135.04 (52)	(Luca et al, 2022)
26	6.497	Dicaffeoylquinic acid methyl ester isomer I	529.1361		530.14341	0.8	C26H26O12	Hydroxycinnamic acids	161.02 (100); 135.04 (18); 179.03 (40); 133.03 (4)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
27	6.827	1,5-Dicaffeoylquinic acid methyl ester	529.1363		530.14365	1.3	C26H26O12	Hydroxycinnamic acids	161.02 (100); 179.03 (51), 135.04 (28); 133.03 (14)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
28	7.190	Ethyl caffeate	207.0678	209.0807	208.07508	1.7	C11H12O4	Hydroxycinnamic acids	133..03 (100); 135.04 (18)	(Cerulli et al., 2024; Barth et al., 2019)
29	7.232	1,5-Dicaffeoylquinic acid ethyl ester	543.1516		544.15891	0.5	C27H28O12	Hydroxycinnamic acids	161,02 (100); 135,04 (27); 133,03 (13); 135,85 (5)	-
30	7.250	3,5-Dicaffeoylquinic acid methyl ester	529.1361		530.14341	0.8	C26H26O12	Hydroxycinnamic acids	179.03 (100); 135.04 (74); 161.02 (41)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
31	7.292	Di-caffeoylquinic acid lactone isomer I	497.1106		498.11790	2.3	C25H22O11	Hydroxycinnamic acids	161.02 (100); 135.04 (29); 133.03 (14); 179.03 (12); 335.08 (6)	(Jaiswal et al., 2011, 2014)
32	7.300	Luteolin	285.0419	287.0550	286.04920	3.2	C15H10O6	Flavones	133.03 (100); 65.00 (26)	(Pagano et al., 2016; Masala et al., 2024b)
33	7.529	3,5-Dicaffeoylquinic acid ethyl ester	543.1515		544.15885	0.4	C27H28O12	Hydroxycinnamic acids	161,02 (100); 135,04 (50); 179,03 (43); 133,03 (28); 363,11 (7)	-
34	7.592	4,5-Dicaffeoylquinic acid methyl ester	529.1362		530.14353	1.0	C26H26O12	Hydroxycinnamic acids	161.02 (100); 133.03 (17); 179.03 (15); 135.04 (11)	(Zhao et al., 2014; Jaiswal & Kuhnert, 2011)
35	7.734	Di-caffeoylshikimic acid isomer	497.1103		498.11759	1.7	C25H22O11	Hydroxycinnamic acids	161.02 (100); 179.03 (68); 135.04 (66); 335.07 (17)	(Jaiswal et al., 2011)
36	7.933	4,5-Dicaffeoylquinic acid ethyl ester	543.1514		544.15873	0.2	C27H28O12	Hydroxycinnamic acids	179,03 (100); 135,04 (97); 161,02 (94)	-
37	8.075	Apigenin	269.0473	271.06024	270.05457	0.4	C15H10O5	Flavones	117.03 (100); 65.00 (25)	(Pagano et al., 2016)

38	8.100	Di-caffeoylquinic acid lactone isomer II	497.1100		498.11735	1.2	C25H22O11	Hydroxycinnamic acids	161.02 (100); 133.02 (59); 335.07 (5); 135.04 (3)	(Jaiswal et al., 2011, 2014)
39	8.446	(9Z)-5,8,11-Trihydroxyoctadec-9-enoic acid	329.2349		330.24216	3.0	C18H34O5	Fatty acids derivates	99.08 (100); 159.00 (78); 223.05 (52)	(Cerulli et al., 2024)
40	8.944	Velutin		315.0862	314.07891	-1.2	C17H14O6	Flavones	272.06 (100); 300.06 (21); 243.06 (21)	(Eissa et al., 2020; S. Liu et al., 2016)
41	9.437	4,7- Dimethoxyflavonol		299.0922	298.08486	0.6	C17H14O5	Flavanols	256.07 (100); 167. 03 (27)	database
42	10.013	Laurylsulfuric acid	265.1490		266.15632	2.2	C12H26O4S	Fatty acids derivates	96.96 (100); 79.95 (20); 149.69 (23)	database

Among the 40 tentatively identified compounds in both ionization modes in ABP PLE extract, the main one resulted to be derivatives of phenolic compounds (n = 28), followed by flavonoids derivatives (n = 9). The bioactive compounds detected in the ABP extract were mainly attributed to benzoic (such as) and hydroxycinnamic acids (such as caffeic acid, mono- and di-caffeoylquinic acids and derivatives, ethyl caffeate), flavonoids and derivatives (such as apigenin, luteolin, kaempferol, velutin and their corresponding glucoside derivatives), which contribute to TPC and TFC values, antioxidant capacity. Most of them may be considered as potential drug candidate for disease treatments, since are reported to exert neuroprotective (Abd El-Aziz et al., 2021b, 2021a; C. Angeloni et al., 2022; Iglesias-Carres et al., 2023; Pagano et al., 2018) and anti-inflammatory activities (Abd El-Aziz et al., 2021a, 2021b; Biel et al., 2019; Iglesias-Carres et al., 2023; Pagano et al., 2016).

Most of the bioactive compounds identified in this study have already been characterized in artichoke (Nguyen et al., 2024; Chileh-Chelh et al., 2024; Luca et al., 2022; El Senousy et al., 2014; Abu-Reidah et al., 2013a; Gouveia & Castilho, 2012;) and its industrial by-products (Masala et al., 2024a, 2024b; Mejri et al., 2020; Pagano et al., 2016; Rejeb et al., 2020; Ruiz-Cano et al., 2014; Sánchez-Rabaneda et al., 2003). However, novel compounds were identified for the first time, including derivatives of caffeoylquinic acids, such as lactone derivatives and ethyl esters. These compounds are likely generated during the pressurized liquid extraction process, which involves high temperatures and solvents like ethyl acetate (EtOAc). Elevated temperatures are also known for their effect on extracting phenolic acids (Perra et al., 2023), free flavonoids or by forming new adducts with higher antioxidant

potentials such as melanoidins (Matei et al., 2012; Plaza et al., 2013). For instance, mono- and di-caffeoylquinic acid lactones, known to be generated as result of thermal processes, such as the thermal decomposition of sugar products or the lactonization of caffeoylquinic acid, observed during coffee roasting (Jaiswal et al., 2010, 2014), was found for the first time in ABP.

The following part focuses on the discussion of tentatively identified metabolites in ABP pressurized liquid extracts obtained under optimized conditions.

Benzoic acid and derivatives (***2, 3, 7***)

In detail, compound **2** was identified in ESI(+) mode as 4-(Methylamino)phenol (C_7H_9NO) providing a pseudo-molecular ion $[M+H]^+$ at m/z of 124.07561 generating a secondary fragment at m/z 108, 80 and 96 confirmed by literature comparison. Compounds **3** was identified as Procatheutic acid ($C_7H_6O_4$) due to the pseudo-molecular $[M+H]^-$ ion observed at m/z 153.02046 and the presence of other weaker fragment at m/z of 109 as described previous study on artichoke leaves' extracts (Chen et al., 2015; Masala et al., 2024b). Compounds **7** was identified as 2-hydroxy-5-methoxybenzaldehyde ($C_8H_8O_3$) presenting a parent ion [M-H]- at m/z 151.04106 and producing the MS^2 at m/z 55, confirmed by spectral database comparison.

*Hydroxycinnamic acids derivatives (**4, 5, 6, 9, 11, 14, 21, 24, 25, 26, 27, 30**)*

The compounds identified belong to the class of hydroxycinnamic acids, such as mono-caffeoylquinic acids (CQAs) and di-caffeoylquinic acids (di-CQAs), found in multiple forms as esters (methyl and ethyl) and lactones. Compounds **4** and **5** were identified as 5- and 3-CQA, respectively, considering their products ions at m/z 191.0566 ([quinic acid−H]$^-$) and 179.0343 ([caffeoyl−H]−) exhibiting slight difference in signal relative intensities. Although the CQA regio-isomers showed a different order of elution, discrimination between 3- and 5-CQA was possible by observing an higher [caffeoyl−H]$^-$ ion intensity for 3-CQA, as described in previous works (Clifford et al., 2003; Pagano et al., 2016). According to Clifford's hierarchical schemes MS^n fragmentation patterns of mono-CQA and di-CQA are strongly influenced by the specific stereochemical arrangements of each substituent on the quinic

acid moiety (Clifford et al., 2003). It was also possible the discrimination among different naturally occurring methyl esters derivative of CQA ($C_{17}H_{20}O_9$) showing a common [M-H]- ion at m/z 368.110735, such as methylated 3- (**6**), 4- (**9**) and 5-CQA (**14**). In this case, the order of elution was considered for the discrimination of the 3 different CQA methyl esters according with data literature (Szymborska et al., 2022; Jaiswal & Kuhnert, 2011). Specifically, methylated form of 3-CQA (**6**) provided a characteristic MS^2 base peak at m/z 161 ($[CA\text{-}H_2O\text{-}H+]^-$) and at m/z 133 ($[CA\text{-}CO_2\text{-}H+]^-$) not detected for the other isomers (Szymborska et al., 2022). In the case of 5-CQA methyl ester (**14**) eluted at RT = 4.486 min, it was identified according to the fragmentation pattern providing a characteristic MS^2 base peak at m/z 179, not observed in the other isomers (Szymborska et al., 2022). Additionally, other isomers of CQAs, such as 1-CQA and 4-CQA, were not detected either in free or in methylated forms, but only as ethylated form on quinate moieties. Compound **11** was identified as methyl 4-methoxycinnamate ($C_{11}H_{12}O_3$) providing $[M+H]^+$ at m/z 193.08612. Further fragmentation ions were produced at m/z 178 (methyl radical) and at *m/z* 149 (loss of CO_2) (Ancillotti et al., 2019).

Compounds **21** and **25** were identified as 3,5-diCQA (isochlorogenic acid A) and 1,4-diCQA providing the same precursor ion ($C_{25}H_{24}O_{12}$) [M-H]- and MS^2 base peak [diCQA-cinnamate-H+]- at m/z of 515.12085 and 353, respectively. Discrimination between the isomers was possible by observing a characteristic peak at m/z 191 attributed to 1,4-CQAs (Clifford et al., 2005).

Compounds **24**, **27** and **30** were identified as 1,4-, 1,5- and 3,5- Dicaffeoylquinic and methyl ester, respectively, generating the same parent ion $[M-H]^-$ 529.13611. The isomer 1,4-CQA methyl ester was distinguished from the others for its characteristic MS^2 base peak at m/z 367 and 317 generated. Besides, other fragment at m/z 349, corresponding to a mono-CQA methylated (15 Da) on quinate moiety, were also observed confirming that is the methylated form. Discrimination was carried out by detecting for 3,5-diCQA methyl ester its typical secondary peak m/z at 349 ($[methyl\ diCQA\text{-}cinnamate+H]^-$), not observed for 1,5-diCQA fragmentation pattern (Clifford et al., 2005; Jaiswal & Kuhnert, 2011). Compound **28** observed at RT 7.19 minutes was identified as ethyl caffeate ($C_{11}H_{12}O_4$), confirmed by its MS^2 spectrum, which displayed a fragment at m/z 161.0224 $[M-H-EtOH]^-$ (Cerulli et al., 2024). As highlighted in previous studies, this effect could be explained by the esterification reactions between chemical groups of carboxylic acids and an alcohol (in our case of EtOAc) occurring

at high temperatures which lead to the ethyl adducts generation (Barth et al., 2019). Compound **34** was annotated as 4,5-Dicaffeoylquinic acid methyl ester ($C_{26}H_{26}O_{12}$) $[M-H]^-$ 529.13623 based on the Clifford's hierarchical scheme for CQAs identification (Clifford et al., 2005; Jaiswal & Kuhnert, 2011).

*Novel discovered compounds (**8, 10, 12, 13, 15, 16, 29, 31, 33, 35, 36, 37, 38**)*

In this section are discussed the identification of compounds found in specific forms such as ethyl esters and lactones of CQAs and di-CQAs and firstly characterized in artichoke by-products extracts. Clifford's hierarchical schemes were employed for the classification and discrimination of CQAs and di-CQAs derivatives and isomers (Clifford et al., 2003). Compounds **8**, **13**, **16**, and **19** were identified as CQA ethyl ester ($C_{18}H_{22}O_9$) all exhibiting the same pseudo-molecular ion [M-H]- at m/z 381.12076. Conversely to the methyl esters detection, it was possible to reveal the four isomers (3-, 1-, 5-, and 4-) of ethylated CQAs. In the same way for CQA methyl esters, 1- and 3-CQA ethyl esters isomers were distinguished based on the order of elution, as already reported in literature (Jaiswal & Kuhnert, 2011); 4- and 5-CQA ethyl isomers were observed to elute after the ethyl esters of 1- and 3-CQA isomers, in contrast to what already reported in the literature but on methylated compounds (Jaiswal & Kuhnert, 2011).

Several adducts and derivatives associated to mono-CQAs and di-CQAs were observed for the first time in artichoke by-products PLE extracts, including methylated, ethylated and lactone derivatives. In detail, the fragmentation of compounds **10**, **12** and **15** gave a parent ion $[M-H]^-$ at m/z 335.084520 thus identifying them as 3-, 4-, and 1-CQLs, respectively, by comparing data obtained with ones reported in literature (Jaiswal et al., 2011, 2014). Besides the identified mono- and di-CQAs, ethylated and lactone forms were also detected. Compounds **29**, **33** and **36** were respectively identified as 1,5-, 3,5- and 4,5-Dicaffeoylquinic all producing same parent ion [M-H]- at m/z 529.13611. They all displayed MS^2 spectra characteristic peaks attributed to characteristic ions at m/z 161, 179, 135, and 363 as already described in literature (Chen et al., 2015). Specifically, the isomer 4,5-diCQA methyl ester was distinguished for its stronger MS^2 ion at m/z 179 (more than the 50% of the base peak, m/z 349) than the one related to methylated 3,5-diCQA methyl ester; 1,5-diCQA methyl form was identified by its weaker MS^1 ion signal at m/z 349 and MS^2 ion at m/z 179 (less than the 10% of the base peak) (Chen et al., 2015; Jaiswal & Kuhnert, 2011).

Compounds **31** and **38** were identified as 3 isomers of di-caffeoylquinic lactone (diCQL) isomers by detecting $[M-H]^-$ ion at m/z 497.11005, with a proposed molecular formula of $C_{25}H_{22}O_{11}$. Previous research evaluated the presence of mono-CQL in roasted coffee matrix as result of high temperature treatment during coffee making (Jaiswal et al., 2014). Compound **37** provided a pseudo-molecular $[M-H]^-$ at m/z 497.11029 was identified as dicaffeoylshikimic acid ($C_{25}H_{22}O_{11}$) due to the characteristic MS^2 base peak generated at m/z 179 ($[CA-H^+]^-$), despite to what is observed in CQL fragmentation where the MS^2 peak base produced is at m/z 161 (Jaiswal et al., 2011).

Compounds **29**, **33** and **36** were identified as 1,5- 3,5- and 4,5-diCQA ethyl esters ($C_{27}H_{28}O_{12}$), respectively, giving the same parent ion [M-H]- at m/z 543. Previous studies profiling diCQAs from *Artemisia argyi* observed ethyl esters derivative of diCQAs in the extracts (Y. H. Zhang et al., 2012). Discrimination among isomers was possible based on the differences related to the relative intensities of MS/MS fragments at m/ 161, 179 and 135. However, as well as methylated derivatives of CQAs and diCQAs, ethylated forms were observed for the first time in ABP PLE extract. As reported by Barth (2019) in a previous work, the presence of such ethylated conjugates could be due to the extraction conditions employed, such as high temperature (180 °C) and the composition of solvent, consisting of a mixture of EtOH and EtOAc (10/90 v/v).

*Flavonoids derivatives compounds (**17, 18, 22, 23, 32**, **37**, **40**, **41**)*

Based on the fragmentation patterns of compounds **17**, **18**, and **23** were tentatively identified as flavonoids glycoside derivatives. Compound **17** was identified as kaempferol-3-rungioside ($C_{27}H_{30}O_{15}$) by detecting the $[M-H]^-$ at m/z 593.1523. Further fragmentation of the kaempferol core occurred providing specific fragment at m/z 285 by loss of glucoside moiety (162 Da) in line with previous research but on different matrices (Man et al., 2022). Also, compound **18** was identified as luteolin-7-O-glucoside (cynaroside) with the proposed molecular formula $C_{21}H_{20}O_{11}$, with a precursor ion $[M-H]^-$ at m/z 447.0943 and showing characteristic MS^2 at m/z 285.0420 (loss of a glucoside unit) (Luca et al., 2022; Masala et al., 2024b). Compound **22** was identified as spherobioside ($C_{27}H_{30}O_{14}$) presenting both pseudo-molecular ion at m/z 577.15649 ($[M-H]^-$) and m/z 579.1713 ($[M-H]^+$) and providing characteristic fragment at m/z 269, then confirmed by database comparison. Besides, by the fragmentation pattern of was possible to observe compound **23** attributed to apigenin-

7-O-glucoside ($C_{21}H_{20}O_{10}$) providing precursor ion $[M-H]^-$ at m/z 431.09964 and a fragment ion at m/z 269 (glucoside loss, 162 Da) corresponding to the aglycone apigenin (Pagano et al., 2016). Compounds **32**, **37**, **40**, and **41** referred to other well-known flavonoids' aglycones. Compound **32** was tentatively identified as luteolin ($C_{15}H_{10}O_6$) due to the observed $[M-H]^-$ ion at m/z 285.0719 and $[M+H]^+$ at m/z 287.0550, the confirmed by comparison with data literature (Abu-Reidah et al., 2013b; Masala et al., 2024a; Pagano et al., 2016). Compound **37** was assigned to apigenin aglycone (C15H10O5), indicated by the pseudo-molecular ion $[M-H]^-$ at m/z 269.0456 and an additional peak at m/z 225, confirmed through literature comparisons (Abu-Reidah et al., 2013b; Masala et al., 2024a; Pagano et al., 2016). Compound 40 was recognized as velutin ($C_{17}H_{14}O_6$), showing a precursor ion [M+H]+ at m/z 315.08621 and a fragmentation ion at m/z 272, corroborated by literature and database comparisons (Eissa et al., 2020; S. Liu et al., 2016). Compound 41 was identified as 4,7-Dimethoxyflavonol (C17H14O5), presenting a precursor ion [M+H]+ at m/z 299.09216 and generating fragmentation ions at 256 and 167, confirmed by matching the fragmentation pattern with spectral databases.

Compound **37** was attributed to apigenin aglycone ($C_{15}H_{10}O_5$) due to the pseudo-molecular ion $[M-H]^-$ at m/z 269.0456 and secondary peak at m/z 225, as verified by comparison with data available in literature (Abu-Reidah et al., 2013b; Masala et al., 2024a; Pagano et al., 2016). Compound **40** was identified as velutin ($C_{17}H_{14}O_6$) giving a precursor ion $[M+H]^+$ at m/z 315.08621, giving a fragmentation ion at m/z 272, then confirmed by comparison with literature data and databases (Eissa et al., 2020; S. Liu et al., 2016). Compound **41** was identified as 4,7-Dimethoxyflavonol ($C_{17}H_{14}O_5$) giving a precursor ion $[M+H]^+$ at m/z 299.09216, and producing fragment ions at 256 and 167, then confirmed by comparing fragmentation pattern with spectral database.

*Other polar and non-polar compounds (**1**, **7**, **9**, **21**, **41**, **45**)*

Compound **1** was identified as Methylmalonic acid ($C_4H_6O_4$), producing a deprotonated molecular ion at m/z 117.02087 ($[M-H]^-$) along with secondary fragments at m/z 99 and 73 (loss of CO_2, 44 Da), verified through comparisons with spectral databases. Compound 7 was identified as 2-hydroxy-5-methoxybenzaldehyde, which yielded a $[M-H]^-$ ion at m/z 151.04106 and was confirmed via spectral comparisons (Caboni et al., 2013). Compound 9 was recognized as 4-hydroxy-N-methylbenzamide ($C_8H_9NO_2$) with a $[M-H]^-$ ion at m/z

150.05719, validated by checking against the spectral database. Compound 21 was tentatively identified as (+)-pinoresinol hexoside ($C_{26}H_{32}O_{11}$), indicated by the precursor ion $[M-H]^-$ at m/z 519.18829 and confirmed through literature data, which showed fragments at m/z 151 and 357 (Abu-Reidah et al., 2013a; Boldizsár et al., 2010; Masala et al., 2024b). Compound 41 was tentatively identified as (9Z)-5,8,11-Trihydroxyoctadec-9-enoic acid ($C_{18}H_{34}O_5$), producing the precursor ion $[M-H]^-$ at m/z 329.23486, confirmed through literature comparisons and exhibiting fragments at m/z 151 and 357 (Cerulli et al., 2024). Lastly, compound 42 was identified as laurylsulfuric acid ($C_{12}H_{26}O_4S$), producing a precursor ion [M-H]− at 265.14902 and exhibiting fragment ions at m/z 96 and 149.

5.2.4. Chemical Characterization of Tomato By-Products PLE Optimal Extract by UHPLC-C18-Q-TOF-MS/MS

Untargeted metabolomics analysis by UHPLC-Q-TOF MS/MS on TBP extracts obtained under optimized conditions. In Table 5.8 are shown the tentative identifications, their retention times, experimental mass, molecular formula and chemical families.

Table 5.8: Chemical profile of TBP PLE extracts obtained by UHPLC-C18-Q-TOF MS/MS ESI (−/+) analyses.

No	RT (min)	Tentative Metabolite name	$[M-H]^-$ (m/z) (theoretical)	$[M-H]^+$ (m/z) (theoretical)	Monoisotopic Mass	Δ ppm	Molecular Formula	Family	MS/MS (*m/z*, *relative abundance*)	Ref
43	1.012	3-Hydroxypicolinic acid	138.0209		139.02694	-3.35	C6H5NO3	Pyridine derivatives	94.02 (100); 66.03 (54)	database
44	1.757	Methyl DL-pyroglutamate		144.0657	143.058244	-1.09	C6H9NO3	Aminoacids derivatives	56.04 (100); 68.93 (51); 85.02 (37)	(Abu-Reidah et al., 2014)
45	3.585	Caffeic acid	179.0361	180,04339	180.04226	-2.28	C9H8O4	Hydroxycinnamic acids	80.02 (100); 53.87 (15); 125.20 (10)	database

46	4.399	4-Coumaric acid	163.0406		164.047345	-3.38	C9H8O3	Hydroxycinnamic acids	177.04 (100); 53.00 (16); 117.03 (8)	database
47	6.855	Feruloyltyramine		314.1384	313.131409	0.99	C18H19NO4	Hydroxycinnamic acids derivatives	77.04 (100); 91.05 (60); 145.02 (47); 121.06 (44)	(Pearce et al., 1998; Zeiss et al., 2019)
48	7.236	Quercetin	301.0363		302.0790.	1.5	C15H10O7	Flavanols	179.03 (100); 63.02 (69); 256.03 (55)	(Chileh-Chelh et al., 2024; Gouveia & Castilho, 2012)
49	7.785	Naringenin	271.0633	273.0758	272.068475	-0.24	C15H12O5	Flavonones	119.05 (100); 65.00 (42)	(Jeong et al., 2023)
50	7.993	(-)-Homoeriodictyol	301.0725		302.07904	-2.65	C16H14O6	Flavanones	108.02 (100); 65.00 (77); 134.03 (54); 136.01 (48)	(Iijima et al., 2008; M. Wang et al., 2023)
51	8.133	Kaempferol	285.0417		286.04774	-3.37	C15H10O6	Flavonols	151.00 (100); 229.05 (47) 257.04 (21)	(Otify et al., 2023)
52	8.148	Peimine		432.3467	431.339944	1.24	C27H45NO3	Steroidal alkaloids	161.12 (100); 414.33 (41); 109.1 (26)	(Z. Wang et al., 2018)
53	8.521	Tomatidine		416.3534	415.345029	-2.70	C27H45NO2	Steroidal alkaloids	161.13 (100); 398.50 (50); 255.20 (31); 70.06 (30)	(Cichon et al., 2017; Santonocito, et al., 2023)
54	8.595	Solasodine		414.3361	413.329379	1.35	C27H43NO2	Steroidal alkaloids	161.13 (100); 255.20 (38);	(Cataldi et al., 2005; Santonocito et

								398.33 (38)	al., 2023)
55	9.701	9-hydroxy-10E,12Z-octadecadienoic acid	295.2271 [H_2O]	294.219495	2.41	C18H30O3	Fatty acids derivatives	277.22 (100); 67.05 (50); 133.10 (25)	database

As well as for compounds identified in ABP, TBP extracts also displayed a wide range of bioactive molecules. These compounds are well-known to exert anti-inflammatory (Coelho, Rodrigues, et al., 2023; Elbadrawy & Sello, 2016; Perea-Domínguez et al., 2018; Salawu et al., 2020; Szabo et al., 2018, 2019) and anti-neurodegenerative effects (Błaszczak et al., 2020; Kapoor et al., 2023b; Lakey-Beitia et al., 2021; Salawu et al., 2020), contributing to the overall antioxidant capacity and bioactive content values measured by spectrophotometric assays. In total, 13 metabolites were tentatively identified in both ionization mode for TBP PLE extract, predominantly including hydroxycinnamic derivatives (e.g., caffeic and coumaric acids), flavonoids and derivatives (e.g., naringenin and (-)-homoeriodictyol), steroid alkaloids (e.g., tomatidine, peimine and solasodine), fatty acid derivatives such as octadecadienic acid, and other less abundant compounds. Identification of each bioactive compounds detected by UHPLC-Q-TOF MS/MS in TBP PLE extract is discussed in section 5.2.8.

The following part focuses on the discussion of tentatively identified metabolites in TBP pressurized liquid extracts obtained under optimized conditions.

*Hydroxycinnamic acid and derivatives (**46, 47, 48**)*

Compounds **46** and **47** were respectively identified as caffeic acid ($C_9H_8O_4$) and coumaric acid ($C_9H_8O_3$), presenting pseudo-molecular ions $[M-H]^-$ at m/z of 179.03609 and 163.0406, respectively. MS/MS fragmentation respectively provided fragment ions at m/z 80 and 77 then confirmed with specatral database comparison. Compound **48** was annotated as feruloyltyramine ($C_9H_8O_3$) presenting a pseudo-molecular ion $[M+H]^+$ at m/z 314.1384 with product ions at m/z 177 and 91, confirmed by literature (Pearce et al., 1998; Zeiss et al., 2019).

*Flavonoids and derivatives (**48, 49, 50, 51, 52**)*

Compounds **48 - 52** were all identified as common flavonoids' aglycone already characterized in TBP. In detail, compound **48** fragmentation generated characteristic ions tentatively attributed to quercetin ($C_{15}H_{10}O_7$), showing a singular MS^1 base peak $[M–H]^-$ presenting m/z at 301.0363 and a secondary fragment at *m/z* 179 (Chileh-Chelh et al., 2024; Gouveia & Castilho, 2012). Compound **49** was identified in both ionization mode as naringenin ($C_{15}H_{12}O_5$) presenting pseudo-molecular ion $[M-H]^-$ at m/z 271.0633 and $[M+H]^+$ at m/z 273.07584 and presenting MS^2 fragment ion at m/z 119 and 65, then confirmed by literature comparison (Jeong et al., 2023). Compound **50** was annotated as (-)-homoeriodictyol ($C_{16}H_{14}O_6$) presenting a precursor ion $[M-H]^-$ at m/z 301.07254, and providing fragment ions at m/z 108, 163 and 179 further confirmed by comparison with literature (Iijima et al., 2008; M. Wang et al., 2023). Compound **51** was identified as kaempferol ($C_{15}H_{10}O_6$), another well-known tomato-related flavonoid, with a parent ion $[M-H]^-$ at m/z 285.0417, providing most abundant fragment ions at m/z the fragments at m/z 51, 229, and 257. Specifically, identification was performed by spectral database comparison and further confirmed by comparison with literature data (Otify et al., 2023).

*Steroidal alkaloids (**52, 53, 54**)*

Compounds **52** - **54** were all identified in positive ionization mode as steroid alkaloids derivatives, phytochemicals that act against herbivores, pathogens, and environmental stress, that recently have gained attention thanks to their antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (Santonocito, Campisi, et al., 2023). Compound **52** was identified as peimine ($C_{27}H_{45}NO_3$) presenting pseudo-molecular ion $[M+H]^+$ at m/z 432.3467 and a characteristic base peak at m/z 161 and product ion at m/z 414, then confirmed with literature data (Z. Wang et al., 2018). Compound **53** was identified as tomatidine ($C_{27}H_{45}NO_2$) presenting parent ion $[M+H]^+$ at m/z 416.35345, providing fragment ions at m/z 398 and 255 (Cichon et al., 2017; Santonocito, et al., 2023). Compound **55** was identified as solasodine ($C_{27}H_{43}NO_2$) presenting parent ion $[M+H]^+$ at m/z 414.33612, producing fragments at m/z 398 and 255 and confirmed by comparison with literature data (Cataldi et al., 2005; Santonocito et al., 2023).

*Other polar and non-polar compounds (**43, 44, 55**)*

Pyridines derivates were also detected in TPB PLE extract: (**43**) 3-hydroxypicolinic acid ($C_6H_5NO_3$) providing pseudo-molecular ions $[M+H]^+$ at m/z 110.06059 and ion $[M-H]^-$ and producing fragment ion at m/z 94, confirmed by spectral database. Amino acids derivatives were also observed. Specifically, compound **44** was identified as methyl-pyroglutamate ($C_6H_9NO_3$) with a precursor ion $[M+H]^+$ at m/z 144.0657, showed product ions at 56 and 68 and confirmed by comparison with literature (Abu-Reidah et al., 2014). Compounds **55** was identified as 9-hydroxy-10E,12Z-octadecadienoic acid ($C_{18}H_{30}O_3$) with a precursor ion $[M+H-H_2O]^+$ at m/z 256.26404 and presenting fragment ion at m/z 277, then confirmed by comparison with spectral database.

5.2.5. Molecular docking

The molecular docking approach is considered an effective tool for identifying specific ligands through the measurement of binding affinity scores with enzymes' active sites. Molecular docking simulations were performed to explore the binding affinity scores of three complexes between AChE, BChE, and LOX and two of the most abundant flavonoids identified in ABP and TBP PLE extracts: apigenin and naringenin. The analysis revealed several possible complexes based on the different flavonoid orientations within the enzyme active sites. Figure 5.4 compares docking complexes and binding affinity scores involving positive controls (galantamine for cholinergic enzymes; quercetin for lipoxygenase) and apigenin. In general, each complex assessed showed good binding affinity scores, suggesting that several interactions within the active sites of the cholinergic enzymes mainly induced by phenolic and flavonoid compounds can promote inhibitory effects. Comparing the binding energy scores of the cholinesterase-galantamine (Fig. 5.4a) and cholinesterase-apigenin (Fig. 5.4d) complexes, the two demonstrated distinct binding affinities, providing better binding scores when apigenin was docked (-8.2 kcal/mol). A hydrogen bond interaction was observed with the residual amino acids TYR121 (peripheral anionic site) and π–π stacking interactions with amino acids TRP83 (catalytic active site) and TYR332 (peripheral binding site).

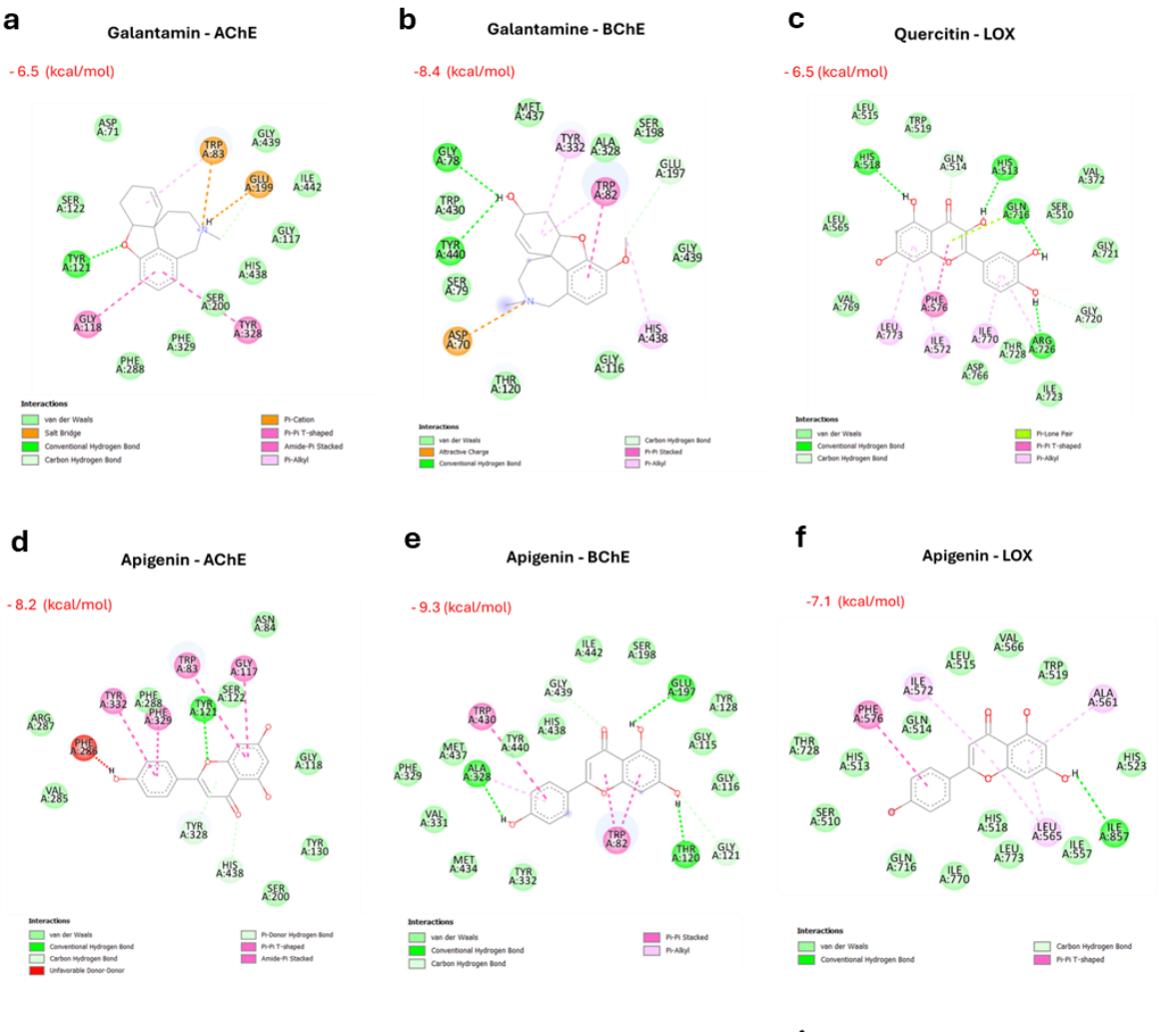


Figure 5.4: Molecular docking simulations of Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE) and Lipoxygenase (LOX) with corresponding positive controls (galantamine for cholinergic enzymes, quercitine for LOX) and apigenin identified in ABP PLE extract. (a) AChE-galantamine; (b) BChE-galantamine; (c) LOX-quercetin; (d) AChE-apigenin; (e) BChE-apigenin; (f) LOX-apigenin

Unfavourable bonds were also observed between PHE286 and the B-ring hydroxyl group. These structural features probably allow enhanced interactions with relatively polar ligands such as apigenin. For the BChE-apigenin complex, docking simulations provided even lower binding scores (-9.3 kcal/mol) than docking with its positive control, suggesting that the flavonoid structure effectively interacts with the active site amino acid residues. Differences in binding affinity scores may be attributed to differences in the active sites, which are in the deep gorge of the two cholinergic enzymes, associated with the BChE catalytic site, which contains fewer hydrophobic aromatic amino acid residues (Nordberg et al., 2013; Sharma et al., 2019). Comparable interactions were mediated by hydrogen bonds, facilitated by the hydroxyl groups of flavonoids and the amino acid residues of the BChE catalytic site

(GLY115, TYR128, and GLU197). Regarding LOX complexes, docking simulations involving apigenin revealed comparable binding affinity scores, as this flavonoid shares similarities in terms of chemical structure with quercetin, which was used as a reference (Fig 5.4c and f). A similar docking approach was employed to assess the binding affinity scores of naringenin with the selected enzyme, and the results are displayed in Figure 5.5. The docking results for naringenin displayed similar binding affinity scores across the selected enzymes (AChE, BChE, and LOX). This supports the hypothesis that the shared chemical structure between these flavonoids contributes to analogous interaction patterns with the enzyme active sites. In the AChE complex, comparable interactions were observed involving TYR residues that mediated hydrogen bond interactions, as well as for AChE-apigenin (see Fig. 5.4a, d). Staking interactions mediated by several amino acids' residues such as PHE, TRP, and HIS were found to stabilize naringenin inside the active site of BChE, allowing better binding affinity scores (see Fig. 5.5b, e).

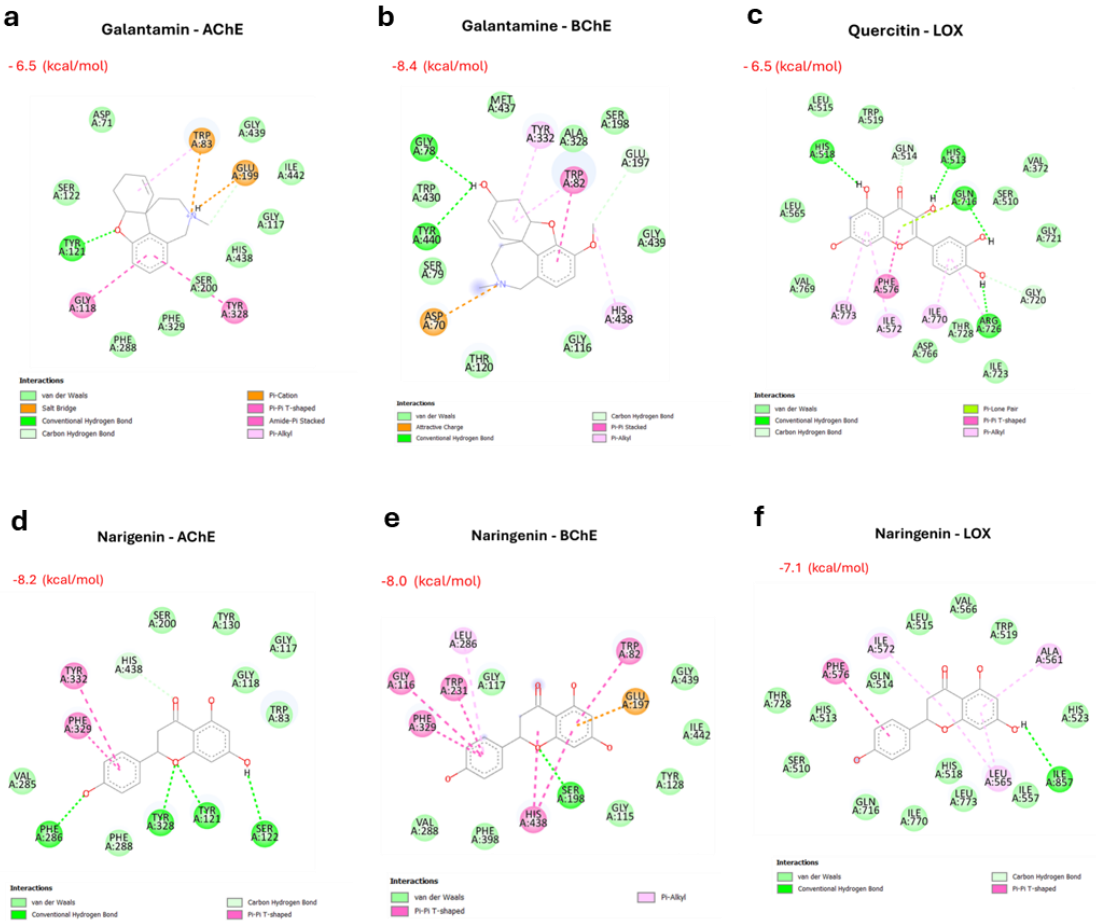


Figure 5.5: Molecular docking simulations of Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE) and Lipoxygenase (LOX) with corresponding positive controls (galantamine for cholinergic

enzyme, quercitine for LOX) and naringenin identified in TBP PLE extract. (a) AChE-galantamine; (b) BChE-galantamine; (c) LOX-quercetin; (d) AChE-naringenin; (e) BChE-naringenin; (f) LOX-naringenin.

The LOX-naringenin complex provided the same scores observed for the complex with apigenin, suggesting that the interactions occurring between flavonoids and enzyme active sites were the same. Most of the interactions described in this study, such as hydrogen bonds and π-π interactions, are reported to reinforce the inhibitory potential of bioactive compounds such as flavonoids against enzymes involved in degenerative disorders. For instance, other bioactive compounds, including coumarin derivatives and flavonoids from different sources, share similarities in chemical structures, thus promoting an inhibitory effect on this enzyme (Li et al., 2023). Since extracts derived from industrial by-products are complex mixtures containing diverse classes of metabolites, these compounds possess chemical groups that can interact through synergistic or antagonistic mechanisms, thereby mediating beneficial biological effects (Olech et al., 2020). These compounds were obtained from agro-food by-products, which are still largely considered unexploited materials. In this regard, their potential reuse within the framework of a circular economy may be essential to maximizing their value while reducing waste and environmental impact.

5.2.6. Conclusions

This research presents a sustainable approach for the valorization of agro-food by-product obtained from processed artichokes (ABP) and tomatoes (TBP) through green pressurized liquid extraction (PLE). By optimizing solvent compositions and temperature via response surface methodology, the most effective conditions - ethanol:ethyl acetate (10:90 v/v) for ABP and 100% CPME for TBP at 180 °C - were determined to enhance the recovery of phenolics and flavonoids. These conditions significantly improved antioxidant capacity, as demonstrated by DPPH and ORAC assays, and maximized the total phenolic and flavonoid content determined by in vitro assays. Untargeted UHPLC-Q-TOF-MS/MS metabolomics revealed a complex chemical fingerprint for both matrices. In ABP, over 40 metabolites were annotated, including canonical hydroxycinnamic acids (e.g., chlorogenic and cynarin), and previously unreported lactone and ethyl caffeoylquinic acid derivatives likely formed under high temperatures and pressures conditions. Similarly, flavonoids such as apigenin, luteolin,

and their glycosides were prominent, contributing to the observed in vitro activities and well-documented for their antioxidant, anti-inflammatory, and neuroprotective functions. Finally, molecular docking revealed characteristic interactions of the most abundant flavonoid identified in ABP and TBP: apigenin and naringenin, respectively. The results demonstrated high affinity for the active site of key enzymes implicated in neurodegenerative processes. The findings demonstrate agri-food by-products represent a valuable resource of bioactive compounds with high antioxidant potential to be potentially reintroduced into new production cycle.

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Chapter 6

POTENTIAL APPLICATION FOR AGRO-FOOD BY-PRODUCTS VALORIZATION

This chapter consolidates insights from multiple studies on the potential applications of agro-food industrial residues, such as artichoke (ABP) and tomato by-products (TBP), as valuable source of bioactive compounds. These matrices, often discarded, were explored for their potential in nutraceuticals, biomedicine, food industries, and sustainable packaging, aiming to deliver functional applications with enhanced economic and ecological value. The valorization of agro-food by-products within the framework of a circular economy represents an innovative and sustainable approach to addressing environmental challenges and maximizing resource efficiency.

The first study investigated the nutraceutical potential of pasta enriched with artichoke-based flour. Analytical characterization using HPAEC-PAD identified and quantified oligosaccharides with prebiotic properties. Functional food products, such as pasta enriched with ABP-derived flour, demonstrated enhanced nutritional profiles and offered innovative solutions for promoting health through diet, showcasing agro-food byproducts' transformative potential in supporting a circular economy.

The second study investigated the in vitro neuroprotective potential and permeability performance of bioactive compounds extracted via pressurised liquid extraction (PLE) from artichoke (ABP) and tomato by-products (TBP). The outcomes from Chapter 5, which revealed high phenolic and flavonoid contents and significant antioxidant activities, were then used to perform in vitro enzymatic inhibition assays against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and lipoxygenase (LOX). These enzymes have been implicated in Alzheimer's disease pathology. The results demonstrated notable neuroprotective activities. In order to further evaluate the ability of these bioactive compounds to reach the target site, the Parallel Artificial Membrane Permeability Assay for the Blood-Brain Barrier (PAMPA-BBB) was employed. This assay assessed their potential to cross an artificial blood-brain barrier, underscoring their neuroprotective promise. The findings were further corroborated by untargeted metabolomic analyses using UHPLC-Q-

TOF-MS/MS, which identified key bioactive constituents with potent antioxidant and anti-inflammatory properties. Taken together, these results underscore the potential of agro-food by-products as a source of neuroprotective compounds with implications for Alzheimer's disease and other neurodegenerative disorders.

A further study examined the encapsulation of carotenoid-rich extracts from tomato waste by-products using state-of-the-art technologies, such as spray-drying and liposome-based methods, with the aim of improving bioavailability and oxidative stability during storage. Liposome-based systems, fortified with inulin, exhibited enhanced preservation of bioactive compounds including carotenoids and terpenoids, which contributes to antioxidant and neuroprotective properties. These findings lend further support to the application of these technologies in the development of functional food products. Another application focused on the development of different bio-based spray formulations enriched with tomato by-product extracts and Vitamin E for active packaging solutions, with the spray application improving the oxidative stability and extending the shelf life of various food products, including meat and vegetable-based items. Oxidative stability assessments and antioxidant capacity tests demonstrated the efficacy of these formulations in mitigating degradation promoted by oxidation processes. The development of biobased active sprays and films for enhancing the shelf life of food is actively being pursued through the “BEST” and the “ECOSISTER” projects, funded by PNRR. The goal is to improve their effectiveness and expand their potential applications in sustainable active packaging systems.

The final study explored tomato by-product powders as a biobased component in wax-based coatings to improve the hydrophobicity of cellulose-based materials. Blends of wax and tomato by-products reduced water absorption and increased contact angles, showcasing their potential in sustainable packaging applications. The addition of tomato by-products, rich in hydrophobic compounds like cutin, contributed to the improved performance of these coatings, offering an eco-friendly alternative to conventional materials. This research on coatings was carried out in collaboration with the German company “Krones” and is now the focus of a PNRR BAC (Bando a Cascata) project of the "MICS, Made in Italy Circolare e Sostenibile” titled “SEAL - Sviluppo di materiali Ecosostenibili per Accessori di stiLe", further advancing the development of innovative and sustainable packaging solutions.

Collectively, these studies highlighted the feasibility and benefits of agro-food by-product valorization, underscoring their role in promoting sustainability, enhancing product functionality, and supporting the circular economy.

6.1. Application in Nutraceutical Field: Artichoke-Based Flour as Natural Ingredient for Functional Food Production

This study is part of the Fil.Pa.Nu project based on a PSR Sicilia funded project leaded by University of Catania (https://carciofiamo.it/fil-pa-nu/). It was aimed at producing nutraceutical pasta enriched with artichoke-based flour, rich in prebiotic compounds. Analysis on carbohydrates fraction confirmed the suitability of artichoke-based samples as a natural ingredient to enhance nutritional properties of food items, thereby promoting a circular economy approach by valorizing agricultural waste. This approach addresses the dual objectives of environmental sustainability and consumer health, establishing a foundation for future functional food innovations.

6.1.1. Materials and Methods

The materials and methods used in this section are consistent with those detailed in Section 3.1.

6.1.1.1. Samples

Artichoke-based samples were collected by University of Catania from the production chain for nutraceutical pasta enriched with their flour (see Table 6.1).

Table 6.1: Samples of artichoke by-products (ABP) collected from Fil.Pa.Nu project

Sample	Description
Liquid 0CV	Juice from blanching
Dry 0CV	Dried artichoke heads
1CV	Artichoke hearts treated with antioxidants solutions

Pasta	Final products: pasta enriched with artichoke-based flour (10% w/w)

6.1.2. Results and Discussion

6.1.2.1. Carbohydrate analysis of pasta enriched with artichoke flour

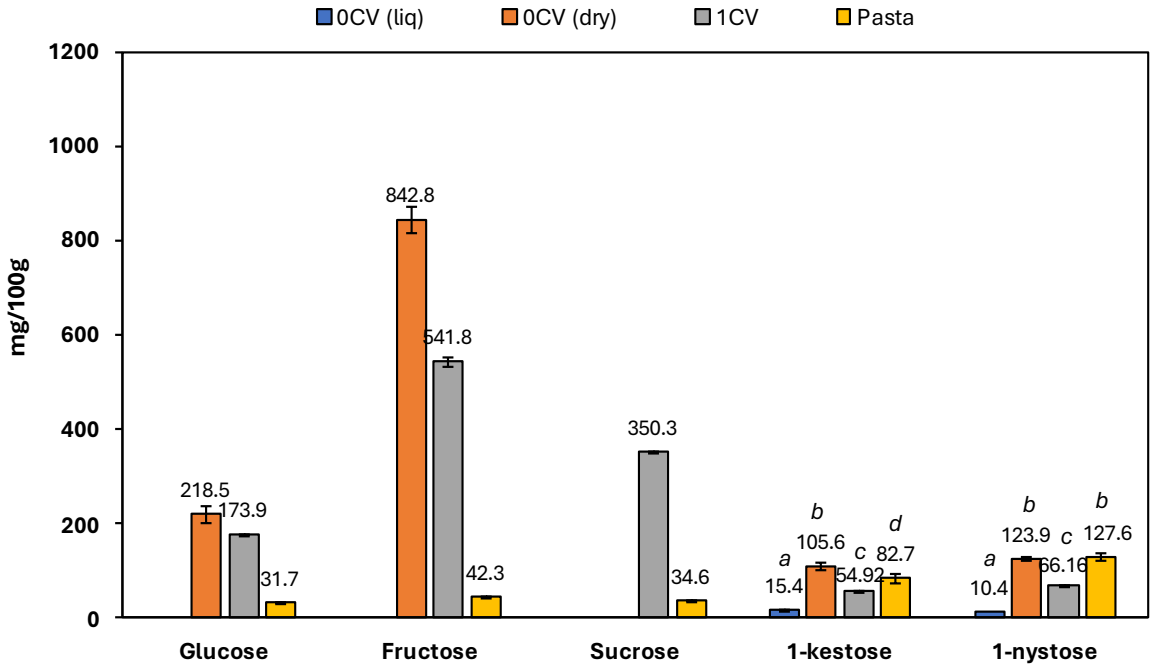
Figure 6.1 illustrates schematic exemplification of nutraceutical pasta production in Fil.Pa.Nu project.

Figure 6.1: Schematic representation of Fil.Pa.Nu production chain.

Several matrices were collected from different sampling points along the transformation chain of *Violetto Ramacchese*, a native variety of Sicily (Italy). Each sample was evaluated for its sugar contents on 100 g of dry-weight samples, and the results are reported in Figure 6.2. Significant differences ($p < 0.05$) in sugar quantification for each sample were observed. Simple sugars such as glucose, fructose, and sucrose were readily quantified in the samples, providing the highest content in dry 0CV (artichokes' head dried and freeze-stored) and 1CV (artichokes' heart cut in half). On the other hand, these sugars were not detected in the extraction juice generated after the boiling step (liquid 0CV). Previous research reported that glucose, fructose, and sucrose in artichoke heads ranged from 12.9 to 71.7 g/kg dry weight. However, these were highly variable depending on cultivar and processing methods (Schütz et al., 2006). As reported in Figure 6.2, related to FOSs quantification, 1-kestose and 1-nystose contents showed high variation across all the samples. The lowest FOSs content was provided by 0CV (liq) and 1CV. In the case of AF-supplemented and 0CV (dry), no

significant difference was observed for each oligosaccharide content. Moreover, AF-supplemented pasta demonstrates a potentially higher FOS content compared to the extraction juice (CV liquid) and shows no significant differences ($p < 0.05$) when compared to dried artichoke heads.

Figure 6.2: (a) Quantification (express as mean ± sd) of polyols, monosaccharides and



oligosaccharides in artichoke samples obtained from technological treatment for the manufacturing of nutraceutical pasta (b) quantification of 1-kestose and 1-nystose.

This is likely because its production involved blending the same extraction juice, flour obtained artichoke residues (collected from other technological treatment points), which are rich in inulin and FOSs, with wheat flour and water to reach proper technological parameters. Incorporating these ingredients into pasta formulations has shown promising results, including increased dietary fiber content, reduced glycemic index, and improved antioxidant activity. An oral dose of at least 3 g/day of fibers like FOS is recommended to confer a nutraceutical benefit (Gibson et al., 2017; Krumbeck et al., 2016; Varney et al., 2017). The sum of 1-ketose and 1-nystose resulting in AF-supplemented pasta was over 230 mg/100g, thus indicating the enhanced prebiotic properties conferred to pasta products.

6.2. Application in Biomedical Field: Evaluation of *in vitro* Neuroprotective Potential and Permeability Performance Across Artificial Blood-Brain Barrier (BBB)

This study was conducted during a period of research visit to CIAL Institute (Madrid, Spain) in the Foodomics research group, and forms part of the work described in Chapter 5.

The objective of this study was to investigate the in vitro evaluation of the neuroprotective potential and permeability performances of bioactive compounds extracted from artichoke (ABPs) and tomato (TBPs) by-products across an artificial blood-brain barrier (BBB). This investigation was undertaken to simulate their potential for neuroprotective application. In order to this end, a series of enzyme inhibition assays were conducted against Acetylcholinesterase (AChE), Butyrylcholinesterase (BChE), and Lipoxygenase (LOX). These enzymes have been linked to neurodegenerative disorders such as Alzheimer's disease. The results for agro-food by-product PLE extracts, obtained under optimised conditions, demonstrated notable in vitro neuroprotective potential.

To assess the capacity of the extracted compounds to cross the BBB, a Parallel Artificial Membrane Permeability Assay – Blood Brain Barrier (PAMPA-BBB) was performed. This assay is regarded as one of the most prevalent in drug discovery, owing to its robust and high-throughput nature (see Figure 6.3). This artificial model is indeed useful in assessing the transport efficiency of these bioactive compounds, demonstrating the ability to permeate across the barrier under physiological conditions (see Figure 6.3). UHPLC-Q-TOF-MS/MS analyses were conducted for the identification of key bioactive compounds, such as phenolic acids and flavonoids, recognised for their antioxidant and anti-inflammatory properties. It is noteworthy that phenolic compounds derived from ABPs, including caffeoylquinic acids, exhibited enhanced permeability and retention when compared to compounds derived from TBPs. These findings underscore the potential for ABP and TBP extracts to play a pivotal role in the field of neuroprotection, particularly in the context of mitigating oxidative stress-related conditions. This evaluation underscores the significance of combining bioactive profiling with permeability assays to understand the therapeutic potential of food-derived compounds in targeting the central nervous system. The results highlighted that ABPs and TBPs could be useful in nutraceuticals designed for brain health, offering an environmentally sustainable and health-focused innovation pathway.

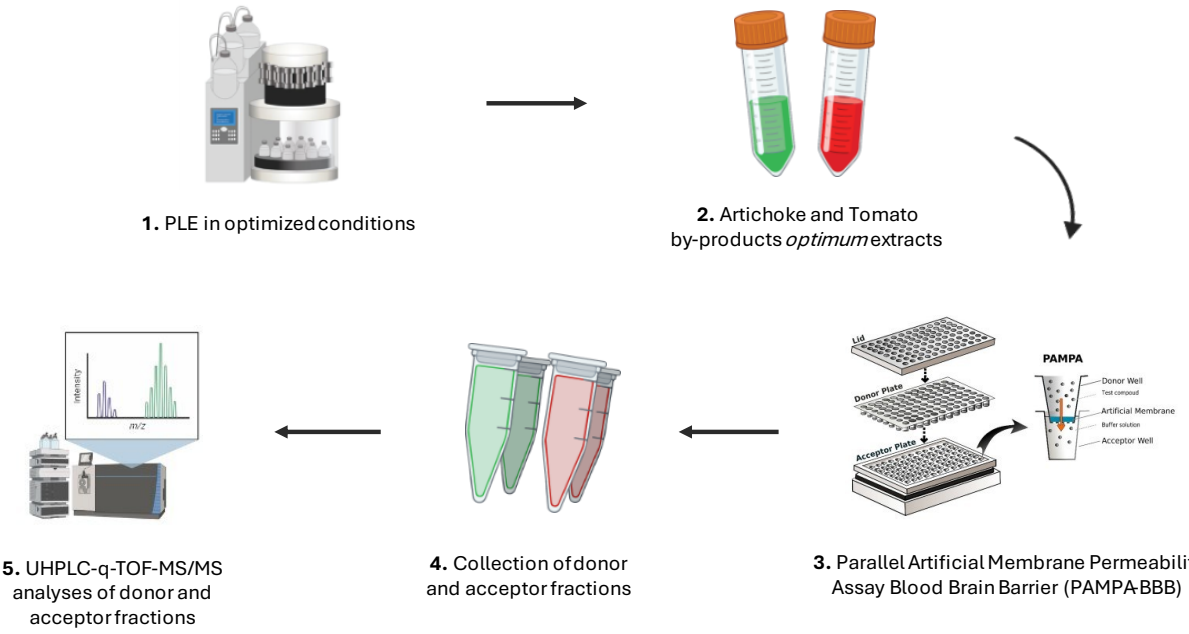


Figure 6.3: Exemplified protocol of Parallel Artificial Permeability Assays – Blood Brain Barrier (PAMPA-BBB) on PLE ABP and TBP extract obtained under optimized conditions

6.2.1. Materials and methods

The materials and methods employed in this study are consistent with those detailed in Chapter 5, Section 5.1. For clarity and coherence, the experimental procedures and protocols are reported in the previous chapter.

6.2.1.1. Pressurized Liquid Extraction (PLE)

The conditions employed for pressurized liquid extraction are consistent with those reported in the Chapter 5, Section 5.1.3.

6.2.1.2. Anti-cholinergic inhibitory assays

The inhibitory activities of artichoke by-products from PLE extracts were assessed using an enzymatic inhibition assay for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), following the fluorescent enzyme kinetic method proposed by Sanchez-Martinez et al. (2021), with some modifications.

In this assay, the inhibitory capacities of the extracts were measured using fluorescent enzyme kinetics. Specifically, 5,5'-di-thio-bis(2-nitrobenzoic acid) (DNTB) reagent was added to each well. Each well was filled with 100 µL of the extract sample at varying concentrations (150 – 1500 µg/mL) in ethanol, along with 100 µL of a buffer solution (150 mM Tris-HCl, pH 8) and 25 µL of 0.8 U/mL AChE or BChE in the buffer. The mixture was incubated for 10 minutes.

The reaction was initiated by adding 25 µL of ABD-F (125 µM) in buffer and 50 µL of ATCI at a concentration equal to the Michaelis–Menten constant (K_M) value in water. Fluorescence was measured at an excitation wavelength of 389 nm and an emission wavelength of 513 nm every minute for 10 minutes at 37 °C. This kinetic measurement was necessary to obtain the V_{mean} value, which corresponds to the mean enzymatic velocity during the measurement. The percentage of inhibition degree (ID%) was calculated through equation (1):

$$ID\% = \frac{V_0 - V_1}{V_0} \times 100 \qquad (1)$$

Where V_0 and V_1 represent the mean reaction rates of enzyme kinetics without and with the extracted sample, respectively, galantamine hydrobromide in pure EtOH was used as the reference inhibitor for both enzymes, while pure EtOH served as a blank control. The K_M value was determined by mixing 100 µL of ATCI at varying concentrations (0.4 - 4 mM) in MilliQ water with 50 µL of pure EtOH and 100 µL of buffer. The reaction was initiated by adding 25 µL of ABD-F (125 µM) in buffer and 25 µL of 0.8 U/mL AChE or BChE in buffer to each well. Mean reaction rates (V_{mean}) and K_M values were calculated using Gen5™ version 2.0 Data Analysis software (BioTek Instruments, Winooski, VT, USA).

6.2.1.3. Lipoxygenase (LOX) inhibitory assays

The LOX inhibitory capacity (i.e. the capacity to inhibit LOX enzymes) of ABP and TBP was determined using a modified version of the protocol proposed by Sánchez-Martínez et al. (2021). The inhibition activity was measured through a fluorescence assay based on enzyme kinetics. The assay comprised 100 µL of extract sample at varying concentrations (100 – 1000 µg/mL) in pure EtOH, 75 µL of fluorescein (1 µM) in buffer (150 mM Tris-HCl, pH = 9), 60

µL of LOX208 U/µL in buffer and linoleic acid (in a concentration corresponding to the K_M value) in pure EtOH, in each well. The fluorescence measurements were recorded at a wavelength of 485 nm for excitation and 530 nm for emission, with a frequency of once per minute for a period of 15 minutes at a temperature of 25°C. The ID% was calculated using the same equation (2) described in the previous section. The K_M value was measured by mixing 100 µL of linoleic acid (6.5 mM) in EtOH, 100 µL of EtOH, 75 µL of fluorescein (1 µM) in buffer and 60 µL of LOX 208 U/µL in buffer, in each well. Quercetin was utilised as the reference inhibitor, while pure EtOH was employed as the blank control.

6.2.1.4. Parallel artificial membrane permeability assay for the blood–brain barrier (PAMPA-BBB)

The PAMPA-BBB in vitro assay was performed in accordance with the protocol described by Sánchez-Martínez et al. (2022). Briefly, 8 mg of porcine brain lipid (PBL) and 4 mg of cholesterol were dissolved in 600 µL of n-dodecane to prepare the BBB solution. A volume of 5 µL of this lipid mixture was carefully applied to the filter membrane of each well in the donor plate to simulate the blood–brain barrier. he donor solution was prepared by mixing 1 mL of plant extract (10 mg/mL in ethanol) with 1 mL of phosphate-buffered saline (PBS, pH 7.4, 10 mM), yielding a total volume of 2 mL. The acceptor plate was filled with 350 µL of PBS buffer in each well, after which the donor plate was gently placed over the acceptor plate, forming a sandwich-like configuration. Subsequently, 350 µL of the donor solution was added to each donor well (upper plate). The assembled plates were then sealed and incubated in the dark at 37 °C for 5 hours under 37% relative humidity. Following incubation, 300 µL from each well of both the donor and acceptor plates was collected, transferred to separate vials, and dried using a SpeedVac system at 40 °C and 13 mBar pressure. The dried residues were reconstituted in 50 µL of pure ethanol and analyzed using UHPLC-Q-TOF-MS, as described in the previous section. Additionally, physicochemical parameters of the detected bioactive compounds - such as the octanol–water partition coefficient (LogP) and topological polar surface area (TPSA) - were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) to support interpretation of their permeability across the artificial membrane.

The in vitro permeability across an artificial BBB is calculated based on the following equation (2), as previously reported by Sánchez-Martínez et al. (2022):

$$P_e = \frac{-\ln\left[1-\frac{C_A(t)}{C_e}\right]}{A\times\left(\frac{1}{V_D}+\frac{1}{V_A}\right)\times t} \qquad (2)$$

In this equation, P_e refers to the permeability of the bioactive compound across PAMPA-BBB in cm/s; A is the effective filter area, which is given by the manufacturer and is 0.3 cm^2; V_D is the donor well volume, which is 0.35 mL; and V_A is the acceptor well volume, which is 0.35 mL; t is the incubation time (s) = 14.400; $C_A(t)$ is the relative area of the compound in acceptor well at time t; $C_D(t)$ is the relative area of the compound in donor well at time t. The equilibrium was calculated according to the following equation (3):

$$C_{equilibrium} = \frac{\left[C_{D(t)}V_D + C_{A(t)}V_A\right]}{(V_D+V_A)} \qquad (3)$$

6.2.2. Results and Discussion

6.2.2.1. In vitro neuroprotective potential evaluation

Acetylcholinesterase (AChE) is a key enzyme involved in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, playing a central role in their pathophysiology (Crous-Bou et al., 2017). Clinical studies have demonstrated the effectiveness of AChE inhibitors in mitigating Alzheimer's symptoms. AChE, along with butyrylcholinesterase (BChE), hydrolyzes acetylcholine, a neurotransmitter critical for cognitive functions (Nordberg et al., 2013). In Alzheimer's, BChE activity was found to increase significantly (40–90%) in affected brain regions, contributing to amyloid β-peptide aggregation and accelerating disease progression (Sridhar & Gumpeny, 2024).

To assess the neuroprotective potential of the ABP and TBP extracts, the IC_{50} values were measured based on the inhibition assay of AChE and BChE enzymes. Lipoxygenase (LOX) inhibition was also performed to evaluate anti-inflammatory ability of agro-food by-products extracts. Lower IC_{50} values relates to a stronger neuroprotective and anti-inflammatory efficacy. In Figure 6.4 are shown the IC_{50} values of ABP and TBP Optimal extract for AChE and BChE inhibition assays.

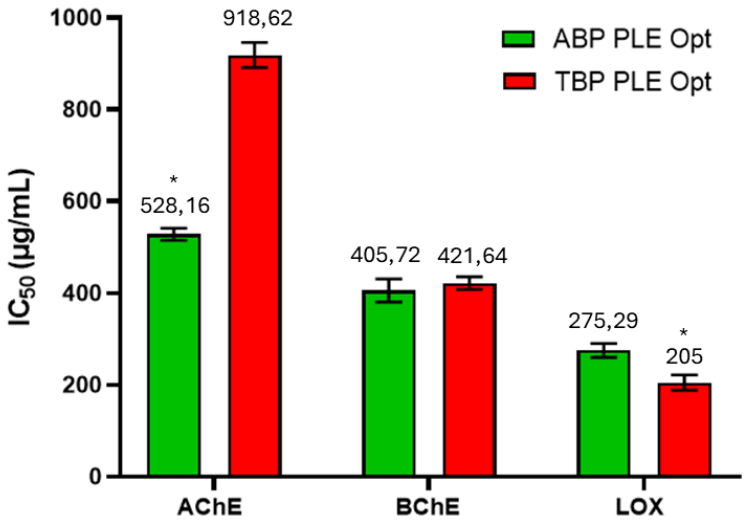


Figura 6.4: Neuroprotective potential evaluation by inhibition assays of AChE, BChE, and LOX for artichoke by-products (ABP) and tomato by-products (TBP) PLE Optimal extract. Galantamine was used as positive control for AChE (IC_{50} = 0.8 ± 0.2) and BChE (IC50 = 3.6 ± 0.2) inhibition; quercetin was used as positive control for LOX inhibition (IC_{50} = 12.3 ± 0.5). Significant differences between ABP PLE Opt and TBP PLE Opt for the same assay ($p < 0.05$) are indicated by an asterisk (*).

Data revealed ABP PLE extract outperformed TBP one in terms of AChE inhibition (IC_{50} = 528.2 µg/mL vs. 918.6 µg/mL), highlighting stronger potential exerted by the bioactive compounds occurring in ABP in mitigating cholinergic dysfunction, a hallmark of Alzheimer's disease. On the other hand, both extracts exhibited comparable BChE inhibition providing no significant differences observed, suggesting comparable efficacy in modulating complementary enzyme of cholinergic pathways. These findings were in accordance with previous research demonstrating that phenolic compounds, such as chlorogenic acid and 3,5-di-O-caffeoylquinic acid (also identified in ABP PLE extract), contribute to anti-cholinesterase activity, with 3,5-di-O-caffeoylquinic acid exhibiting strong AChE inhibition potential (Iglesias-Carres et al., 2023). Furthermore, flavonoids like quercetin and luteolin, abundant in ABP, were found to effectively block cholinesterase active sites, as reported by Rejeb et al. (Rejeb et al., 2020). Other studies also highlighted correlations of chlorophylls and carotenoids, also present in ABP and TBP extracts, with in vitro AChE and BChE inhibition, supporting their potential in neuroprotective treatments (Turkiewicz et al., 2019).

Concerning the anti-inflammatory potential, ABP PLE extracts provided better performances in terms of LOX inhibition (IC_{50} = 275.3 µg/mL vs. 205 µg/mL), demonstrating its higher *in vitro*

anti-inflammatory capacity, which is considered critical for alleviate oxidative stress and inflammation-induced disease. As previously reported, several anti-inflammatory effects may be promoted by flavonoids, such as apigenin, quercetin, and naringenin, which were found to inhibit LOX active site through their structural features like ortho-dihydroxyl groups and other specific functional groups (Ribeiro et al., 2020; Rondanelli et al., 2019; Sinha et al., 2019).

Although agro-industrial by-product extracts generally exhibit reduced inhibition potential compared to their corresponding commercialized products (compounds loss during processing), the optimized PLE extracts demonstrated remarkable neuroprotective potential. This retention of bioactive compounds highlights the importance of refining extraction and processing methodologies to preserve their functional properties. By optimizing these processes, agro-industrial by-products can be reused into sustainable and renewable sources of health-promoting substances, offering significant opportunities for industrial applications and the valorization of agro-food by-products and waste.

6.2.2.2. Blood Brain Barrier (BBB) *in vitro* Permeability Evaluation of Agro-Food By-Products

The blood-brain barrier (BBB) represents one of the main structures protecting the central nervous system from potentially harmful substances, while allowing selectively crossing essential biomolecules. Evaluating the permeability of bioactive compounds across the BBB is crucial in neurotherapeutic research, as the efficacy of potential drugs depends on their ability to reach target sites within the brain. Cholinesterase and lipoxygenase inhibitors require strong lipophilic abilities to diffuse across the BBB, promoting the inhibition of the enzyme. In this study, in vitro parallel artificial membrane permeability assay for the blood–brain barrier (PAMPA-BBB) was employed to explore bioactive compounds' permeability potential in artichoke and tomato industrial by-products to cross the artificial BBB layer. Previous studies highlighted the significant influence of molecular weight (M_W) and lipophilicity (LogP) parameters on permeability performances. In this regard, these factors were considered in our investigation. Table 6.2 displays the experimental permeability coefficients (Log Pe, calculated as described in Section 6.2.1.3), lipophilicity values (LogP, obtained from PubChem), and the chemical families of compounds capable of crossing the artificial blood-brain barrier (BBB).

Table 6.2: LogP and experimental PAMPA-BBB values of bioactive compounds identified in ABP and TBP PLE extracts obtained under optimized conditions.

Sample	Metabolite name	$[M-H]^-$ /$[M+H]^{+*}$ (m/z)	Family	LogP	PAMPA-BBB Log (Pe) (cm/s)	RSD Log (Pe) (%)	Cross BBB potential
ABP	Apigenin	269.04727	Flavones	2.49	-3.88	0.30	+++
ABP	Protocatechuic acid	153.02046	Hydroxybenzoic acid	0.40	-4.09	0.08	+++
ABP	Laurylsulfuric acid	265.14902	Fatty acid derivatives	1.96	-4.18	0.28	+++
ABP	4-Hydroxy-N-methylbenzamide	150.05719	Hydroxyphenols	1.12	-4.21	0.06	+++
TBP	Naringenin	271.06332	Flavanones	2.4	-4.30	0.40	+++
TBP	Acacetin	283.06271	Flavones	2.1	-4.39	0.70	+++
TBP	9-hydroxy-10E,12Z-octadecadienoic acid	295.22888	Fatty acids derivatives	5.3	-4.41	0.80	+++
ABP	Ethyl caffeate	161.02574	Hydroxycinnamic acids	1.38	-4.42	0.86	+++
ABP	Quercetin	301.03635	Flavonols	1.50	-4.50	1.09	+++
TBP	(-)-Homoeriodictyol	301.07254	Flavanones	2.4	-4.51	0.20	++
TBP	Kaempferol	285.04169	Flavonols	1.9	-4.53	0.26	++
ABP	Luteolin	285.0419	Flavones	2.12	-4.64	0.91	++
TBP	Coumaric acid	163.0406	Hydroxycinnamic acids	1.5	-4.61	0.77	++
ABP	2-Hydroxy-5-methoxybenzaldehyde	151.04106	Hydroxyphenols	1.58	-5.06	0.25	++
TBP	3-Hydroxypicolinic acid	138.02094	Pyridine derivatives	1.3	-5.11	0.99	++
ABP	3-Caffeoylquinic acid lactone	161.02567	Hydroxycinnamic acids	-0.10	-5.25	0.50	++
ABP	Neochlorogenic acid	353.08905	Hydroxycinnamic acids	-0.10	-5.38	0.94	++
ABP	Chlorogenic acid	353.08957	Hydroxycinnamic acids	-0.10	-5.39	0.47	++
ABP	3-Caffeoylquinic acid methyl ester	367.10489	Hydroxycinnamic acids	0.30	-5.56	1.28	+
ABP	4-Caffeoylquinic acid lactone	335.07864	Hydroxycinnamic acids	-0.10	-5.67	0.26	+

ABP	1,4-Dicaffeoylquinic acid	515.12085	Hydroxycinnamic acids	-0.10	-5.88	0.15	+
ABP	3-Caffeoylquinic acid ethyl ester	381.12076	Hydroxycinnamic acids	0.70	-5.95	0.74	+
ABP	1-Caffeoylquinic acid ethyl ester	381.12082	Hydroxycinnamic acids	0.70	-5.96	1.39	+
ABP	Spherobioside	577.15649	Isoflavonoid O-glycosides	-0.50	-6.27	1.28	+
ABP	1,5-Dicaffeoylquinic acid methyl ester	529.13635	Hydroxycinnamic acids	0.30	-6.28	0.29	+
ABP	4-Caffeoylquinic acid lactone	161.0256	Hydroxycinnamic acids	-0.50	-6.29	0.06	+
ABP	3-Caffeoylquinic acid lactone	335.07913	Hydroxycinnamic acid	-0.50	-6.37	0.10	+

*PAMPA-BBB results based on Könczöl et al. (2013) +, log Pe > −6.5; ++, log Pe > −5.5; +++, log Pe > −4.5.

Table 6.2 shows that both the PLE conditions and the differences between matrices have strongly influenced the chemical profiles of agro-food by-product extracts, resulting in a lower abundance of compounds capable of PAMPA-BBB diffusion in TBP samples. Based on these outcomes, the highest Log Pe values (< 4.50) for ABP PLE extract were primarily associated with phenolic compounds, such as protocatechuic acid and ethyl caffeate, as well as flavonoids, including apigenin and quercetin, highlighting their potential to cross the BBB and contribute to neuroprotective effects. Regarding the TBP PLE extract, other flavonoids, such as naringenin, acacetin, and phenolic compounds like coumaric acid and hydroxypicolinic acid, effectively cross the artificial BBB. For instance, bioactive compounds such as protocatechuic acid (LogP = 0.40) and quercetin (LogP = 1.50) demonstrated high permeability despite their distinct chemical structures. This effect can be attributed to their comparable lipophilicity degrees, as LogP and Log (Pe) quantified, which describe a compound's ability to cross lipophilic barriers such as the blood-brain barrier (BBB).

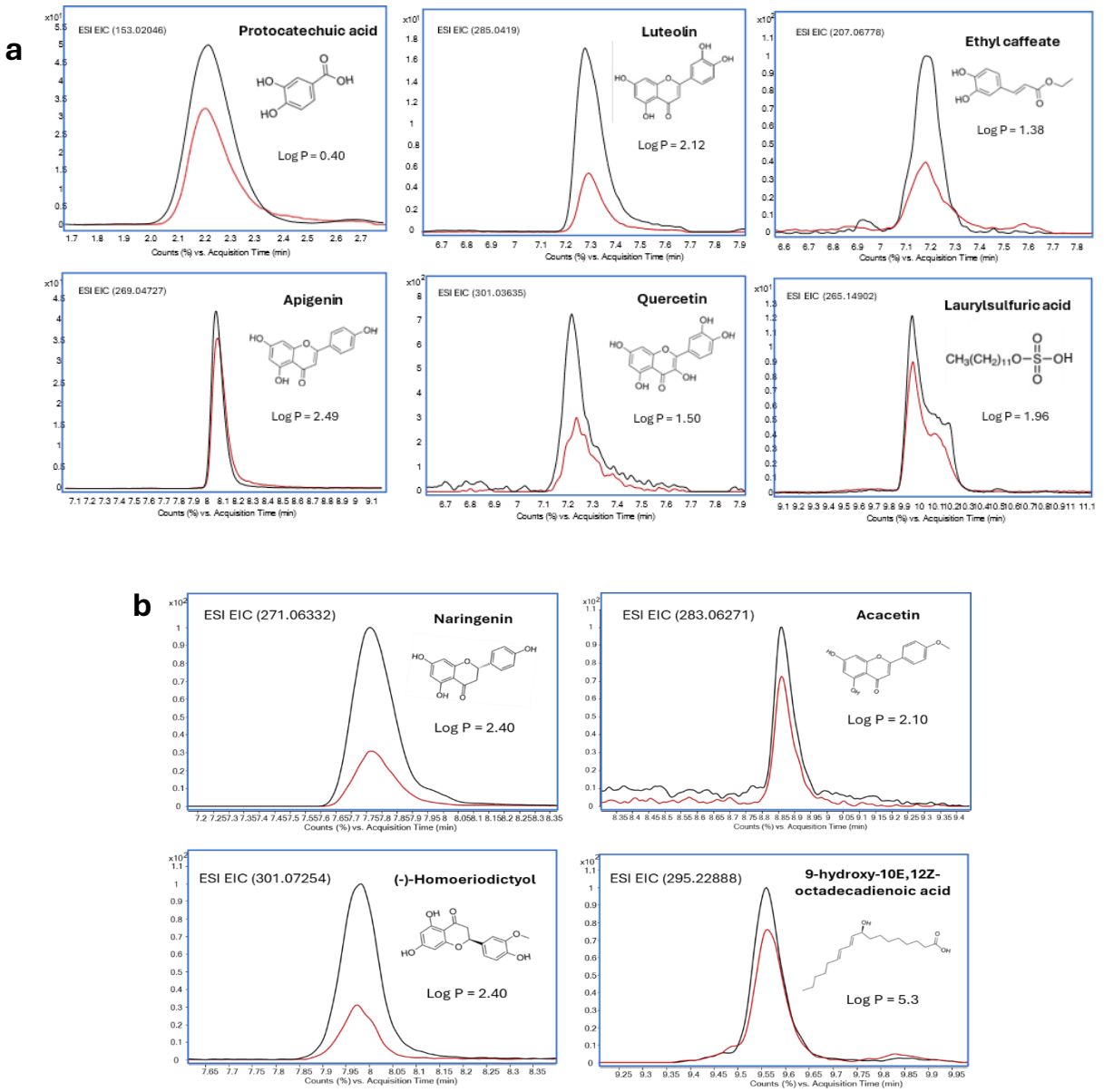


Figure 6.5: Representative extracted ion chromatograms of bioactive compounds identified in (a) ABP and (b) TBP PLE extracts obtained under optimised PLE conditions (see Chapter 5, Section 5.2, Table 5.6). Black lines of the chromatograms refer to the donor fractions; red lines refer to the acceptor fractions.

Indeed, LogP values between 0 and 3 are reported to present an enhanced ability to cross BBB (Könczöl et al., 2013; Sánchez-Martínez, Valdés, et al., 2022). Conversely, highly lipophilic molecules like N-lauryl diethanolamine (LogP 4.20), found in TBP extract, exhibited slightly lower diffusion (++), aligning with studies suggesting moderate lipophilicity optimizes BBB crossing. Molecular weight (M_W) is another key parameter affecting the diffusion performances of several biomolecules. Previous research highlighted that compounds having M_W below 500 Da could effectively cross the BBB (Hitchcock, 2008). In

our case, compounds such as apigenin (M_W = 270.24 Da) and ethyl caffeate (M_W = 194.18 Da) found in ABP provided high experimental diffusion values (LogPe = -3.88 and LogPe = -4.42, respectively). Larger molecules, such as di-caffeoylquinic acids (di-CQAs) or glycosylated flavonoids, demonstrated limited permeability, likely due to their larger molecular size and higher polarity, as previously described by Sánchez-Martínez et al. (2022). Additionally, it has also been reported that compounds with topological polar surface area (TPSA) below 90 Å tend to diffuse better through BBB (Hitchcock, 2008; Sánchez-Martínez, Valdés, et al., 2022). Flavonoids such as apigenin (TPSA = 90 Å) or naringenin (TPSA = 90 Å), provided the best diffusion performance in terms of measured Log (Pe). At the same time, glycosylated compounds like spherobioside, characterized by higher TPSA, exhibited lower ability, even when MW and LogP were within the proper ranges. As demonstrated in the study by Wrobel-Biedrawa et al. (2022), low-molecular-weight compounds, including protocatechuic acid and analogous phenolic acids, have been detected in brain tissues following in vivo experiments on mice, underscoring the potential for bioactive compounds derived from agro-industrial sources. Lauryl sulfonic acid derivatives, a type of fatty acid derivative, have also exhibited high BBB permeability (Log Pe = -4.18), indicating their capacity to penetrate the lipophilic environment characteristic of the BBB, suggesting their potential for bioactive use. Figure 6.5 presents the representative extracted ion chromatograms of bioactive compounds that have been identified in ABP and TBP. These chromatograms illustrate the highest diffusion abilities through an artificial blood-brain barrier (BBB). This investigation highlighted the significant potential of agro-food by-products as a valuable source of bioactive compounds with numerous health benefits and promising applications in the biotechnological and nutraceutical fields. The investigation further demonstrated the practical suitability of bioactive compounds, such as apigenin, quercetin, and phenolic compounds identified in artichoke and tomato by-products PLE extracts, for targeting the central nervous system in the treatment of neurodegenerative diseases, as evidenced by their ability to cross the blood-brain barrier (BBB). These molecules are worthy of consideration as potential drug candidates, owing to their optimal physicochemical properties, including molecular weight, balanced lipophilicity (Log P), and optimal diffusion rates to cross the BBB.

6.3. Encapsulation Strategies to Optimise Bioavailability of Bioactive Compounds Obtained from Tomato By-Products

This study was carried out during the visiting period spent at CIAL Institute (Madrid, Spain) in the Foodomics research group under the supervision of Prof. Miguel Herrero, Dr. Lidia Montero, and Dr. Victor Manuel Amador-Luna.

Bioactive compounds (BCs) found in agro-food industrial residues are widely recognized as underutilized resources with significant nutritional and biotechnological potential. However, their incorporation into food products to enhance nutritional value is often a challenge due to low solubility, limited bioavailability, and sensitivity to environmental factors such as heat, oxygen, and light. Encapsulation has been employed for decades in food science to protect sensitive beneficial biomolecules, prolong food shelf life, and control the release of functional ingredients. Techniques such as microencapsulation, nanoencapsulation, and liposomal systems have demonstrated outstanding potential for protecting BCs from degradation in an industrial scalability context.

This research aimed to evaluate the stability performance of two encapsulation techniques, spray-drying and liposomal encapsulation, applied to BCs-rich extracts obtained through ultrasound-assisted extraction (UAE) from tomato by-products (TBP). The study investigated the efficiency of these techniques in retaining bioactive compounds, including phenolics, carotenoids, and terpenoids, over time. Several spectrophotometric assays were preformed measuring carotenoid (TCC) and terpenoid content (TTC), total antioxidant activities (by DPPH assay), and evaluating *in vitro* neuroprotective potential through an acetylcholinesterase inhibition assay. This study also considered the evaluation of terpenoid compounds for their recognized antioxidant and neuroprotective properties (Amador-Luna et al., 2024; Suárez-Montenegro et al., 2021). The results highlighted the greater performance of liposome-based systems, which provided enhanced protection against oxidative degradation and effectively maintained the bioactivity of the encapsulated compounds over time. In contrast, spray-dried powders demonstrated high stability but contained lower levels of bioactive compounds, resulting in reduced antioxidant and neuroprotective potential.

Future investigation will focus on assessing the in vitro digestibility and bio accessibility of all samples using the INFOGEST protocol (Brodkorb et al., 2019). This standardized

approach will allow monitoring of the release of key BCs, such as carotenoids, during simulated digestion across various reactors. Additionally, advanced MS-based techniques will be used to obtain chemical profiles of the samples undergone through the different digestion steps. Encapsulation methods, such as nanoemulsions and liposomal systems, have proven effective for stabilizing bioactive compounds during digestion and enhancing their targeted delivery.

6.3.1. Materials and Methods

6.3.1.1. Reagents and Materials

MilliQ water, ethanol (99%), and methanol (99%) were obtained from VWR (Barcelona, Spain). The following analytical standards were acquired from Sigma-Aldrich (Madrid, Spain): 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), disodium phosphate (Na2HPO4), monopotassium phosphate (KH2PO4), chloroform (ChCl3), Trizma hydrochloride (Tris-HCl), fluorescein sodium salt, linoleic acid, gallic acid, Folin-Ciocalteu reagent, quercetin, limonene, and all-trans-β-carotene. Additionally, 4-(amino-359 sulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F), galantamine hydrobromide, and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were sourced from TCI Chemicals (Tokyo, Japan). All assays conducted in 96-well microplates were performed using a spectrophotometer and a fluorescence microplate reader (Cytation 5 imaging reader with auto-disperser, BioTek Instruments, Winooski, VT, USA).

6.3.1.2. Extraction procedure

The technology employed for the extraction procedure of the active compounds from the tomato by-products was Ultrasound-Assisted Extraction (UAE). This method was selected due to its effectiveness in extracting apolar compounds such as carotenoids. Specifically, 1 g of TBP powder was dissolved in 72 mL of pure EtOH; then UAE was performed at 65°C for 20 min following the protocol described by Li et al (2022). After that, supernatant was collected and centrifugated (Sorvall Lynx 6000 Superspeed Centrifuge, Thermo Scientific) at 10.000 rpm for 5 min at 4 °C. After centrifugation, the extract was carefully transferred into

round flask bottles and concentrated by means of rotary evaporator (Buchi Rotavapor R-210).

6.3.1.3. Encapsulation procedures

The encapsulation was carried out using methodical steps designed by the Foodomics group. Two liposomal encapsulation methods were employed by mixing TBP extract with:

1) Sunflower lecithin: in this method, 10 g of sunflower lecithin was added to 495 mL of Elix water. The mixture was heated to 54°C while stirring at 1500 rpm for 5 minutes. Next, 10 mL of the TBP extract (4.64 mL of TBP extract mixed with 20 mL of 50% ethanol) was added. The solution was then stirred at 1380 rpm for 30 minutes

2) Sunflower lecithin with addition of inulin: this method followed the same procedure as the first, with the addition of 7.5 mL of inulin. The solution was stirred at 1380 rpm for 30 minutes.

After preparing both mixtures, they were placed in an ultrasonic bath for 15 min to ensure proper homogenization of the liposomal encapsulated systems. The resulting solutions were then lyophilized, with the obtained matrix stored at -20°C overnight. The freeze-drying process was conducted after pre-cooling the samples to remove moisture content and ensure the stability of encapsulation, both with and without inulin. This controlled process optimized the liposome formulation.

6.3.1.4. Spray drying

The encapsulation system by spray drying technique was also evaluated, following an optimized protocol adapted from Corrêa-Filho et al. (2019) with some modifications tailored to the specific requirements of the study. First, Milli-Q water was heated to 54°C under magnetic stirring equipped with a stir bar, and 3.1 g of inulin (encapsulating agent) was dissolved into the solution. The mixture was stirred at 1350 rpm for 5 min to allow complete dissolution of inulin. Simultaneously, in a 50 mL falcon tube, a separate mixture was prepared by combining 4.64 g of TBP extract with 20 mL of 100%. The mixed solution was added to the inulin solution previously prepared in Milli-Q. The resulting solution was stirred at 1350 RPM for 30 min to ensure thorough homogenization. After homogenization, the

mixture was further processed using a laboratory-scale Mini Spray Dryer S-300 (Buchi Labortechnik AG), transforming the liquid solution into a dry powder. Specifically, spray-dryer set up is reported in Table 6.3. This spray drying step effectively encapsulated the extract within the inulin matrix, yielding a stable system. Finally, the encapsulated matrix was collected and stored for further analysis.

Table 6.3: Parameters for spray-drying treatment

Parameter	Value
Inlet Temperature (°C)	122
Outlet Temperature (°C)	79
Feed Flow Rate (L/h)	1
Drying Air Flow Rate (m^3/h)	29
Atomization Pressure (mbar)	58

6.3.1.5. Stability study

Stability evaluations of the TBP extract and encapsulated samples were conducted over 9 weeks. The samples were kept in a custom-designed column oven (at a controlled temperature of 40 °C) developed by researchers at the Foodomics Laboratory to simulate accelerated aging conditions. This oven maintained a controlled temperature, allowing a faster stability assessment compared to ambient conditions. Analyses on total carotenoid and terpenoid contents, antioxidant activities, and neuroprotective potential were conducted.

6.3.1.6. Total Carotenoid Content (TCC)

The total carotenoids content (TCC) was measured using spectrophotometric assays (Synergy HT, BioTek Instruments, Winooski, VT, USA) in accordance with the protocol proposed by Amador-Luna (2024). The extract (0.05 mg/mL) was dissolved in cold acetone/water (90:10, v/v) after an overnight incubation under dark exposure at 4 ºC. The mixture was then subjected to centrifugation at 1500 rpm for 10 min at 4 ºC. The resultant upper layer was collected and further diluted with 5 mL of acetone/water (90:10, v/v). The

absorbances were measured at 480 and 750 nm (turbidity). TCC values were obtained by using Britton (1985) equation (4).

$$\text{TCC}\ (\%) = \text{A} \cdot \frac{10^6}{A_{1cm}^{1\%} \cdot 0.03 \cdot [M^{ext}]} \cdot 100\% \qquad (4)$$

Where: A refers to sample absorbance corrected for turbidity (A_{480} - A_{750}) (UA); $\text{A}_{1\text{cm}}^{1\%}$ refers to the molar absorptivity coefficient of β-carotene (2500·100 mL/g cm); $[M^{ext}]$ refers to sample concentration (g/mL).

6.3.1.7. Total Terpenoid Content (TTC)

Total terpenes contents (TTC) were evaluated performing the protocol provided by Amador-Luna (2024). Briefly, 200 µL of dry extract dissolved in EtOH (concentration of 250 mg mL^{-1}) was added to 1.5 mL of $ChCl_3$ and mixed for 30 min using a vortex. Then, 100 µL of sulfuric acid was added to each sample, keeping the solution in an ice bath. The mixture was carefully mixed and incubated for 2 hours in darkness. At the same time, a calibration curve of limonene was prepared at different concentrations (30 - 500 mg mL^{-1} in EtOH). Each calibration point was treated in the same way as for the samples. After the incubation, a supernatant was removed, and a reddish precipitate was collected. Finally, the precipitate was diluted in 1.5 mL of EtOH and analyzed in an absorbance spectrophotometer (Synergy HT, BioTek Instruments, Winooski, VT, USA) at 528 nm.

6.3.1.8. DPPH assay

Total antioxidant capacity was evaluated by means of spectrophotometric analysis able to measure the radical scavenging activity. The followed protocol was the one proposed by Amador-Luna (2024). In brief, 990 µL of DPPH 0.1M were mixed with 10 µL of the sample and after 30 min the absorbance was measured at 516 nm.

6.3.1.9. Cholinesterase inhibitory assay

The anti-cholinesterase potentials were evaluated by in vitro inhibition assays of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) enzymes with a fluorescent assay (based on Ellman's method). The protocol for the analyses was proposed by Sánchez-Martínez et al. (2022). In brief, stock solutions of both enzymes were freshly prepared in buffer Tris-HCl (150 mM, pH 8). A volume of 100 µL of the buffer was mixed with 25 µL of AChE or BuChE enzyme, with concentrations calculated to represent the Km value (in MilliQ water). In addition, 100 µL of each sample was added at seven distinct concentrations in EtOH/H2O (1:1, v/v), and the mixture was dispensed into each well of the microplate. Following a 10 min period of incubation, an automated dispenser, which was connected to the microplate reader, introduced 25 µL of ABD-F (125 µM) in buffer, and 50 µL of ACth (or BCth) was added and agitated to finally obtain the average kinetic speed. This was measured at λex = 389 and λem = 513 nm for 15 minutes at the minimum interval possible at 37 ºC. Galantamine was utilised as a reference inhibitor. The results obtained for each sample were expressed as equivalents of galantamine.

6.3.1.10. Statistical analysis

Results were analyzed using SPSS vs.19 (IBM) and expressed as average ± standard deviation (SD). The results represent the average of three replicates in the case of bioactivity assays. A one-way ANOVA analysis with p-values < 0.05 followed by Tuckey's test was performed to evaluate statistical differences.

6.3.2. Results and discussion

6.3.2.1. Total Carotenoid Content (TCC) evaluation

The stability studies assessed the total carotenoid content (TCC)in TBP extract over 9-week monitoring period. Their corresponding encapsulated samples were also considered. Initially, the TBP extract provided the highest TCC (~40.27 mg/100 mL), followed by spray-dried TBP (~32.45 mg/100 mL), liposome-encapsulated TBP (~32.45 mg/100 mL), and liposomes enriched with inulin (~30.60 mg/100 mL) as showed Figure 6.6a. Figure 6.4b displays the trend over the 9-week monitoring period, showing a rapid degradation for TBP extract reaching undetectable values by week 5. This effect can be related to its higher

susceptibility to oxidative and environmental stress without a protective treatment through encapsulation systems (X. Liu et al., 2024; Munin & Edwards-Lévy, 2011).

The outcomes of the spray-drying treatment presented significantly lower initial values (see Fig. 6.6a), stabilizing at approximately 10 mg/100 mL throughout the monitoring period. The lower TCC values observed can likely be attributed to thermal degradation caused by the elevated temperatures employed during the spray-drying process (see conditions in 6.3.1.4). For instance, several studies reported that spray drying inlet temperatures between 110°C and 200°C affect carotenoid content and encapsulation efficiency due to increased exposure to thermal stress (Eun et al., 2020; Guergoletto et al., 2020; Šeregelj et al., 2021). Similarly, the antioxidant activity of the encapsulates is compromised at higher inlet temperatures, attributed to the degradation of antioxidant compounds during drying (Eun et al., 2020; Šeregelj et al., 2021). Furthermore, it has been demonstrated that prolonged exposure to oxygen during the drying process can contribute to carotenoid degradation, underlining the pivotal role that process conditions can play in preserving the bioactive properties of the matrix (Guergoletto et al., 2020). Liposome-based encapsulation demonstrated significantly improved carotenoid retention, maintaining TCC levels above 25 mg/100 mL throughout the study, highlighting the efficacy of liposomes in protecting sensitive compounds from oxidation and environmental degradation.

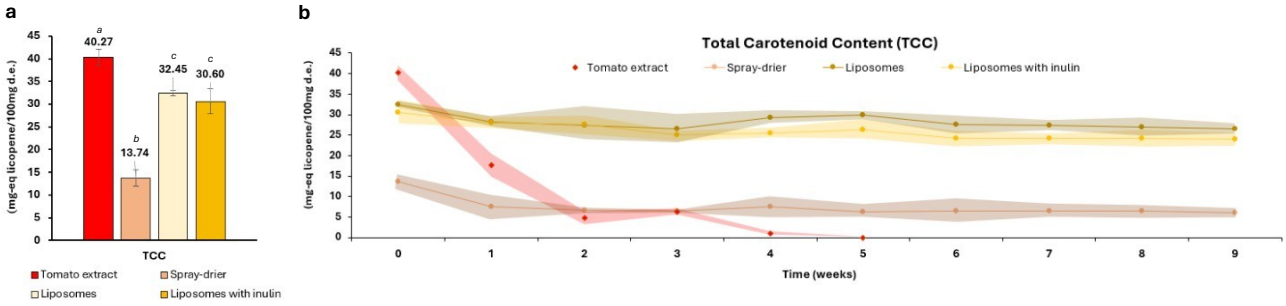


Figure 6.6: Stability evaluation of Total Carotenoids Content (TCC) over 9 weeks of storage.

Notably, liposomes enriched with inulin offered the highest stability, retaining TCC near 30 mg/100 mL after nine weeks but without providing any significant difference compared to the normal liposome-based encapsulation (Fig. 6.6b). The addition of inulin may enhance the protective barrier of liposomes and, at the same time, can act as a prebiotic, allowing controlled release in the gastrointestinal tract in vivo application. In addition, despite the

absence of statistical differences between the two liposome-based encapsulation, previous research has reported that inulin has a positive effect on structural integrity, thereby preserving its antioxidant properties (Rezagholizade-Shirvan et al., 2024; Xue et al., 2022). This finding aligns with results observed in encapsulation studies involving prebiotics and bioactive compounds from agro-food by-products and waste.

6.3.2.2. Total Terpenoid Content (TCC) evaluation

The terpenoid content (TTC) stability was also assessed for each encapsulation system and compared to the raw TBP extract over a 9-week storage period (see Figure 6.5a). It was observed that TBP crude extract recorded the highest TTC (~6907.4 mg equivalent limonene/100 mL), followed by inulin-enriched liposomes (~5726.3 mg/100 mL), conventional liposomes (~5551.0 mg/100 mL), and spray-dried TBP (~635.2 mg/100 mL). The observed differences can be attributed to the distinct encapsulation methodologies employed in each case. Specifically, the spray-drying technique typically involves the use of elevated inlet temperatures, a process that has been demonstrated to exert a significant impact on the bioactive compound content, as previously observed for TCC (Guergoletto et al., 2020).

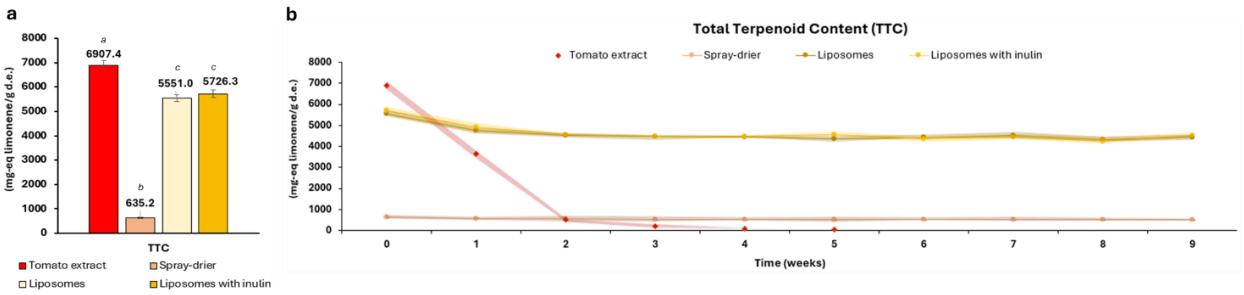


Figure 6.7: Stability evaluation of Total Carotenoids Content (TCC) over 9 weeks of monitoring period.

Figure 6.7b displays the stability trend over the monitoring period of the four samples. Similarly to carotenoid contents, TTC related to TBP extract showed a drastic reduction by week 5, probably due to properties of these BCs, which are highly prone to oxidation, as well as observed for carotenoids. However, the incorporation of inulin showed no significant improvement compared to the conventional liposome system. This effect may be due to an

unbalanced inulin-to-liposome ratio that not provided better performance, despite being recognized for its ability to strengthen encapsulation systems (Munin & Edwards-Lévy, 2011; Xue et al., 2022). In comparison with the TBP extract, spray drying offers a stable level of preservation over time, although the lower average TTC.

6.3.2.3. Cholinesterase inhibitory evaluation

The acetylcholinesterase (AChE) inhibition capacity of tomato by-products (TBP) was also evaluated. In Figure 6.8a, the initial values are reported in terms of µg-eq of galantamine/g dry extract. TBP tomato extract exhibited the highest AChE inhibition capacity at T0 (22.1 µg equivalent galantamine/mL), followed by liposomes (18.4 µg/mL), liposomes enriched with inulin (17.6 µg/mL), and spray-dried TBP (4.3 µg/mL).

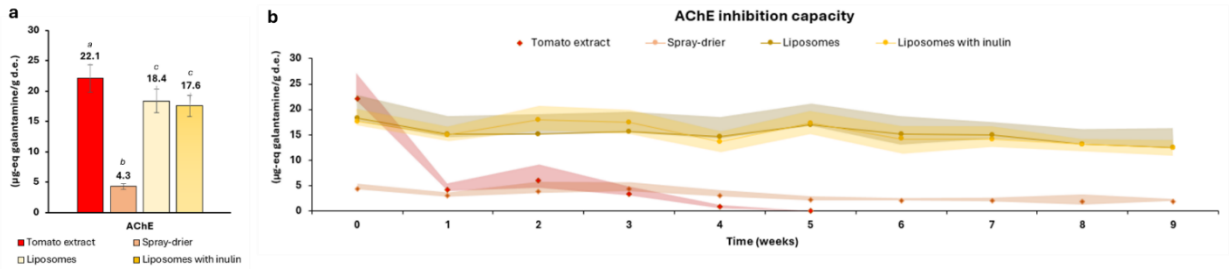


Figure 6.8: Stability evaluation of AChE inhibitory capacity over 9 weeks of monitoring period.

Figure 6.8b displays the trend of values over the monitoring period of 9 weeks. Rapid reduction was observed in TBP extract, with levels lowering significantly after only 3 weeks and becoming undetectable by week 5. This behavior may be related to the activity's loss of BCs with high antioxidant potential over time. Oxidation phenomena are known to degrade these compounds which contribute to a reduction of their biological effectiveness (S. Angeloni et al., 2021; Colovic et al., 2013; Sánchez-Martínez, Valdés, et al., 2022). Spray-dried TBP showed an initial low AChE inhibition capacity, further decreasing to negligible levels within three weeks, likely due to the thermal and oxidative stress generated during the drying process, which is known to degrade bioactive compounds (Guergoletto et al., 2020).

In contrast, liposomal encapsulation effectively preserved AChE inhibitory activity, maintaining levels above 15 µg/mL throughout the 9-week storage period. The stability

provided by liposomes can be attributed to their ability to encapsulate and protect sensitive compounds from oxidation and environmental degradation, consistent with prior studies on liposomal efficacy in stabilizing bioactives (González-Peña et al., 2023; Rezagholizade-shirvan et al., 2024). Liposomes enriched with inulin demonstrated similar stability performance, providing comparable AChE inhibition results. However, the incorporation of inulin did not significantly enhance the performance compared to conventional liposomes, possibly due to suboptimal inulin-to-liposome ratios (see Fig. 6.8b).

6.3.2.4. DPPH assay evaluation

The stability over time of DPPH radical scavenging activity was also monitored over 9-week monitoring periods for all the conditions. Figure 6.9a reports initial values recorded before the monitoring, evaluating the antioxidant potential of different formulations. As observed in the previous analyses, TBP extract exhibited the highest antioxidant activity, suggesting that the extraction process helped to enrich the sample of BCs with high antioxidant potential. The spray-dried TBP provided a significantly lower initial DPPH inhibition capacity (45.2 µmol Trolox equivalents/g dry extract) respect to the extract. Encapsulation systems, however, demonstrated considerably higher stability over time when compared to the other formulations. Liposome-based samples maintained stable DPPH inhibition capacity (~276.6 µmol Trolox equivalents/g d.e.) over the storage period, effectively preserving the antioxidant activity of TBP. Liposomes enriched with inulin showed a slight better performance (~291.5 µmol Trolox equivalents/g d.e.). However, the difference was not statistically significant, likely due to the inulin-liposome ratio not being optimized for further enhancement.

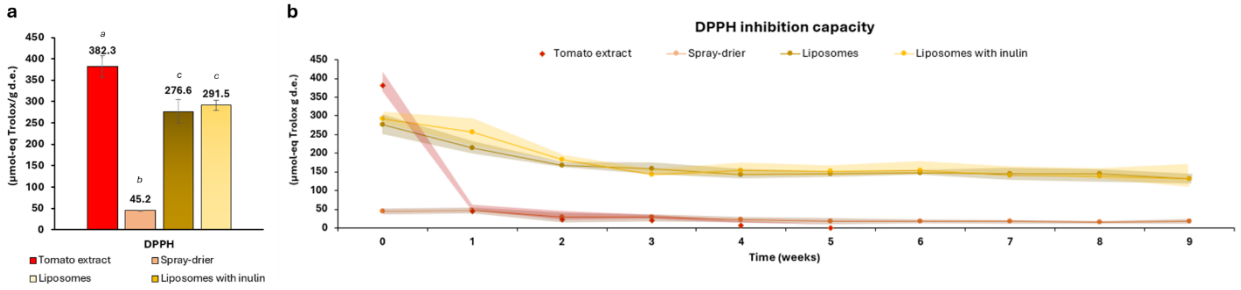


Figure 6.9: Stability evaluation of AChE inhibitory capacity over 9 weeks of monitoring period.

Figure 6.9b reports the results of stability evaluation over time. A strong reduction in DPPH activity was observed within the first three weeks, with radical scavenging capacity reaching undetectable values. This loss can be attributed to the rapid degradation of antioxidant compounds, such as phenolics and carotenoids, in the absence of protective mechanisms, a phenomenon frequently reported for unprocessed plant-based extracts during storage (X. Liu et al., 2024; Pinho et al., 2022). For the formulation obtained through the spray drying technique, antioxidant activity was found to decrease further, stabilizing at values close to zero by week 3. As confirmed by the previous evaluation, the encapsulated sample provided the highest results regarding antioxidant activity preservation over time (see Fig. 6.9b). Inulin may still contribute to structural stability and synergistic antioxidant effects, as suggested by prior studies on encapsulation systems (X. Liu et al., 2020; Soukoulis & Bohn, 2018). The results obtained demonstrated the efficacy of liposome-based encapsulation, both with and without inulin, in stabilising the evaluated parameters over time. These systems effectively protected against oxidative degradation, ensuring the preservation of bioactive compounds such as carotenoids and terpenoids, which have the capacity to exert biological effects, including enhanced neuroprotective potential (via AChE inhibition). The advantages shown by liposome-based encapsulation systems include their ability to enhance the bioavailability of bioactive compounds, protect sensitive ingredients from environmental and oxidative stress, and enable the controlled release of these compounds during digestion or processing (Correâ-Filho et al., 2019; X. Liu et al., 2024; Munin & Edwards-Lévy, 2011; Rezagholizade-Shirvan et al., 2024). These characteristics render liposome-based systems particularly well-suited to enhance the shelf-life, efficacy, and health benefits of functional food products, while concomitantly addressing challenges associated with the stability and delivery of sensitive bioactive ingredients.

6.4. Applications in the Field of Packaging: Active Spray Formulation for Food Shelf-Life Enhancement

The extension of shelf-life for perishable food products represents a critical challenge within the food industry, with the preservation of food from oxidative degradation being of particular

concern. Since oxidative degradation represent one of the primary factors affecting food quality. Oxidative processes can lead to undesirable changes in flavor, texture, and color, which can ultimately reduce the commercial viability of the product. Antioxidant additives are a common solution to this problem, and biobased formulations represent a novel approach that is in line with sustainability goals.This section examines the use of a natural polysaccharide-based antioxidant spray to enhance the shelf life of various food items, including meat. GRECI Industria Alimentare spa provided hydro-soluble aromas with antioxidant activity. Numerous experiments have been conducted to demonstrate the spray's ability to extend the shelf life of treated food products.

Additionally, TBP water extracts have been utilized to prepare spray solutions that substitute an antioxidant aroma with active compounds derived from by-products, supporting a circular economy approach. However, only preliminary experiments were carried out. A safety assessment procedure to check the absence of genotoxic effects by the active formulations has been conducted by Ames test performed in collaboration with Prof. Victoria Krauter of Department of Applied Sciences at FH Campus of Wien.

Spray formulations have been developed and tested in collaboration with the Chef of the experimental kitchen of GRECI Alimentare Spa. Designed as active ingredients in some food recipes with a potential market, the versatility of these antioxidant solutions in practical culinary applications they has been demonstrated.

6.4.1. Materials and methods

6.4.1.1. Chemicals and Reagents

The following substances were used in the experiment: water (Milli-Q), ethanol 96%, methanol 99%, sodium alginate, glycerol, sodium carbonate, d-(+)-gluconic acid-σ-lactone (GDL), Folin-Ciocalteu reagent, gallic acid, and DPPH (1,1-diphenyl-2-picrylhydrazyl), which were purchased from Sigma-Aldrich (Steinheim, Germany).

6.4.1.2. Samples

Fresh meat samples, including beef hamburger, veal fillet, horse fillet, and beef fillet, were supplied by GRECI Industria Alimentare S.p.A. (Ravadese, Parma, Italy). Slices (approximately 15 × 6.5 × 1.5 cm, length × width × height) and hamburgers (15 × 1.5 cm,

diameter × height) were carefully treated with a volume of spray of approximately 0.02 mL/cm^2. The shelf-life of the meat, stored at 4°C and covered with aluminum foil, was monitored from across 96 hours, with measures recorded every 24 hours. A control sample for each type of meat was stored under the same conditions.

6.4.1.3. Spray preparation

A biobased spray formulation was prepared according to the protocol proposed by Grimaldi et al. (2022), with certain modifications. In summary, a 1% w/v sodium alginate solution was prepared in 1 L of distilled water at 70 °C under vigorous stirring. Glycerol was then added in a quantity of 0.3 g/g of sodium alginate. In addition, a suspension of $CaCO_3$ (0.04 g/g of alginate) and GDL (5.4 g/g of $CaCO_3$) in 100 mL of distilled water (with an approximate pH of 5.0) was prepared independently. The suspension was then added to the alginate solution under vigorous stirring and kept at 40 °C for 30 min. A biobased antioxidant alginate principality (Vitamin E) – supplied by the company GRECI Industria Alimentare spa (Ravadese, Parma, Italy) – was incorporated (0.2 % w/v) into the spray solution, which was then stirred at room temperature for 15 minutes (in order to achieve proper homogenisation). The resulting formulation was then stored in a plastic flask equipped with a sprayer tool for application.

6.4.1.4. Total Antioxidant Capacity (%TAC)

The primary and secondary shelf-life of the sprays was determined using the DPPH assay, following the protocol outlined in section 3.1.10 of Chapter 3. The antioxidant activity of the spray was monitored over a storage period of 6 months, and measures recorded every 2 months, and again after one year. Each time, an unopened bottle of spray was analyzed to assess its primary shelf-life, while the already opened bottles were evaluated for their secondary shelf-life. Each time, an unopened bottle of spray was analyzed to assess its primary shelf-life; while the already opened bottles were evaluated for their secondary shelf-life.

6.4.1.5. Oxitest measures

The Oxitest Instrument (Velp Scientifica, Italy) was used to evaluate the oxidative stability of minced meat containing about 25% of fat. For the experiment, 30 grams of minced meat enriched with 5% (w/w) of hydro-soluble basil aroma was placed in each titanium sample holders. The oxygen pressure was set at 6 bars, and the temperature was maintained at 90 °C. The Induction Period (IP), expressed in minutes, was measured for each sample.

6.4.1.6. Ames test

Measurements were conducted at the FH Campus of Wien using a bacterial mutagenesis assay. The test involved plating bacteria on a solid agar medium containing minimal histidine. A disc infused with the test substance was placed at the centre of the plate, allowing the substance to diffuse into the medium. Trace amounts of amino acids in the medium enabled the detection of mutations that require multiple cycles of cell duplication to manifest phenotypically, such as point mutations on a single DNA helix. To evaluate mutation frequency, the distance from the disc was measured after 48 hours of growth, with any color change from purple to yellow being noted. These results were compared to a control plate, where bacteria were grown on the same medium without the suspected mutagen, to account for the effects of spontaneous revertant.

6.4.1.7. Color analysis

Color variation was evaluated on treated and untreated samples including horse meat, beef meat and beef hamburgers. Color was measured using colorimeter operating in CIE L*a*b* color space (TECHKON Spectrodens, Königstein, Germany). The instrument is equipped with a LED light source. The measurement aperture was 3 mm, and two optical configurations were adopted (0° and 45°). The spectral range used was 400 - 700 nm in 10 nm steps, with a spectral resolution of 10 nm and the pixel distance sensor < 3 nm. Before the measurement, the colorimeter was calibrated using a white reflectance standard supplied by the manufacturer. A customized perforated jig was used to record the color coordinates in the same points allowing a repeatable positioning. Three measurement of color coordinates were recorded, and the software adopted was TECHKON Spectro Connect 2.5.

Color variation (ΔE) in CIE L*a*b* color space were calculated as follow:

$$\Delta E_{ab} = \sqrt{(L_2 - L_1)^2} + (a_2 - a_1)^2 + (b_2 - b_1)^2$$

where (L_2 – L_1), (a_2 – a_1), (b_2 - b_1) are the difference in terms of lightness, green-red and blue-yellow coordinates, respectively.

6.4.1.8. Statistical analysis

All statistical analysis in this study was conducted using Microsoft Excel. The resulting data were expressed as the mean ± standard deviations (SD) of a minimum of three independent replicates. To evaluate significant differences among the treatments and time points, a one-way analysis of variance (ANOVA) was conducted, followed by post-hoc pairwise comparisons using Tukey's test ($p < 0.05$).

6.4.2. Results and discussion

6.4.2.1. Oxidative stability of meat samples enriched with hydro-soluble basil aroma

The antioxidant activity of hydro-soluble basil aroma has been evaluated by Oxitest reactor. The study involved minced meat with a fat content of 25%. Both treated meat, which was infused with hydro-soluble aroma, and untreated meat (referred to as TQ) were tested. The induction period (IP) for the TQ sample was approximately 430 ± 6 minutes, while the treated meat exhibited an IP of about 455 ± 4 minutes. This indicates that at the initial assessment (T0), the meat enriched with hydro-soluble aroma demonstrated a slightly longer IP and, therefore, greater oxidative stability compared to the untreated sample. When the minced meat treated with hydro-soluble aroma was stored at 4 °C, the IP values after 24 and 96 hours showed further improvement, measuring 485 ± 22 and 522 ± 32 minutes, respectively. Over time, the treated meat exhibited a slight increase in oxidative stability, ranging from 5% to 17% compared to the control. Conversely, the untreated control samples showed a steady decrease in IP, dropping to about 90 minutes after 96 hours of storage. These results underscore the protective effect of the aroma on minced meat during oxidation. Significant enhancement in terms of oxidative stability were observed in the treated meat after 24 and 96 hours of storage at 4 °C.

6.4.2.2. Spray preparation and its shelf-life

The spray was prepared following a previously reported procedure, packaged in plastic spray bottles, and stored at room temperature. Its shelf life was evaluated by monitoring antioxidant activity, with the %TAC parameter selected as the key indicator of the spray's antioxidant properties. Measurements were conducted using the DPPH assay at two-month intervals over six months and again after one year. Both primary and secondary shelf lives were assessed, with no significant differences observed (data not reported), indicating comparable performance regardless of whether the bottles were opened or sealed.

The lowest reduction in antioxidant activity was recorded at four months under both conditions. However, by six months, %TAC values showed a significant reduction of approximately 70%, at which point the spray could no longer be considered an effective antioxidant formulation. The results obtained suggested that shelf-life of the spray product is guaranteed for up to four months for potential market delivery.

6.4.2.3. Genotoxicity evaluation of spray formulations

The Ames test is a widely used and cost-effective method for assessing mutagenesis in live cells by monitoring histidine production in His^- bacterial clones. Figure 6.10 displays results on active spray formulations enriched with TPB extract. The tested samples (P1 at varying concentrations: 100%, 32%, 10%, 3.2%, 1%, and 0.32%) did not demonstrate mutagenic activity. The absence of mutagenicity is supported by the lack of significant increase in the number of revertant colonies compared to the negative control (C-: DMSO), as shown in both the purple (A) and yellow (B) test plates. The addition of a positive control (C+: 2-AA) successfully induced a mutagenic response above the positive threshold, confirming the sensitivity and validity of the assay.

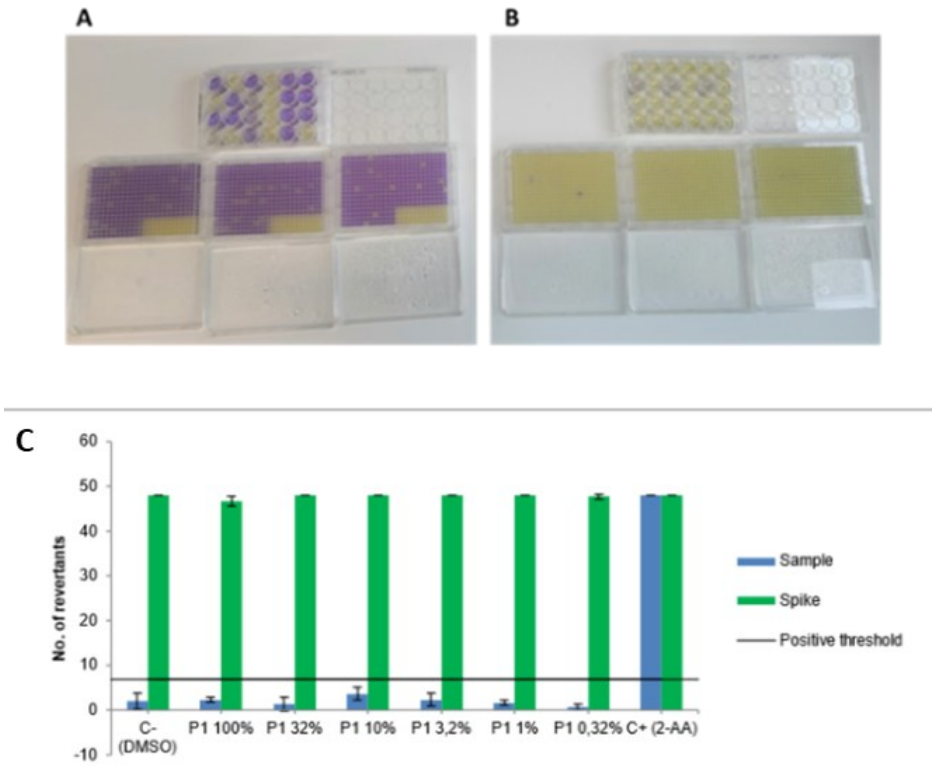


Figure 6.10: Negative Ames test response obtained for sprays A) without spike (purple); B) with spike (yellow); C) Ames test results for active sprays enriched with TBP extract.

In contrast, the tested sprays remained below the positive threshold even when spiked (yellow plate, B), further confirming their non-mutagenic profile. These findings indicate that the antioxidant-enriched sprays derived from industrial by-products do not exhibit genotoxic effects, supporting their potential application as a safe product.

6.4.2.4. Color variation analysis

Experiments were conducted to assess the impact of hydro-soluble basil-enriched spray and vitamin E spray on the shelf-life of various meat types in collaboration with the R&D department and the culinary staff at GRECI Industria Alimentare spa. The color changes in meat samples, specifically horse meat, veal, and beef hamburgers, were evaluated by measuring the color variation (ΔE) in the CIE L*a*b* color space. This color space is significant as color coordinate variations are closely related to the human ability to distinguish different colors (Hernández Salueña et al., 2019). Figure 6.11 illustrates the results of color variation when applying the hydro-soluble basil aroma spray to different

meats. For pork hamburgers (Fig. 6.11a) and veal (Fig. 6.11b), the antioxidant spray treatment resulted in significantly lower ΔE values, indicating a reduction in color changes within these types of meat. This suggests that the treatment effectively mitigates oxidation-related color reductions. As is widely recognized, oxidation processes and storage conditions have a profound impact on the chemical properties of food, including its color stability (Dai et al., 2014; Moya et al., 2021; Śmiecińska & Daszkiewicz, 2021).

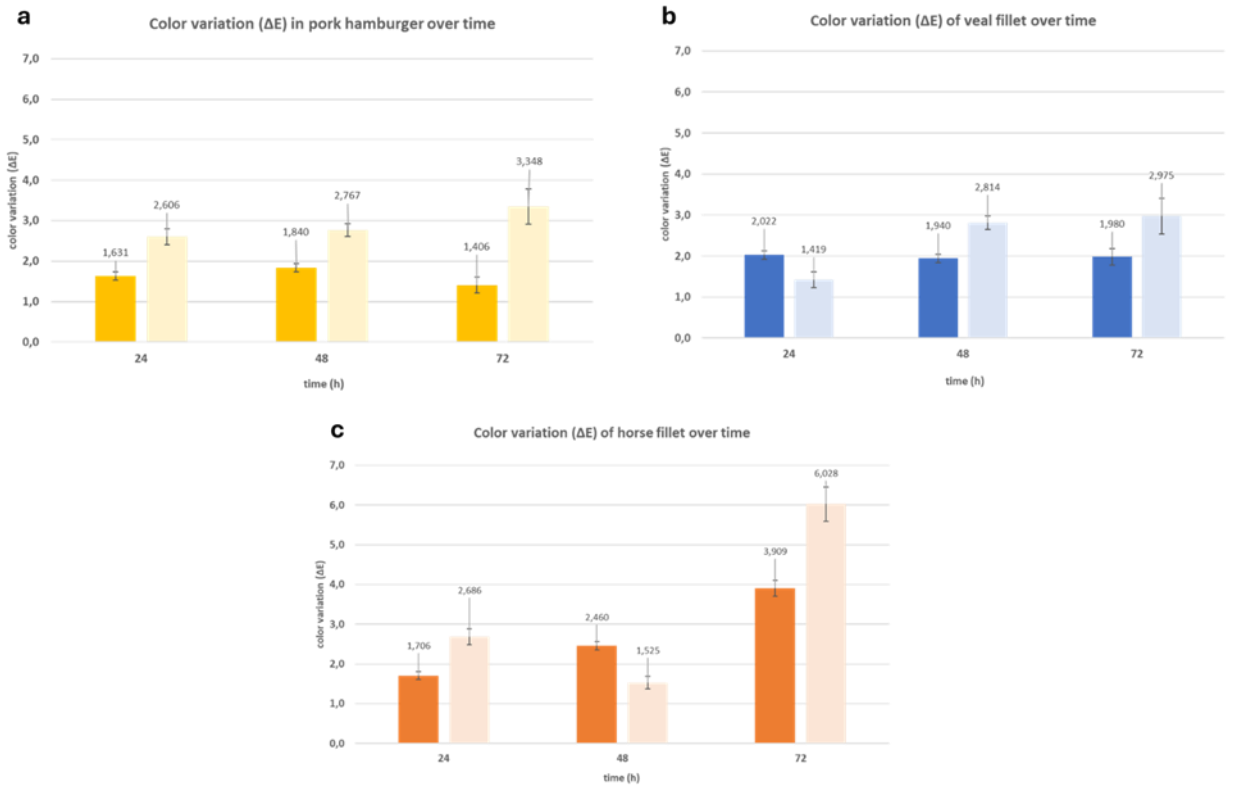
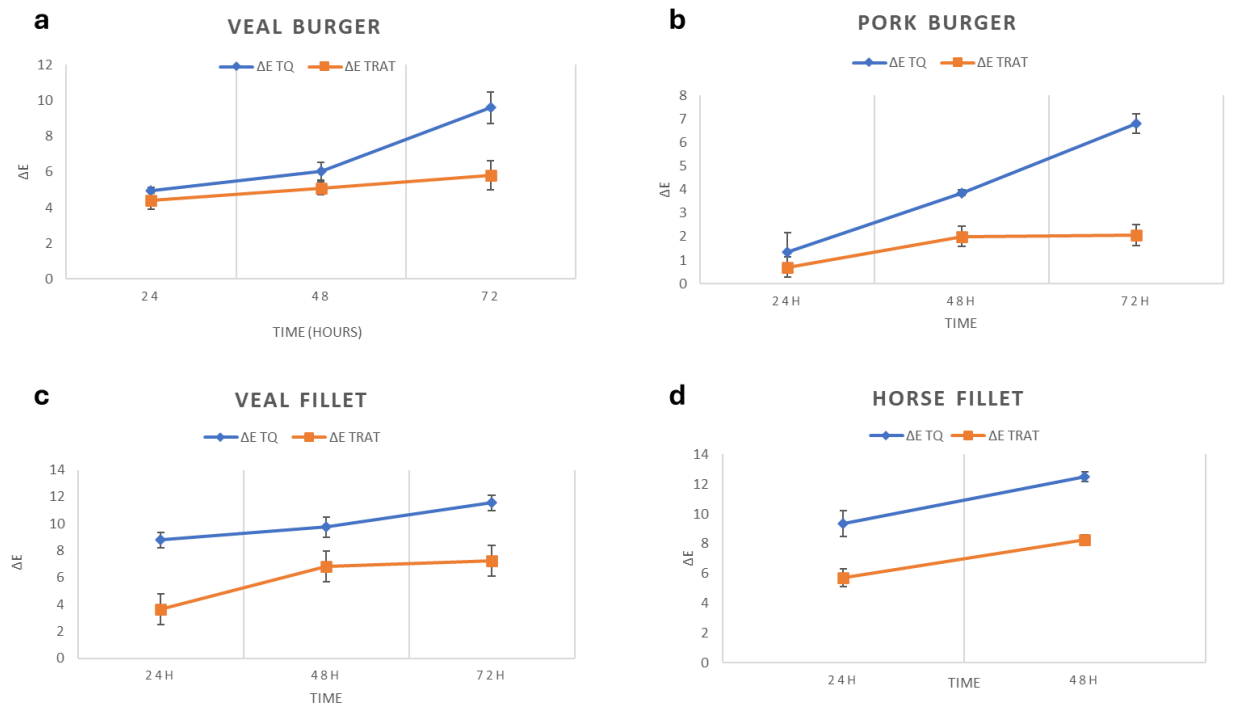


Figure 6.11: Color variation (ΔE) values of treated and untreated (a) pork burger, (b) veal meat and (c) horse meat during storage. Light yellow and blue bars refer to TQ of (a) pork and (b) veal, dark bars refer to TR; light orange refers to TR, dark orange refers to TQ of horse meat.

In contrast, the color variation observed in horse meat showed a more substantial increase in ΔE after 72 hours for both the treated and untreated samples compared to their initial stable values (Fig. 6.11c). Although the ΔE values for the horse meat samples remained lower only at the 24-hour mark, statistical analyses did not confirm significant differences between treatments. This lack of significant difference is likely due to the accelerated formation of meta-myoglobin, compounds associated with the browning phenomena typically observed in horse meat (López-Pedrouso et al., 2023; Tikhonov et al., 2024).

Based on these outcomes, the company partner GRECI Industria Alimentare spa, proposed to test also a formulation with an antioxidant that does not affect the organoleptic properties of the meat. For this reason, *Solution 2* involving vitamin E as active principle for the biobased antioxidant spray was applied on pork burger, veal burger, veal fillet, and horse fillet.

Figure 6.12: Color variation (ΔE) values of (a) veal burger, (b) pork burger, (c) veal fillet, and (d) horse fillet after 72 hours of storage at 4 °C



As shown in Figure 6.12, the graphs depict the color variation (ΔE) over time for treated and untreated meat samples. The data indicate that meat color changes (blue lines) follow a linear trend, confirming the strong impact of oxidation on color parameters over time. Notably, treated samples (orange lines) exhibited significantly lower ΔE variation, particularly depending on the type of meat where the spray was applied. For treated veal burgers (Figure 6.12b), a gradual increase in ΔE was observed compared to the control. At T_{72}, the spray-treated burger displayed significantly lower color changes than the untreated counterpart. While, in Figure 6.12c, the untreated veal burger showed a constant linear increase in ΔE over time, the spray-treated burger demonstrated a period of stability between T_{48} and T_{72}. Conversely, treated veal samples initially recorded an increase in ΔE during the first 48 hours, followed by stabilization until T_{72}. The untreated veal control continued to exhibit a steady increase in ΔE throughout the same period. For horse fillet, reported in Figure

6.12d, significant color change was observed only until T_{48}, as browning and an unpleasant odor developed afterward. Both treated and untreated horse meat showed a linear change in ΔE up to T_{48}, then color stability was no longer maintained. These findings underscore the effectiveness of the vitamin E spray as active principle in mitigating color changes in specific types of meat, such as veal, while revealing its limitations in other cases, like horse meat, which was ineffective in maintaining color stability despite treatment.

Additionally, microbiological safety tests of the vitamin E spray, conducted according to ISO 4833-1:2013 standards, measured the Total Viable Count (TVC), and confirmed its safety, with results showing <1 UFC/mL. These findings further underscore the potential of vitamin E spray as a natural and effective solution for enhancing the quality and shelf life of certain meat products.

Innovative approaches combining agro-food extracts and polymer composites can be also investigated for future works. Examples of applications can be the development of sustainable absorbent pads characterised by hydrophobicity and antioxidant or antimicrobial properties (Cabrera-Villamizar et al., 2025; Alchera et al., 2022). Such materials present a promising solution for reducing environmental impact while simultaneously improving functional performance in sustainable applications.

The evaluation of the TBP spray is ongoing as part of the project “BEST - Bioactive compounds to Extend food Shelf-life through Innovative Technologies” funded within the PRIN PNRR (Italy’s National Recovery and Resilience Plan).

6.5. Tomato By-Products as Natural Filler for Hydrophobicity Enhancement of Cellulose-Based Materials

This work investigated the use of tomato by-products (TBP) as natural fillers for improving the hydrophobicity performances of cellulose-based materials. Cellulose, while widely used in packaging as a sustainable alternative to plastic, suffers from limited performance due to its lack of resistance to humidity and liquids. To address these limitations, sustainable solutions have been developed over the years, such as by exploiting cutin obtained from TBP, as realised by *Tomapaint* (https://www.it.tomapaint.com/).

Formulations composed of beeswax and TBP were prepared in two ratios (2:1 and 4:1, m/m) were prepared to coat virgin and recycled paper samples, then applied via thermal pressing. These ratios were deliberately selected based on preliminary investigations aimed at balancing the hydrophobicity enhancement of beeswax with the mechanical and thermal stability of TBP. The 2:1 ratio represents a moderate beeswax loading, hypothesized to synergistically reduce water permeability while maintaining acceptable processability. Conversely, the 4:1 ratio was prepared to investigate higher beeswax content to test the upper limit of wax-based hydrophobicity reinforcement. The selection of TBP was driven by the need to tune and improve the processability of wax, which is ineffective for application on the paper surface due to limitations related to mechanical and thermal instability. The hydrophobic properties of these coatings after application were assessed through water uptake (%WU) tests and contact angle (CA) measurements. Results revealed that TBP, rich in hydrophobic components like cutin, significantly reduced water absorption when combined with wax. The wax/TBP (2:1) blend achieved a reduction in water uptake exceeding 80%. However, increasing the TBP content in the coating formulation led to unfavourable outcomes, with a reduction in hydrophobic performance as reflected by higher water uptake (WU) values and lower contact angle measurements. Contact angle analyses highlighted a discrete ability to repel water. Specifically, the only wax demonstrated the best performance, recording the highest values (more than 100°). However, the inclusion of TBP led to reduced CA values. Despite this reduction in hydrophobic performance, the use of TBP was essential to improve the processability of cellulose-based materials, making them more adaptable for industrial applications. The study demonstrates the potential of wax/TBP blends as sustainable, water-resistant materials for eco-friendly packaging. Further research is needed to evaluate their mechanical properties and long-term stability for broader industrial applications.

6.5.1. Materials and Methods

6.5.1.1. Reagents and Samples

Virgin and recycled paper samples were provided by the German company Krones. Ethanol (96%) and acetic acid (5%) were supplied by Carlo Erba, while tomato by-products (TBP) were sourced from GRECI Alimentare S.p.A. Commercially available beeswax blocks were also used. The tested samples were cut into paper discs with a diameter of 5 cm for analysis

6.5.1.2. Coating Preparation and Applications by Thermal Pressing

Beewax and TBP were separately grounded by planetary mill (Pulverisette 0, Fritsch, Idar Oberstein, Germany) to achieve a fine powder. As shown in Figure 6.13, wax/TBP were combined in two different ratios (2:1 and 4:1).

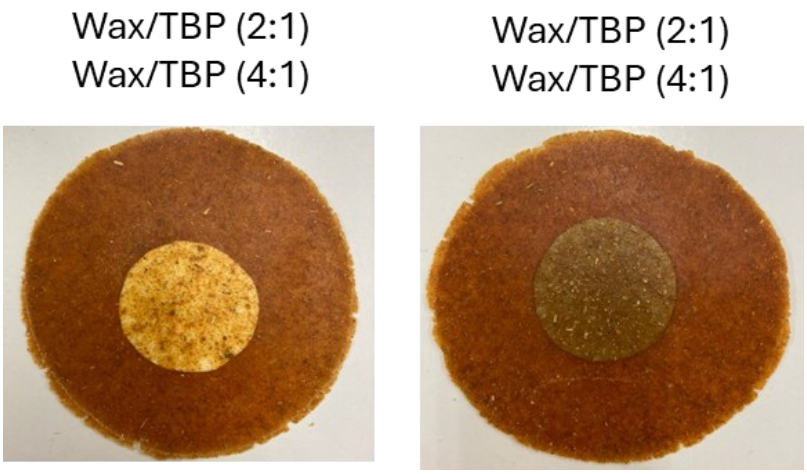


Figure 6.13: Wax/TBP coating formulations applied to virgin (left) and recycled (right) paper

Wax/TBP coating formulation was prepared by thermal pressing. Parameters used for film formation were as reported in Table 6.5:

Table 6.5: Press-performing conditions for film preparation.

Coating composition	Ratio w/w	T	Strength	Cooling T	Plate thickness
	(g/g)	(°C)	(Kg)	(°C)	
Wax/Tomato By-Products (TBP)	2:1	45	2000	35	Plane
Wax/Tomato By-Products (TBP)	4:1	45	2000	35	Plane

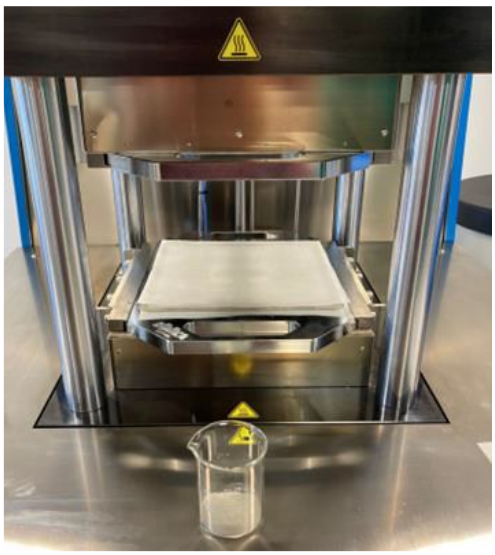


Figure 6.14: Laboratory thermal press for coating application

The film was pressed upon both virgin and recycled paper discs under same the conditions specified in Table 6.5 on both sides of the samples (see Figure 6.14). Additionally, a benchmark coating composed of only wax was prepared and pressed upon the paper for comparison.

6.5.1.3. Water Uptake Test

The water uptake (%WU) test was performed following protocol described by Heydari et al. (2023). Each sample was cut into a disk of 5 cm in diameter. Both control and treated paper disks were oven-dried at 60 °C till reaching constant weight. Each sample was dipped into 250 mL of Milli-Q water for 24 h at room temperature. The water uptake has been calculated as follows:

$$\%WU = \frac{W_f - W_i}{W_i} \times 100$$

where W_i refers to the weight before water immersion and W_f is the final weight after 24 h of dipping. For each coating, WU values were recorded in triplicate for both virgin and recycled paper samples.

6.5.1.4. Contact Angles Measurement

The surface contact angle (CA) measurements were performed by OCA25 equipment (Data Physics Instruments). In brief, 1 µL of water was dropped onto paper samples. The measure

has been conducted in three different points of the same sample. The results were determined by measuring the contact angle degrees: CA values higher than 90° refer to hydrophobic surface; CA lower than 90° defines for hydrophilic surface (see Fig. 6.15).

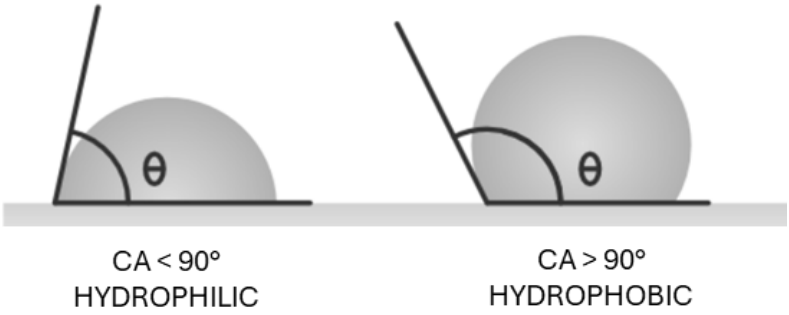


Figure 6.15: Contact Angle (CA) angles for determination of surface's hydrophobicity.

6.5.1.5. Statistical Analysis

A statistical analysis of data was performed by using Microsoft Excel, and differences among mean values were processed through a t-test analyses. Significance was defined at $\alpha = 0.05$.

6.5.2. Results and discussion

6.5.2.1. Water Uptake

Water uptake (WU) analyses were performed on paper samples coated with different formulations, including neat paper, wax-based coating, wax/TBP (2:1), and wax/TBP (4:1). The results reveal distinct trends in the effectiveness of the coatings in reducing water absorption, critical for their application in water-resistant materials.

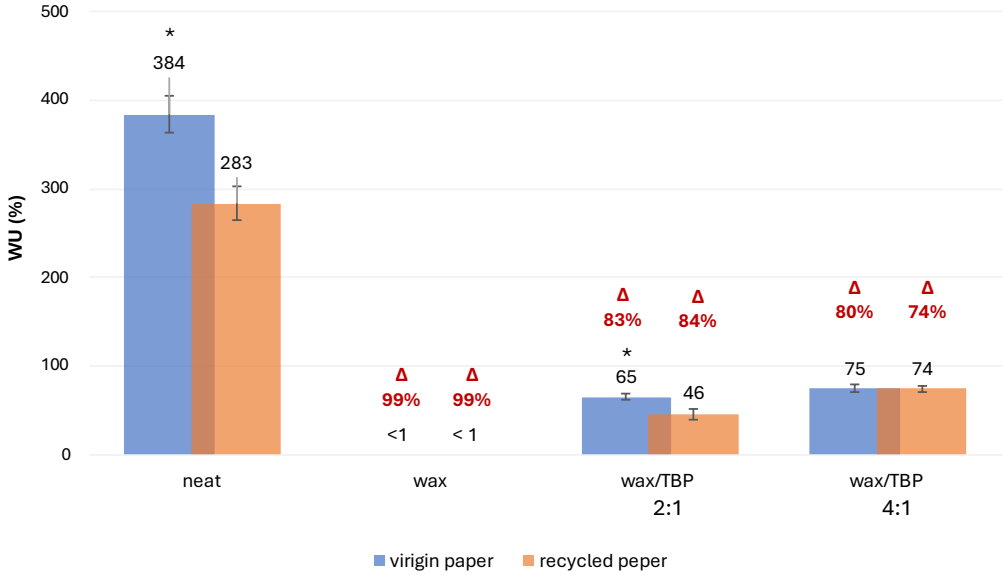


Figure 6.16: Water Uptake (%WU) values for neat paper, beewax, wax/TBP (2:1) and (4:1) coatings. Asterisk (*) indicates statistically significant differences between samples (virgin vs recycled paper).

Figure 6.16 displays %WU rates for all the samples assessed. The results revealed distinct trends in coatings efficacy in reducing water absorption, which is critical for applying these materials in packaging applications. Neat paper (without treatments) exhibited the highest WPA for virgin and recycled paper, demonstrating its hydrophilic nature and poor water resistance. The analyses were conducted on neat paper considered as a baseline for evaluating the impact of the coatings . In comparison, paper samples coated with pure wax showed the highest reduction in WU, underscoring the hydrophobic nature of wax and its ability to function as a barrier against water and humidity. However, the use of wax alone as coating agent is greatly limited due to its processability challenges, such as brittleness, thermal and mechanical instability, and poor resistance to manufacturing processes (Jahangiri et al., 2024; Wu et al., 2021). To address these limitations, the inclusion of agro-food by-products powders, such as TBP, was explored as a strategy to enhance the processability and functionality of the coatings while maintaining hydrophobic performance. Tomato by-products are known to be rich in bioactive compounds, including cutin, a natural polymer with hydrophobic properties that forms a protective barrier in plant cuticles (Heredia-Guerrero et al., 2017; Hood et al., 2021). The presence of cutin and other hydrophobic molecules in TBP likely contributed to enhance water resistance in the wax/TBP blends. The wax/TBP coatings provided a significant reduction ($p < 0.05$) in terms of water

uptake percentage when compared to both neat paper and pure wax-coated samples. The neat paper, used as a control, showed the highest %WU, with values exceeding 90% for both virgin and recycled substrates. The pure wax already reduced water uptake by approximately 50%, whereas the addition of TBP further enhanced hydrophobic performance. Specifically, the wax/TBP (2:1) formulation led to a reduction in water uptake of over 80%, confirming its effectiveness as a water-resistant barrier. Notably, although the wax/TBP (4:1) coatings also reduced water uptake substantially, the difference between the two wax/TBP ratios was minimal. This observation is supported by statistical analysis: a one-way ANOVA, followed by Tukey's HSD post-hoc test, revealed no statistically significant difference ($p > 0.05$) in %WU between the 2:1 and 4:1 blends for both virgin and recycled paper. These results indicate that increasing TBP content beyond the 2:1 ratio does not yield additional benefits in water resistance. In general, as illustrated in Figure 6.15, the addition of TBP to wax coatings enhances performance, but an optimal threshold appears to exist at the 2:1 ratio, beyond which no further hydrophobic gains are observed. Finally, these findings demonstrated the potential of wax/TBP blends as functional coatings for enhancing water resistance in paper-based materials. By using agro-food by-products powders as natural fillers, the amount of wax can be reduced, thus offering additional advantages in terms of processability, thermal resistance, thereby making the materials more suitable for industrial applications.

6.5.2.2. Contact Angles Measurement

Contact angle (CA) measurements were performed on the samples to further assess the wettability performance of the biobased coatings. The contact angle is a key indicator of surface hydrophobicity, with higher CA values signifying the improved ability to resist to water: values higher than 90° refer to hydrophobic behaviour; CA lower than 90° defines for hydrophilic surface. Figure 6.17 illustrates the values related to the CA measurement of treated and untreated paper samples. The neat paper was the only sample where contact angle was not measurable, as the water droplet was immediately absorbed at the beginning of the experiment. This behaviour reflects the highly hydrophilic nature of the uncoated virgin and recycled paper. Wax materials showed a significant increase in contact angle values, demonstrating their ability to repel water, thus enhancing surface hydrophobicity. Concerning wax/TBP coating blends, both the 2:1 and 4:1 ratio exhibited comparable and

slightly lower contact angles than the pure wax, suggesting that including TBP in the coating significantly reduced hydrophobicity on the surface.

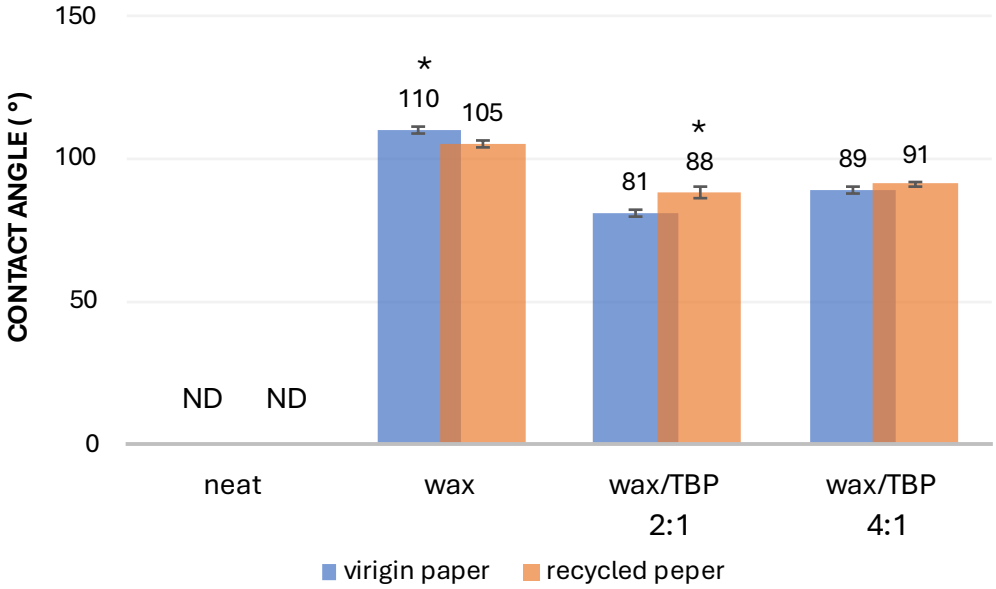


Figure 6.17: Contact angles values recorded for neat paper, wax, wax/TBP (2:1) and (4:1) coatings. Asterisk (*) indicates statistically significant differences between samples (virgin vs recycled paper).

This effect could be due to the heterogeneity of TBP matrix, which present components that may improve wettability behaviour. However, the presence of hydrophobic components such as cutin in the TBP contributed to maintaining a degree of hydrophobicity, effectively balancing the influence of the hydrophilic compounds (Hood et al. 2021; Heredia-Guerrero et al. 2017).

Further investigations on these coatings' mechanical and long-term stability properties could provide valuable insights for their broader application in sustainable packaging solutions.

6.6. Conclusions

The investigations presented in this chapter collectively underline the transformative potential of agro-food by-products deriving from artichoke and tomato processing chains—as high-value matrices for applications in the field of nutraceutical, biomedical, and sustainable packaging. These approaches explored not only offer novel pathways for waste reuse but also represent a substantial contribution to the development of a resilient and circular economy strategies.

The enrichment of pasta with artichoke-based flour demonstrates a successful model of functional food innovation, where prebiotic-rich formulations were achieved through the integration of agro-food by-products. This nutraceutical application supports dietary health while simultaneously repurposing materials that would otherwise be discarded, thereby achieving a dual objective of promoting human wellness and environmental sustainability. From a biomedical standpoint, the extraction of bioactive compounds from artichoke and tomato by-products, using pressurized liquid extraction (PLE) (described in Chapter 5), provided extracts that demonstrated in vitro neuroprotective activities. The optimized extracts showed discrete inhibitory activity against cholinesterase and lipoxygenase enzymes, which are key biomarkers involved in neurodegenerative diseases. Furthermore, the in vitro parallel artificial membrane permeability assay for the blood–brain barrier (PAMPA-BBB) was used to assess the ability of the bioactive compounds extracted through PLE optimization to cross an artificial layer simulating the blood-brain barrier. Among the compounds extracted from artichoke by-products, flavonoids such as apigenin, luteolin, and caffeoylquinic acid derivatives like ethyl caffeate exhibited high permeability performances. Similarly, extracts from tomato by-products were rich in flavonoids such as naringenin, acacetin, and homoeriodictyol, which also showed strong potential to cross the BBB. These findings highlight the therapeutic potential of phenolic- and flavonoid-rich extracts for addressing central nervous system disorders. They also reinforce the value of agro-food waste as a source of bioactive compounds, warranting further pharmacokinetic studies and clinical investigation.

In the context of nutraceutical application, encapsulation technologies such as liposome-based systems and spray-drying were successfully applied to carotenoid-rich tomato by-product extracts. The liposomal systems tested (liposome and liposome with inulin) provided higher oxidative stability, neuroprotective potential, and sustained antioxidant potential over time. These preliminary results underscore the advantages of the encapsulation systems in maximizing the efficacy, stability, and bioavailability of bioactive compounds occurring in tomato by-products extract. Moreover, this technology represent a concrete alternative for the development of novel functional foods and nutraceuticals, particularly those targeting oxidative stress and neurodegenerative conditions, while also contributing to the sustainable valorization of agro-industrial by-products.

Tomato by-products extracts were also used as active principle into biobased active sprays formulations for food shelf-life enhancement. The development of such formulations

demonstrated enhanced oxidative stability in meat products and promising color retention effects, verified through Oxitest analysis and ΔE colorimetry assessments, respectively. Importantly, genotoxicity assessments via Ames test confirmed the safety of these novel spray systems, supporting their commercial viability and consumer acceptance.

Finally, the use of tomato by-products as natural fillers in wax-based coatings exemplifies an upcycling pathway for the reuse of tomato by-products into sustainable packaging solutions. The addition of powders obtained from tomato by-products to beeswax allowed the improvement of the hydrophobicity in terms of water uptake and contact angle of cellulose-based materials.

Together, the works presented in this chapter highlight sustainable strategies to effective exploit agro-food by-products as valuable source of bioactive compounds in several applications. The outcomes align with the principles of the circular economy, in which resource efficiency, innovation, and sustainability need to be pursued. Looking forward, further optimization and scaling of these approaches may boost a broader adoption of sustainable practices within the agro-food and bioeconomy sectors, ultimately contributing to a more resilient and regenerative production model.

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Chapter 7

CONCLUSIONS

This doctoral research comprehensively demonstrated the significant potential of agro-food by-products as valuable sustainable resources, aligning with the principles of the circular economy. The project explored analytical strategies to characterize bioactive compounds recovered from artichoke and tomato industrial residues, optimized green extraction processes, and assessed innovative applications across the food, nutraceutical, and packaging sectors.

The analytical characterization of artichoke by-products (ABP) revealed a high content of dietary fibers, including fructo-oligosaccharides (FOSs), and phenolic compounds with significant antioxidant potential. Advanced analytical techniques, such as Fourier-transform infrared spectroscopy (FTIR-ATR) and high-performance liquid chromatography (HPLC-SEC and HPAEC-PAD), provided valuable insights into the chemical composition and bioactivity of these by-products derived from various technological treatments. In collaboration with GRECI Industria Alimentare Spa, it has been estimated that approximately 200 mg of prebiotic compounds can be extracted from 100 g of fresh artichoke by-products. With an annual production of 200 tonnes of these residues, the estimated yield is 400 kg/year. These findings highlight the potential for creating new economic value while promoting the sustainable use of agro-food by-products. Additionally, these analytical methods have also been used in the Fil.Pa.Nu project to evaluate the fibers content of artichoke flour enriched pasta for the development of functional foods.

Concerning tomato by-products (TBP), complementary studies evaluated their nutritional properties, particularly focusing on Total Phenolic Content (TPC) and Total Antioxidant Capacity (%TAC) after different drying process. Seasonal abundance of TBP generated during industrial processing, prompted investigations into innovative storage methods, emphasizing the influence of drying temperature and packaging on bioactive compounds preservation. Carotenoid levels, including β-carotene and lycopene, were also monitored using UHPLC-QqQ/MS to assess their stability and retention during storage under different conditions.

Optimization by Response Surface Methodology (RSM) of pressurized liquid extraction (PLE), proved to be crucial in enhancing the recovery efficiency of bioactive compounds and sustainability of bioactive compounds extraction. Comprehensive analytical characterization, including spectrophotometric and enzymatic inhibition assays, revealed that the extracts demonstrated strong inhibitory effects against enzymes linked to Alzheimer's disease pathology, such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and lipoxygenase (LOX). An untargeted metabolomic analysis using UHPLC-Q-TOF-MS/MS revealed a wide range of phenolics and flavonoids in PLE extracts obtained through optimized conditions, which highlighted anti-inflammatory and neuroprotective properties. The PAMPA-BBB assay indicated that several compounds could effectively cross the blood-brain barrier. In silico molecular docking simulations also showed significant interactions between the active sites of AChE, BChE, and LOX with the predominant flavonoids in the extracts, reinforcing their potential as therapeutic agents.

The research also explored practical applications of agro-food by-products in different fields. Encapsulation techniques were developed to stabilize and enhance the bioavailability of carotenoids-rich extract for nutraceutical and food applications. A bio-based antioxidant spray, acting as active packaging, successfully extended the shelf life of perishable foods (meat and vegetable-based product), offering a natural alternative to synthetic additives in food industry. Additionally, sustainable coatings formulation including tomato by-products powders, improved the hydrophobicity and functionality of cellulose-based materials, contributing to advancements in eco-friendly packaging solutions.

These innovations highlight the dual role of agro-food by-products in promoting environmental sustainability while creating economic value. By integrating scientific advancements with industrial applications, this research demonstrates the opportunities offered by transforming waste into high-value resources.

Altogether, this work proved that agro-food industrial residues could be transformed from unemployed materials into resources of high industrial relevance, contributing concretely to the circular bioeconomy model.

FUTURE PROSPECTS

Agro-food by-products represent an undervalued resource with incredible potential for valorisation in a context of circular economy, addressing critical environmental and economic challenges. Based on the results of this research, future developments need to be considered to advance the valorization of agro-food by-products. Building upon the results of this doctoral thesis, several future developments can be envisioned to advance the valorization of agro-food by-products.

A priority will be to scale up environmentally friendly extraction technologies, such as Pressurised Liquid Extraction (PLE) previously presented, from laboratory to industrial scale, ensuring environmental and economic sustainability through comprehensive life cycle assessments and techno-economic analyses. However, a critical aspect that must be considered on these technological advancements is the rigorous evaluation of the safety and toxicological aspects associated to the agro-food by-products. Indeed, they are chemically complex matrices that may contain residual pesticides, heavy metals, mycotoxins, or microbial contaminants arising from agricultural practices, storage, or processing. Therefore, future investigations must intensify efforts on comprehensive risk assessment protocols, including contaminant profiling, microbiological screening, and advanced toxicological studies, such as cytotoxicity and mutagenicity assays, to ensure the safety and regulatory compliance of products destined for food, nutraceutical, or packaging applications.

Furthermore, although the in vitro antioxidant and neuroprotective activities reported in this thesis are promising, in vivo validation through animal models and clinical trials remains essential to confirm bioavailability and health benefits in real biological systems. Another key direction involves the development of advanced encapsulation and delivery systems, such as nanostructured lipid carriers and bio-based hydrogels, to further enhance the stability and controlled release of bioactive compounds. Expanding the valorization approach to other industrial residues - such as citrus peels, grape pomace, and cereal brans - would also allow broader application and promote a more comprehensive circular economy model.

In the field of packaging, the integration of bioactive extracts into bio-based films to create smart packaging solutions, capable of antioxidant release or spoilage detection, represents

a highly innovative prospect. In our case, innovative solutions such as active sprays and sustainable coating formulations offer significant advantages in packaging, providing environmentally friendly and functional alternatives to conventional materials. However, as previously discussed, the incorporation of bio-additives derived from agro-food by-products into existing products is still considered challenging due to technological and safety concerns that require multidisciplinary cooperation and continuous technological advancement to overcome.

Looking ahead, the development of innovative packaging solutions within a circular economy will be a key focus for an innovative start-up company created in collaboration with the University of Parma, ULISSE Solutions Srl, of which I am a founding partner. This effort is aimed at advancing sustainable packaging technologies, while strengthening collaboration with local companies to promote continuous technology transfer through the promotion of innovative approaches for industrial applications.

LIST OF PUBBLICATIONS DURING PhD PROGRAM

Scientific Publications

1. Messinese, E., Pitirollo, O., Grimaldi, M., Milanese, D., Sciancalepore, C., & Cavazza, A. (2023). By-Products as Sustainable Source of Bioactive Compounds for Potential Application in the Field of Food and New Materials for Packaging Development. Food and Bioprocess Technology 2023, 1, 1–22. https://doi.org/10.1007/S11947-023-03158-2

2. Pitirollo, O., Messinese, E., Grimaldi, M., Barbanti, D., & Cavazza, A. (2025). Effects of a Biobased Antioxidant Gel on Meat Shelf-Life: Oxidative Stability and Color as Quality Parameters. Gels 2025, Vol. 11, Page 279, 11(4), 279.vhttps://doi.org/10.3390/GELS11040279

3. Messinese, E., Valdes, A., Cavazza, A. & Cifuentes, A.: "Exploring Neuroprotective Potential of Bioactive Compounds Obtained from Artichoke By-Products by Pressurized Liquid Extraction (PLE) via Response Surface Methodology (RSM)" (under submission)

4. Pitirollo, O., Gallo, M., Fontanarosa M., Messinese, E., Grimaldi, M., Milanese, M., Sciancalepore, C. & Cavazza, A.: "A review on sustainable coating systems for the development of hydrophobic cellulose materials for food packaging" (under preparation)

Contributions to Congresses During PhD Program

1. **Poster presentation:** Messinese, E., Pitirollo, O., Grimaldi, D., Cavazza, A.: *"Analytical assessment of meat shelf life: evaluation of the effect of novel biobased antioxidant treatments*" - XXIX Congresso della Divisione di Chimica Analitica della Società Chimica Italiana (SCI); 11-15 Sept 2022

2. **Oral communication, presenting author:** Messinese, E., Pitirollo, O., Grimaldi, D., Cavazza, "Agro-food by-products as sustainable resource: analytical approaches for bioactive compounds characterization" – 4° Workshop: I chimici per le Biotecnologie; 01 July 2022

3. **Poster contribution:** Cavazza, A., Pitirollo, O., Messinese, E. & Grimaldi, M. (2023). "Analytical evaluation of wooden and biobased items for food contact", 3rd International CIRCUL-A-BILITY CONFERENCE: Re-thinking Packaging for Circular & Sustainable Food Supply Chains of the Future, 11 – 13 Settembre 2023, Madrid, Spagna.

4. **Poster contribution:** Cavazza A., Mattarozzi M., Messinese E., Pitirollo O., Grimaldi M., Piergiovanni M., Riboni N., Bianchi F., Careri M., "Integrated analytical approach for the characterization of bio-based food contact materials XXX Congresso della Divisione di Chimica Analitica della Società Chimica Italiana, 17 – 21 settembre 2023, Vasto, Italia.

5. **Poster contribution:** Messinese E., Pitirollo O., Grimaldi M., Darecchio E., Ornaghi P., Manzoli M., Cavazza A., "Analytical assessment of meat shelf-life: evaluation of the effect of novel bio-based antioxidant treatments", XXX Congresso di Chimica degli Alimenti, 29 – 31 maggio 2023, Marsala, Italia.

6. **Co-author of oral communication:** Cavazza A., Grimaldi M., Messinese E., Milanese D., Pitirollo O., Sciancalepore C., Corradini C., "Integrated strategies against food waste: byproducts exploitation for shelf-life extension and packaging development", XXX Congresso di Chimica degli Alimenti, 29 – 31 maggio 2023, Marsala, Italia.

7. **Co-author of oral communication:** Pitirollo O., Messinese E., Grimaldi M. & Cavazza A., "How to reuse agro-industrial by-products as new source for novel applications". 4th Symposium on Circular Economy and Sustainability, 19 – 21 Giugno, 2023, Heraklion, Greece.

8. **Co-author of oral communication:** Pitirollo O., Fontanarosa M., Messinese E., Grimaldi M. & Cavazza A., "SPRAY4PACK: active edible packaging to prolong food shelf-life", 3rd International CIRCUL-A-BILITY CONFERENCE: Re-thinking Packaging for Circular & Sustainable Food Supply Chains of the Future, 11 – 13 September 2023, Madrid, Spagna.

9. **Co-author of oral communication:** Grimaldi M., Sciancalepore C., Nottiani D. G., Cavazza A., Pitirollo O., Messinese E., Milanese D., "NEATMAT: development of biocomposite materials

using natural fillers", 3rd International CIRCUL-A-BILITY CONFERENCE: Re-thinking Packaging for Circular & Sustainable Food Supply Chains of the Future, 11 – 13 Settembre 2023, Madrid, Spagna.

10. **Co-author of oral communication:** Pitirollo O., Messinese E., Fontanarosa M., Grimaldi M., Cavazza A.: "Development of food packaging in the context of circular economy", 5th Symposium on Circular Economu and Sustainability, 17-19 June 2024, Chania, Greece.

11. **Co-author of oral communication:** " Cavazza A., Pitirollo O., Grimaldi M., Messinese E., Fontanarosa M., "Technology transfer from the synergy between academic research and industry: development of innovative packaging in a context of circular economy", XXVIII Congresso Nazionale della Società Chimica Italiana, 26 – 30 August 2024

12. **Oral communication, presenting author:** Messinese E., Cavazza A., Valdes A., Cifuentes A.: "Optimization of an innovative approach for green extraction of bioactive compounds from artichoke and tomato industrial by-products", XXIII Meeting of the Spanish Society of Chromatography and Related Techniques, 23 – 25 Oct 2024, Pamplona, Spain.

13. **Co-author of oral communication:** Pitirollo O., Fontanarosa M., Grimaldi M., Marino C., Messinese E., Riboli M., Sciancalepore C., Manconi E., Tamborrini P. & Cavazza A. "New packaging materials from agro-industrial by-products", ILSI - 8th International Symposium on Food Packaging, 1- 4 Apr 2025, Dubrovnik, Croatia.

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