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PhD Thesis

ENHANCEMENT OF WASTE DERIVED FROM AGRO-INDUSTRIAL ORIGIN

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PREFACE

This Ph.D. thesis is submitted as a requirement for obtaining the Ph.D. Degree in “Nutraceuticals, Functional Foods and Human Health” at the University of Naples Federico II (Italy). It is written based on three years of research conducted by the author, Lidia Ciriaco, at the Department of Pharmacy in Naples, under the supervision of Prof. Paolo Grieco (tutor), at Damor Farmaceutici (Naples) under the supervision of Dr. Barbara Maglione (co-tutor), and at the Department of Analytical Chemistry in Granada (Spain) under the supervision of Prof. Ana María García Campaña and co-supervised by Dr. Laura Carbonell-Rozas and Dr. Alfonso Narváez.

Lidia Ciriaco

Naples, October 2025

"La cosa importante è di non smettere mai di interrogarsi. La curiosità esiste per ragioni proprie. Non si può fare a meno di provare riverenza quando si osservano i misteri dell'eternità, della vita, la meravigliosa struttura della realtà. Basta cercare ogni giorno di capire un po' il mistero. Non perdere mai una sacra curiosità."

Albert Einstein

ABSTRACT

Triticum vulgare, or *aestivum*, is an annual herbaceous plant belonging to the genus *Triticum*, family *Poaceae*. The aqueous extract of *Triticum vulgare* (TVE) is obtained from the whole germinated plants, and it is involved in tissue repair processes, stimulating chemotaxis and fibroblast maturation. During the production process of this aqueous extract, a significant quantity of solid and liquid waste is produced, and to date, no purpose has been found for these wastes. Waste management is an emerging challenge, and agro-industrial residues offer opportunities beyond traditional disposal.

My PhD project focused on the analysis and characterization of wastes from TVE production (Damor Farmaceutici, Italy), with the aim to valorize them according to circular economy principles (Reduce, Reuse, Recycle).

In the first part of the project, I analysed and characterised, from a chemical-physical and biological point of view, the solid waste (SW) and liquid waste (LW), to assess their biocompatibility and possible reuse with a view to sustainable valorisation. Advanced techniques, including ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS), revealed that ferulic acid was the predominant phenolic compound in SW (89.782 mg/kg), while dihydroferulic acid prevailed in LW (6.24 mg/L). These compounds represented 55.87% and 84.39% of the total bioactive compounds detected in the solid and liquid

fractions, respectively. Antioxidant activity was higher in SW (8.598 mmol Trolox/kg in ABTS; 7.262 mmol Trolox/kg in FRAP), consistent with its higher phenolic content (TPC). Finally, cytotoxicity assessment was conducted using *in vitro* preclinical models of human skin (HaCaT and HDFa lines) by exposing cells to the samples for 24, 48 and 72 hours. No cytotoxic effects were observed even at the highest concentration tested (100 µg/mL) at 72 h.

Based on these promising results, during the second part of the PhD project, I focused more attention on the solid waste. Although traditional extraction techniques, such as organic solvent extraction, are widely used for their efficiency, they present different disadvantages such as long extraction times, high solvent consumption and environmental drawbacks.

In order to overcome these limitations and to reduce environmental impact, new-generation solvents such as natural deep eutectic solvents (NADES) have recently emerged. Eight different NADES formulations with varying physicochemical properties (pH, polarity, and viscosity) were investigated. The extraction efficiency was assessed through ferulic acid recovery analysis by UHPLC-Q-ToF-HRMS. The highest recovery of ferulic acid was achieved with the NADES composed of betaine:1,3-butanediol:water (1:3:10.6, molar ratio), attributed to its moderate pH, low viscosity, and water content. The extraction process parameters were optimized through Response Surface Methodology (RSM). Results showed that the extraction yield was comparable to that obtained using a conventional hydroalcoholic mixture. Non-targeted

analysis revealed the presence of different bioactive compounds, depending on the extraction method used.

Overall, this work highlights the potential of wheat-based pharmaceutical waste as a source of bioactive compounds. Solid waste, rich in ferulic acid and antioxidants and free of cytotoxic effects, shows the most promising characteristics, while liquid waste, although less concentrated, remains a source of valuable molecules. The study also demonstrates the feasibility of NADES as a sustainable and effective alternative to traditional solvents, enabling the production of ready-to-use extracts for food and nutraceutical applications.

In conclusion, the results of my PhD thesis underline the possibility and importance of valorising waste by exploiting its bioactive compounds to reduce environmental impact and promote health benefits.

The thesis is structured as follows. **Chapter 1**, organized into six sections and related subsections, introduces the general framework of waste management and by-product valorization, with a focus on bioactive compounds, wheat-derived wastes, and extraction/characterization techniques. **Chapter 2** outlines the research objectives, while **Chapters 3 and 4** present the experimental results for the specific aims. **Chapters 5 and 6** provide reflections and conclusions, respectively. Finally, **Chapter 7** serves as the Appendix.

A complete list of abbreviations is provided at the end of the thesis.

LIST OF PUBLICATIONS

The present work has been reflected in 2 different articles published within the following academic journals:

Paper I

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Paper II (Submitted)

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Other publications:

1. Di Lorenzo, E., Romano, F., **Ciriaco, L.**, Iaccarino, N., Izzo, L., Randazzo, A., Musto, P. and Di Maio, E. (2025). **Periodic cooking of eggs.** *Communications Engineering*, [online] 4(1).
2. Di Matteo, A., Paolillo, A., **Ciriaco, L.**, Silva, J.L. da, Pascale, S.D. and Izzo, L. (2025). **Effect of Cooking and in vitro Digestion on the Polyphenols and Antioxidant Properties of Asparagus officinalis L. cultivars.** *Foods*, 14(13), p.2367.

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Chapter 1

Introduction

Waste management is a worldwide problem and nowadays it's a topic of great interest, which affects both the environment and human health (Roy *et al.*, 2023). The introduction chapter provided a comprehensive overview of the topic, focusing on the growing production of agro-industrial waste.

It dealt with the main environmental and economic challenges associated with waste management, highlighting the urgency of adopting more sustainable practices. A central theme was the strategic importance of by-product valorisation in the framework of a circular economy, which aims to transform waste into valuable resources. Therefore, this chapter proposed a review of the most relevant examples of valorization strategies reported in recent literature, together with a discussion of the classification, chemical composition of agro-industrial waste and its bioactive potential. Particular focus will be placed on wheat, one of the most widely cultivated crops worldwide, highlighting both its economic relevance and the potential applications of wheat-derived extracts. Furthermore, classical and innovative extraction techniques for bioactive compounds will be discussed, with specific attention to sustainable approaches and the use of green solvents, such as natural deep eutectic solvents (NADES).

1.1 Waste management and circular economy: General Background and Research Impact

Waste management is described as the set of strategies and methods developed to recognize, regulate and manage various forms of waste throughout their lifecycle, from initial production to final disposal. Effective application of practices such as reduction, reuse and recycling can significantly reduce environmental impacts, taking into account both direct emissions and the waste of resources (Mubaslat, 2021). As a consequence, successful waste management can be considered the first step towards a circular economy, where materials are no longer considered waste, but exploitable and precious resources (MacArthur, 2013).

Circular economy (CE) refers to a type of economy that concerns industrial processes and economic activities in order to restore or regenerate products by eliminating waste and maintaining their higher value (Roy *et al.*, 2023). It is in contrast with the linear economy approach, based on a “take-make-dispose” model (Geisendorf and Pietrulla, 2018). Early contributions include the book written by Boulding, who emphasised its necessity to ensure human survival on Earth (Boulding, 1973) and later, Pearce and Turner corroborated that the classical linear economy must be replaced by a circular system (Pearce and Turner, 1989). In Europe, the first CE policies date back to Germany with the Waste Management Act of 1972 (BGBI I S. 873) (Licht, 1972) and the concept of extended producer responsibility (Andersen, 2007). Nowadays, CE is often regarded as an innovative model that integrates economic, social, and

environmental dimensions of sustainable development and involves all actors in the economic system to encourage them to adopt more sustainable practices (Planing, 2015; Ghisellini, Cialani and Ulgiati, 2016).

On an operational level, this economy is applied both on a micro scale, as in individual enterprises, and on a macro scale, i.e. throughout the entire economic system, and its adoption requires specific skills and appropriate decision-making tools (Golinska *et al.*, 2015). One comprehensive definition of CE is provided in the European Union's Circular Economy Action Plan: "In a circular economy the value of products and materials is maintained for as long as possible; waste and resource use are minimised, and resources are kept within the economy when a product has reached the end of its life, to be used again and again to create further value" (European Commission, 2015).

The concept of CE is frequently associated with the 3R principles: "R's: reduce, reuse and recycle" (Madaan *et al.*, 2024). Reduction concerns eco-efficiency, i.e. creating value by reducing environmental impact (Hupples and Ishikawa, 2005), with indirect benefits for social welfare as well (Ness, 2008). Reuse implicates designing goods for disassembly and remanufacturing at a local scale, also to reduce transport and packaging (Stahel, 2013), supported by consumer and business involvement (Lenzen *et al.*, 2007; Watson, 2008). Recycling alludes to the reuse of waste materials for new applications, but excluding energy recovery from waste (European Union, 2008). Finally, the CE also encourages the employment of renewable energy (Ellen MacArthur Foundation, 2015; European Commission, 2015).

However, the circular economy is often reduced to waste management based on the 3Rs, neglecting fundamental aspects such as product design, waste prevention at source, and efficient and responsible resource consumption (Ghisellini, Cialani and Ulgiati, 2016). It is therefore essential to apply the principles of the circular economy and the 3Rs throughout the entire life cycle of materials to preserve their value and retain resources within the economic system. In short, the circular economy must be regenerative, that is, capable of renewing resources and materials and avoiding waste and depletion.

In recent times, the circular economy has become a fast-growing sector that has attracted considerable research interest. Scientific research is increasingly concentrating on creating innovative methods for resource recovery and valorization, as well as waste reduction through circular strategies.

From anaerobic digestion, for the conversion of organic waste into renewable energy (Ragazzi *et al.*, 2017), to the cultivation of microalgae for the production of high-value products (Rahman, 2020) and extraction of bioactive compounds from different waste (Rifna, Misra and Dwivedi, 2023), research provides concrete solutions to minimise environmental impact, recover valuable resources and limit the use and consumption of natural resources.

1.1.1. The Growing production of Agro-Industrial waste

Agro-industrial waste (AIW) refers to the wide range of organic and inorganic residues generated throughout agricultural production and food processing activities. This includes by-products such as peels, husks, shells, stems, and other non-edible materials. According to the European Commission (2008), it is essential to distinguish between “waste”, i.e. materials that have no purpose or value at the time of their generation, and “by-products”, which, although secondary to the main output, may have an economic or functional value and be reintegrated into other production cycles (European Parliament and Council, 2008). The term “food waste” broadly encompasses losses and discards, occurring across the entire food supply chain, while food loss (FL) mainly occurs in the upstream stages of the food chain, such as sowing, cultivation, harvesting, initial processing, industrial processing and distribution (Azelee *et al.*, 2020; Espro *et al.*, 2021).

Global waste generation has become a major issue due to the growth of the global population and the corresponding increase in demand for agricultural and food products (Thyberg and Tonjes, 2016). The Food and Agriculture Organization of the United Nations (FAO) estimates that one-third of all food produced for human consumption worldwide is lost or wasted, approximately 1.6 billion tonnes per year (FAO, 2011), with fruits, vegetables, roots, and tubers accounting for up to 50% (Ravindran *et al.*, 2018). More recent data indicate that in 2022 alone, 1.05 billion tonnes of food were wasted, corresponding to 19% of food available to consumers, while an additional 13%

was lost along the supply chain (FAO, 2022; UNEP, 2024b). Households are the main contributors, responsible for about 60% of global food waste (631 million tonnes), compared to 28% from food services (290 million tonnes) and 12% from retail (131 million tonnes). On average, each person wastes 79 kg of food per year, enough to provide 1.3 meals a day to every hungry person (UNEP, 2024a). The FAO further assessed that food waste is distributed across different stages of the supply chain: farming (11–23%), industrial manufacturing (17–19%), retail distribution (8–17%), and end-user consumption (over 50%) (FAO, 2019; Amicarelli and Bux, 2021).

All these data are collected in the Food Waste Index (UNEP, 2024b), which analyses updated global data on waste, proposing multi-stakeholder cooperation through Public Private Partnerships (PPP) as a possible solution. This report, first published in 2021, monitors national progress to reduce food waste by half by 2030 in line with Sustainable Development Goals.

The Sustainable Development Goals (SDGs) are 17 global objectives adopted in 2015 by all United Nations Member States to promote global prosperity, protect the environment, and ensure the well-being of future generations by 2030 (Agenda 2030) (Moyer and Hedden, 2020). These goals are applied worldwide, guiding sustainable development policies in both developed and developing countries. In particular, SDG 12.3 aims to halve *per capita* global food waste by 2030.

Beyond the agri-food system, industrial sectors also contribute significantly to global waste generation. In the pharmaceutical sector, waste generation is

estimated to be between 25 and 125 times the weight of drugs produced, totalling 18 million tonnes per year (Belal, Shukla and Balasubramanian, 2025).

This highlights the significance of waste generation not only in food-related systems but also in all industrial processes, emphasising the importance of developing effective waste management strategies to reduce environmental and human health impacts.

1.1.2. Environmental and Economic Issues Related to Waste Management

Improper waste management is a major global environmental threat, contributing to soil and water pollution, biodiversity loss and climate change (UNEP, 2021; Mohajan, 2025). Globally, approximately 38 per cent of municipal solid waste (MSW) is not treated safely, but managed through uncontrolled landfilling or unregulated incineration, causing the emission of Methane (CH₄) and carbon dioxide (CO₂), two powerful greenhouse gases (GHGs) and also black carbon and non-methane volatile organic compounds (UNEP, 2024a). For every tonne of waste disposed of, about 28.4 kg of methane is released, a gas with 25 times the warming potential of CO₂ (Kumar and Samadder, 2022). On a global scale, landfills are responsible for 3% - 9% of anthropogenic methane emissions (Aryan *et al.*, 2023).

In addition to MSW, agricultural waste also contributes significantly to environmental degradation. Indeed, in developing countries, this waste is

also often burned or abandoned, with negative impacts on human, animal and environmental health (Perwez and Al Asheh, 2025).

Among the various waste streams, food waste (FW) has received growing attention due to its substantial environmental impact and complex treatment options. The environmental impact of FW varies according to the treatment technology used. The most impactful method is landfilling, followed by composting, incineration and anaerobic digestion (AD), while use as animal feed is the least impactful (-347 kg CO₂ eq/tonne) (Moult *et al.*, 2018; Roy *et al.*, 2023). In fact, industrial exploitation of FW in the US could avoid 190 million tonnes CO₂ eq/year (RedCorn, Fatemi and Engelberth, 2018).

Decentralised AD, with domestic use of biogas, is the option with the lowest environmental impact (-238.4 kg CO₂ eq/tonne) (Tian *et al.*, 2021). Anaerobic digestion offers advantages over incineration, which is often inefficient due to the high moisture content of FW. Composting and the use of food waste as animal feed also provide notable environmental benefits, while thermal treatments can still provide significant benefits, particularly in terms of energy recovery (Guven, Wang and Eriksson, 2019).

The environmental footprint is also high in other industrial sectors. The extraction and processing of raw materials generate environmental costs estimated at between EUR 400-5000 billion per year, derived mainly from greenhouse gas emissions, acidification and particulate matter (Arendt, Bach and Finkbeiner, 2022). Concerning the nutraceutical and food supplements industrial field, the production of nutraceutical products can cause

deforestation, heavy water consumption and plastic waste, but the choice of recycled plastic packaging reduces the demand for new plastic, containing greenhouse gas emissions and pressure on natural resources (European Environment Agency, 2021). The pharmaceutical sector contributes about 52 Mt CO₂ annually, which is the equivalent of emissions from 11 million cars, while also generating more than 300 Mt of single-use plastics. Moreover, the release of APIs (Active Pharmaceutical Ingredients) into aquatic environments contributes to antibiotic resistance and contamination of aqueducts and soils near factories (Belkhir and Elmeligi, 2019; United Nations Environment Programme (UNEP), 2019).

In addition to environmental damage, the mismanagement of waste also has a significant economic impact, with high costs. The global cost of food waste is enormous, with estimates reaching \$680 billion in developed countries and \$310 billion in developing countries (Roy *et al.*, 2023). Thus, treatment costs per tonne vary widely depending on the technology used: they range from \$79/t for incineration, \$89/t for composting, up to \$122/t for anaerobic digestion (AD), and \$138/t for landfilling (Slorach *et al.*, 2019).

However, it is also possible to have an economic viability thanks to the treatment of these wastes, which depends on the methods of valorisation adopted. For example, anaerobic digestion (AD), when used only to produce methane, may be unprofitable (Chen *et al.*, 2017). Conversely, when biogas is fed into the electricity grid or used in transport, the economic benefits increase significantly. A study by Guo and Yang (2019) shows that an AD plant capable

of processing 200 tonnes of waste per day can generate more than USD 10,500 per day, thanks in part to public subsidies (Guo and Yang, 2019).

A notable example of this variability is the Hong Kong International Airport, where on-site incineration emerged as the most efficient solution, combining the highest energy recovery with the lowest life-cycle cost (HKD 461.73/tonne) (Lam *et al.*, 2018). Decentralised composting may also be more cost-efficient (~\$100/t), while centralised systems often face operational limitations (Pai, Ai and Zheng, 2019).

Beyond individual technologies, integrated strategies such as multi-stream biorefineries are emerging as particularly promising from both economic and environmental perspectives, enabling the production of high-value products from FW, with far greater returns than traditional uses like energy, feed, or fuel (Tuck *et al.*, 2012; Engelberth, 2020). Nevertheless, tackling the full scope of environmental and economic impacts linked to waste requires a broader, systemic approach (Sund *et al.*, 2025). In Europe, emissions from facilities such as chemical factories and coal-fired power stations are estimated to cause annual externalities ranging from EUR 268 to EUR 428 billion, equivalent to nearly 2% of the EU's GDP (Gross Domestic Product) (European Environment Agency, 2024). The estimated annual lifecycle costs of plastics alone, including indirect effects on the environment, human health, and the economy, are close to \$4 trillion worldwide (European Environment Agency, 2024).

According to European legislation, the pharmaceutical and cosmetics industries are obliged to pay up to 80 per cent of the urban wastewater

treatment costs (EUR 1.2 billion per year). This not only applies the ‘polluter pays’ principle but also drives innovation, pushing companies to reduce water consumption, reduce waste, and adopt more sustainable practices, aligning economic responsibility with environmental sustainability (European Commission, 2022).

In conclusion, circular economy strategies offer a way to align environmental, economic and social benefits (Čuček, Klemeš and Kravanja, 2012; Ghisellini, Cialani and Ulgiati, 2016).

1.1.3. The Strategic Role of By-Product Valorization in a Circular Economy Framework

One of the most pressing environmental issues of the 21st century is the sustainable reuse of biomass from agro-industrial wastes for the manufacture of food, bioactive compounds, supplements, biomaterial medications, and energy from renewable sources (Freitas *et al.*, 2021).

The conversion of these by-products into reusable materials could contribute significantly to pollution mitigation (Mo *et al.*, 2018; Omo-Okoro, Daso and Okonkwo, 2018). The agricultural and food sectors generate large amounts of waste, which can be transformed into raw materials for innovative products, stimulating a more sustainable production model (Ng *et al.*, 2020).

In the framework of the circular economy, waste is redefined from a burden (with a negative perception) to a potential resource to be reintegrated into

production cycles. In this perspective, technologies such as composting, anaerobic digestion for biogas production, or the development of new sustainable materials assume a strategic role.

The main advantages of sustainable waste management include:

- Reduction of raw material consumption;
- Containment of climate-changing emissions;
- Recovery of useful nutrients for agriculture (UNEP, 2024a);
- Stimulation of innovation and environmental awareness.
- Growth of employment opportunities in recycling and reuse industries.

On the waste management scale, preventing *a priori* waste formation and encouraging its reuse continue to be the most effective measures, avoiding more expensive and energy-intensive procedures.

The reuse of agro-industrial waste represents a significant economic advantage, as it allows low- or zero-cost by-products, which involve disposal costs for companies, to be transformed into high-value-added ingredients (Banerjee *et al.*, 2017). Numerous case studies confirm the economic feasibility of this approach. For example, pectin and d-limonene can be extracted from orange peels, generating net profits of up to \$10.5 million from 50,000 metric tonnes of biomass, even considering the minimal cost of the raw material (Kleofas *et al.*, 2014). Another concrete example of industrial valorisation is the production of starch and pectin from mango residues in India, processing just 6% of national production is valued at around USD 7 million, while 12,000

tonnes of pectin alone could reach about USD 118 million, with a quality comparable to commercial grades (Sandhu and Lim, 2008; Nagel *et al.*, 2014). Furthermore, the interest of important companies, for example in the cosmetics field, such as L'Oréal, The Estée Lauder Companies and many others, in polyphenols extracted from fruit waste, used in the formulation of innovative cosmetic products, demonstrates how the valorisation of organic waste can translate into new, highly profitable market opportunities, in addition to the ethical significance of this practice (Krzyżostan, Wawrzyńczak and Nowak, 2024).

Overall, it is emphasized once again that the valorization of agro-industrial waste emerges as a strategic driver for sustainability, promoting innovation while generating environmental, economic, and social benefits within the framework of a circular economy.

1.1.4. Classification of Wastes: Agricultural, Food, and Pharmaceutical Origins

Waste classification is an essential step for the efficient management, recovery and sustainable treatment of waste materials. In particular, this categorisation by origin makes it possible to identify the environmental criticalities and valorisation potential associated with each type.

1. *Agricultural Waste*

Agricultural waste results from different activities, such as cultivation, livestock farming, agro-industrial primary processing and rural household life (Anastopoulos and Kyzas, 2014; Mo *et al.*, 2018). According to FAO (2019) classification and recent studies (Wang and Tester, 2023), these wastes can be distinguished into:

- Field residues: plant waste such as straw, stubble, leaves, stems, and twigs left in the fields after harvesting (Aravani *et al.*, 2022);
- Process residues: husks, pomace, cores, seeds, roots, molasses, straw, bagasse and bran resulting from the processing of agricultural products (Senthilkumar *et al.*, 2020);
- Farming wastes: manure, urine, wash water and livestock slurry, which can generate harmful gases and environmental contamination if not properly treated (Bala *et al.*, 2023);
- Aquaculture wastes: non-consumed feed and organic materials excreted by animals, which contribute to water pollution (Hashim *et al.*, 2023).

In addition, the improper use of pesticides, herbicides and fertilisers leads to hazardous waste, such as contaminated containers and residual substances, often disposed of inappropriately, with serious consequences for soil, water and human health (Rajmohan, Chandrasekaran and Varjani, 2020). The valorisation of agricultural waste, which is often rich in cellulose, lignin and hemicellulose, includes its use for the production of bioenergy, organic

fertilisers and animal feed, although these resources are still largely underused today (Senthilkumar *et al.*, 2020).

2. Food Waste

Food waste, as previously mentioned, is divided into two main categories: food loss (FL), which occurs during the production and distribution chain due to process inefficiencies, and food waste generated at the retail, household, or food service levels (UNEP, 2024a).

A further distinction refers to the composition of these wastes. Edible fractions refer to food that, despite being suitable for consumption, is nonetheless discarded, whereas inedible fractions comprise the unavoidable components, such as peels, bones, shells, and kernels.

Food waste can also come from the agri-food industry, which produces solid, liquid and gaseous waste containing organic substances such as carbohydrates, proteins and lipids. If managed correctly, this waste can become a valuable source of bioenergy or be used for the extraction of bioactive compounds and the development of new high-added-value products (Barba *et al.*, 2019).

3. Pharmaceutical Waste

Pharmaceutical waste, although quantitatively smaller, is among the most environmentally critical due to the presence of persistent, poorly degradable and potentially toxic active ingredients. They originate from various sources:

pharmaceutical industries, healthcare facilities and pharmacies (Kadam *et al.*, 2016). They can be classified as:

- Hazardous waste: cytotoxic drugs, antibiotics, hormones, chemotherapeutic agents and some controlled substances that require special treatment (Dar, Maqbool and Rasool, 2019);
- Non-hazardous waste: expired or unused nutraceuticals or over-the-counter medications (e.g. functional foods and analgesics), considered to have a low environmental impact;
- Chemo waste: waste from cancer therapies, containing highly active residues and contaminants.

Besides medicines, contaminated materials such as syringes, drips, masks, PPE, bottles, contaminated absorbents and pharmaceutical containers also fall into this category (Kümmerer, 2010). Residual APIs are frequently detected in aquatic environments, even after conventional treatments in sewage treatment plants, with removal rates below 10% for compounds such as atenolol and carbamazepine (Berthod *et al.*, 2017). This can compromise water quality, soil fertility and ecosystem balance (Sreekanth *et al.*, 2014).

In conclusion, a systematic classification is essential to plan effective waste management strategies, with the aim of minimising environmental impacts, maximising resource recovery, and fostering more sustainable waste management practices across different sectors.

1.2 Valorization Strategies in Recent Literature

Valorization is an environmentally friendly and sustainable process that turns waste into valuable products (Pathak *et al.*, 2022). Agricultural waste must be modified in a more feasible and effective way using specific valorization procedures to be used appropriately. Therefore, it is first necessary to apply a series of treatments to overcome the structural complexity of lignocellulosic materials and allow their chemical analysis (Dey *et al.*, 2021). In fact, cellulose, hemicellulose, and lignin compose the majority of the non-edible portion, which includes product off-cuts like leaves, stems, and other lignocellulose portions. Pre-treatments, which are essential at this stage, may include physical processes (such as grinding, ultrasound, pyrolysis, microwaves, irradiation, steam explosion), chemical (wet oxidation, ozonolysis, acid or alkaline hydrolysis) and/or biological (use of enzymes or microorganisms) (Prasad, Padhi and Ghosh, 2023).

In the last decades, the valorization of agro-industrial waste has taken a central role in the development of sustainable processes, with applications in numerous industrial sectors, including food and nutraceuticals, pharmaceuticals and cosmetics, energy, chemicals, the environment, advanced materials and tissue engineering (Kumar *et al.*, 2022).

Food and Nutraceutical Sectors

The food and nutraceutical sectors represent one of the most consolidated applications for waste and by-products. In particular, the peels of different fruits, such as pomegranate, grape, and apple, among others, processed into powder, have been used as natural colouring agents in functional foods (Goula *et al.*, 2017; Neha Gupta *et al.*, 2019). Carotenoids extracted from tomatoes and melons have shown beneficial effects on the skin and immune system (Benmeziane *et al.*, 2018). Other residues have been applied for the formulation of new functional foods, including soybean residues, rich in protein, fiber and isoflavones (Nile *et al.*, 2020), and edible mushroom waste, which represents a source of polysaccharides and bioactive compounds with immunostimulant and anti-inflammatory properties (Wang, 2020). Polyphenols extracted from different waste matrices, such as fennel, pea pods and broad bean hulls, have, in some cases, been encapsulated in gastro-resistant capsules, improving bioavailability and bio-accessibility (Castaldo, Izzo, *et al.*, 2021; Castaldo, Izzo, Gaspari, *et al.*, 2022; Castaldo, Izzo, Lombardi, *et al.*, 2022). Furthermore, pectin extracted from lemon peel is also used in nanotechnology to encapsulate bioactive compounds (Panwar *et al.*, 2021). Red orange by-product shows high amounts of anthocyanins (in particular, cyanidin-3-glucoside), flavanones, and hydroxycinnamic acids. Its extracts demonstrated antioxidant activity and the reduction of lipid accumulation in HepG2 cells, suggesting a role against hepatic steatosis (Chiechio *et al.*, 2021; Burò *et al.*, 2022).

Spent coffee ground (SCG) and tea residues have a high concentration of polyphenols, melanoidins and caffeine, with marked antioxidant activity, and SCG has also been incorporated into enriched biscuits, maintaining sensory characteristics comparable to conventional products (Zuorro and Lavecchia, 2012; Castaldo, Lombardi, *et al.*, 2021). Another source of theobromine and caffeine is represented by Cocoa bean husks (Mazzutti *et al.*, 2018).

In the bakery sector, the integration of waste such as pomegranate peels, goji berry residues and tegument has enabled the production of muffins and biscuits enriched in polyphenols, fiber and protein, without compromising their sensory characteristics (Chávez-Santoscoy *et al.*, 2016; Bora, Ragaee and Abdel-Aal, 2019).

The use of vegetable oils from by-products is also growing. For example, grape seed oil, derived from grape pomace, has shown positive effects on the lipid profile in animal models, increasing HDL (high-density lipoprotein) and reducing LDL (low-density lipoprotein) (Beres *et al.*, 2017).

Numerous industrial initiatives evidence the interest in exploitation on a commercial scale (Espro *et al.*, 2021). In Denmark, the company Kaffe Bueno recovers antioxidants and fatty acids from SCG by means of supercritical CO₂ extraction, with the goal of reaching an annual production of 1,200 tonnes by 2026 (Choi and Koh, 2017). In Norway, Repolar Pharmaceuticals uses spruce resin from naturally damaged trees to produce Abilars, a healing ointment based on resin acids and lignans with antimicrobial properties (Jokinen and Sipponen, 2016).

In Italy, the company H&AD uses bergamot waste for the extraction of polyphenols through ultrafiltration and absorption, obtaining the active ingredient Bergacyns, whose clinical efficacy has been documented for its hepatoprotective effect (Musolino *et al.*, 2020).

Pharmaceutical sector

Beyond food and nutraceutical applications, agro-industrial residues are also increasingly recognised as valuable sources for the pharmaceutical sector. Lignocellulosic residues (LRs) and food loss (FL) are gaining increasing interest as sustainable sources for the production of Bioactive Compounds (BACs) and APIs due to their richness in functional molecules, but also for the development of innovative delivery systems.

Mango peels, for example, have been used for the extraction of pectin, which is employed in the formulation of biodegradable release systems (Chaiwarit *et al.*, 2020), while silk proteins have been used to produce electrospun nanofibers capable of sustained release of lafutidine (Nangare *et al.*, 2020). Extracts from seeds and skins of Pinot noir grapes have shown antimicrobial activity, suggesting their possible use in the prevention of intestinal infections (Klančnik *et al.*, 2017). By-products from different *Citrus* species exhibit hepatoprotective, anti-inflammatory, anti-ageing and anti-cancer properties due to the presence of flavonoids, essential oils and tannins (Mahato *et al.*, 2018). In addition, phenolic extracts from potato peels and bran have demonstrated anti-inflammatory, antimicrobial and immunomodulatory

properties (Fermoso *et al.*, 2018; Joly *et al.*, 2020). Compounds with antiproliferative activity, obtained from mango seeds and active on HT-29 tumour cells, also confirm the industrial interest in waste employment (Ballesteros-Vivas *et al.*, 2019).

Bio-based precursors can also be used in the synthesis of conventional APIs. Furosemide can be obtained from furfurylamine, derived from furfural from lignocellulosic residues (Minio *et al.*, 2024); the same furfural can then also be used to synthesise ancrolol (a beta-blocker). Paracetamol, on the other hand, can be produced from p-hydroxybenzamide derived from lignin, or from β -pinene, a by-product of the paper industry (Hallamasek, Ubbenjans and Lapkin, 2024). Phenylephrine can be synthesised from cardanol, derived from cashew nutshell liquid, avoiding complex functional protection steps (Shi, Kamer and Cole-Hamilton, 2019).

Several APIs derived from renewable resources are already listed in prominent international pharmacopoeias, including Ph. Eur. and USP-NF. Among these, succinic acid, produced by fermentation, is used in the synthesis of prodrugs and pharmaceutical formulations (Jansen and van Gulik, 2014). L-(+)-lactic acid, obtained by fermentation of sugars, is approved for pharmaceutical, veterinary, food and hygiene applications (Li *et al.*, 2021). Non-essential amino acids such as aspartic and glutamic acid, as well as polyols such as glycerol, sorbitol and xylitol, are now also obtained from biomass and find use as excipients, osmotic agents and laxatives, respectively (Takkellapati, Li and Gonzalez, 2018).

Further applications include bioactive peptides extracted from protein by-products, such as meat and milk processing residues, which have shown biological regulatory activities, suggesting potential use in antioxidant and antimicrobial formulations (Lemes *et al.*, 2016). Metabolites derived from redox enzymes present in plant residues have also proven useful as biomarkers for neurodegenerative or cardiovascular diseases and for the potential treatment of neurodegenerative disorders (Yang and Lee, 2015; Angeloni *et al.*, 2023).

Finally, agricultural wastes such as peanut shells, cobs and cocoyam husks have proven to be valuable substrates for the fermentative production of antibiotics such as tetracycline, rifamycin B and oxytetracycline by specific strains of *Streptomyces* (Asagbra, Sanni and Oyewole, 2005; Kashif and Shahid, 2022).

Cosmetics and skin-care treatments

In line with the European Green Deal directives and the growing demand for bio-based formulations, the cosmetic industry has adopted increasingly sustainable strategies, including the extraction and reuse of agro-food waste (Krzyżośtan, Wawrzyńczak and Nowak, 2024).

Agro-food waste, including fruit peels, seeds and stones, grape marc, olive pomace, sugar cane bagasse, coffee grounds, and wheat straw, is recognized as a source of valuable compounds (Osorio, Flórez-López and Grande-Tovar, 2021).

Among concrete examples, the pomace waste from *Arbutus unedo* L., rich in polyphenols, has been exploited as a natural ingredient in the development of skincare products against oxidative stress and premature ageing (Di Lorenzo *et al.*, 2025). Peels of *Citrus reticulata* yield essential oils with strong antibacterial properties, in some cases superior to those of synthetic antibiotics in the treatment of acne. Citrus peels also have anti-melanogenic properties (Bae *et al.*, 2012; Hou *et al.*, 2019). Vine by-products, such as stems and shoots, are rich in resveratrol, a phenolic compound with anti-ageing and depigmenting properties (Ferreira, Bottini and Fontana, 2023), and brewer's spent grain is a source of ferulic acid, used in regenerative and photoprotective cosmetics, often delivered through liposomal nanosystems (Bucci *et al.*, 2020).

Various plant-derived residues, including peels from tomato, potato, avocado, various fruits and beet leaves, are notable sources of phenolic acids such as caffeic and chlorogenic, which exhibit strong antioxidant properties and contribute to UV protection (Martínez-Inda *et al.*, 2023). Extracts from mango peels and tomatoes, owing to their high content of vitamins and phenolic compounds, are widely employed in formulations aimed at enhancing skin defense mechanisms and mitigating oxidative stress (Dias *et al.*, 2021). Similarly, extracts of pomegranate by-products, rich in flavonoids, tannins, and fatty acids such as punicic acid, have demonstrated anti-inflammatory, antimicrobial, brightening, and regenerative activities (Ko, Dadmohammadi and Abbaspourrad, 2021).

Fermentation, is an effective technology for converting waste into biocompatible cosmetic ingredients: *Citrus unshiu* peels fermented with *Schizophyllum commune* yields extracts with photoprotective properties (Bae *et al.*, 2012), while tilapia skins (and other fish waste) fermented with *Lactobacillus*, have been used for collagen production, with stimulating effects on cell proliferation without cytotoxicity (Khan *et al.*, 2023).

Furthermore, Costa *et al.* identified bioactive peptides from yeast residues (*Saccharomyces cerevisiae*) normally used for β -farnesene production, with the ability to modulate relevant skin biomarkers (Costa *et al.*, 2023).

By-products and waste of the coffee industry are very interesting and widely studied. Extracted compounds such as chlorogenic acids and caffeine exert anti-inflammatory, antimicrobial and anti-ageing effects, also contributing to photoprotection and stimulation of hair growth (Rodrigues, Oliveira and Alves, 2023). They are used in scrubs and moisturisers and are also the subject of patents for anti-ageing, moisturising and photoprotective applications (de Mello *et al.*, 2023).

Other fruit and vegetable by-products present several properties and recovery opportunities: banana peels are a source of starches (Bhavani *et al.*, 2023); carrot peels, rich in β -carotene, offer anti-ageing effects and are used as natural pigments (Ikram *et al.*, 2024); potato peels, containing chlorogenic acid, show antibacterial and antioxidant activities and, when fermented, can produce biosurfactants and lactic acid (Ansari *et al.*, 2014) (Kowalczewski *et al.*, 2022). Apple residues, which account for about 25 per cent of the fruit's

weight, are rich in pectin, triterpenes, phenolic acids and flavonoids, useful for their antioxidant, antibacterial and anti-inflammatory actions, used in the management of acne, wounds and the regulation of sebum production (Barreira, Arraibi and Ferreira, 2019). Avocado waste (seeds, peel and spent pulp) is also rich in fiber, polyphenols and monounsaturated fatty acids such as oleic and palmitoleic, which are used in protective and photoprotective oils (Araújo *et al.*, 2018). Olive tree wastes, including leaves, pomace and vegetation waters, contain compounds such as hydroxytyrosol and oleuropein, known for their antioxidant, antimicrobial and anti-inflammatory properties, which strengthen the skin barrier and protect against UV rays, proving useful in the treatment of atopic dermatitis, psoriasis and eczema (Dauber *et al.*, 2023).

Finally, grape marc, consisting of seeds, skins and stems, is rich in polyphenols, unsaturated fatty acids and vitamins and has been widely exploited as a functional ingredient in anti-ageing and antioxidant products (Teixeira *et al.*, 2014). In the dairy sector, whey (a by-product) is a source of protein and fermentable lactose, used to moisturise and regenerate the skin (Alfano *et al.*, 2022).

In conclusion, the use of agro-industrial wastes represents a strategy that can improve the bioavailability and efficacy of cosmetic formulations, while reducing environmental impact.

Livestock sector/feed industry

Besides its use for the pharma sector and cosmetics, agro-industrial waste is gaining attention in the livestock and feed industry as it represents one of the most sustainable strategies to reduce organic waste while obtaining alternative raw materials for feed production (Malenica, Kass and Bhat, 2023). García *et al.*, (2005) examined the chemical and microbiological compositions of several solid organic waste types, including meat, fish, vegetables, and household trash, as well as the presence of dioxins, furans, polychlorinated biphenyls, and mineral content. The results showed that fruit and vegetable by-products might be used to prepare animal feed. Subsequently, Esteban *et al.*, (2007) confirmed the possible use of these wastes as an alternative to traditional raw materials in pig feed, thanks to high carbohydrate content (~65%), although the high fiber content may limit digestibility and suggested a drying pre-treatment due to the high water content (~88%) in vegetable by-products.

San Martin, Ramos and Zufía, (2016) assessed the feasibility of reusing vegetable waste from the food industry in feed production, analysing its safety, nutritional value, drying technology and environmental impact. After the drying process, these by-products are suitable for livestock use, although coffee and wine residues require attention due to the presence of nitrites. According to EPEA Technical Committee, their homogenous composition allows for their unified management, with up to 6% inclusion in feed. From

an environmental point of view, the process is sustainable if optimised, especially compared to practices such as landfill or incineration.

Several residues have shown specific benefits. Pumpkin residues used in animal feed have good nutritional value and are rich in bioactive compounds, including carotenoids, polyunsaturated fatty acids, proteins, vitamins, minerals and polysaccharides. Incorporating pumpkin waste into ruminant diets has demonstrated beneficial effects on milk and meat composition, increasing the levels of lutein, violaxanthin, and α - and β -carotene. It also exhibits anti-inflammatory, antimicrobial, and antioxidant properties that improve animal health (Valdez-Arjona and Ramírez-Mella, 2019; Boldea *et al.*, 2021).

In a recent study, Kanakai *et al.*, (2023) included pellets based on fermented agro-industrial waste (composed of fermented citrus residues with yeast by-products), adding 4 % to 6 % of these pellets into the diets of beef cattle, and found a significant increase in protein absorption, digestibility, ruminal microbial population and propionate concentration, suggesting a sustainable potential for the valorisation of agro-industrial waste as a formulated feed.

In conclusion, agro-food by-products represent a valuable alternative for feed production, provided that adequate treatments such as drying are applied to ensure safety, nutritional quality and digestibility.

Energy sector

To produce energy, lignocellulosic wastes, including rice straw, sugar cane bagasse, and maize wastes, are frequently utilized. Biomass is commonly classified into two groups: woody biomass and non-woody biomass. Woody biomass, consisting of compact lignocellulosic materials, is traditionally used for thermal energy production in industrial and domestic settings, while non-woody biomass is employed in various energy conversion processes as a result of its simpler preparation. Conventional combustion generates electricity through the release of high-pressure steam in boilers, while gasification of straw and maize produces syngas, a mixture mainly of CO and H₂, which may be utilized for both mechanical and thermal energy (Maitlo *et al.*, 2022). The pyrolysis process, on the other hand, allows lignocellulosic residues to be transformed into aromatic compounds, generating bio-oil (a liquid fuel), biochar (used in agriculture as a soil conditioner), and gases such as CO, CO₂, H₂, and CH₄ (Dai *et al.*, 2020).

Moreover, pyrolysis also allows non-woody biomass to be converted into fuel oils, while anaerobic digestion and fermentation are used to obtain hydrogen and biogas from wet biomass (Akbarian-Saravi, Mobini and Rabbani, 2020). In addition, biomass rich in polysaccharides (starch, pectin, cellulose, chitin) is used for the production of bioethanol through enzymatic hydrolysis, fermentation and saccharification (Kumar *et al.*, 2020).

Chemical sector and biorefineries

In addition to energy production, agro-food waste can be converted into building blocks and/or chemical intermediates for the synthesis of chemical compounds. Biorefineries represent an integrated model that can convert waste into sugars, oils, chemical compounds, energy and bioactive molecules. Especially agricultural countries like Brazil have successfully implemented this model (Ahmad *et al.*, 2020; Fonseca, Costa and Cruz, 2020).

Chemical transformation of biomass permits the obtaining of compounds such as furfural, polysaccharides and polyols. For example, furfural, derived from hydrodistillation in an acidic environment, can be converted into solvents or precursors for pesticides and drugs (Zang, Shah and Wan, 2020). Some by-products, such as cholesterol extracted from meat industry by-products such as brains and bone marrow, are used for the synthesis of vitamin D3 (Jayathilakan *et al.*, 2012).

Biomedical and tissue engineering

Numerous waste products have also been exploited for the production of tissue engineering scaffolds. Scaffolds containing regenerated cellulose nanofibers have shown good results in terms of cell adhesion and proliferation for bone engineering (Chakraborty, Adhikari and Saha, 2019). Hydrogels from coconut shell or celery cellulose have been used for the controlled release of fatty acids (Thinkohkaew, Rodthongkum and Ummartyotin, 2020; Yan *et al.*, 2020).

Environmental sector

Beyond biomedical uses, agro-industrial residues have also been explored for environmental purposes, like as water purification. Indeed, lemon peel can remove up to 89% copper ions from industrial wastewater, offering a low-cost and environmentally sustainable solution (Meseldzija *et al.*, 2019).

Innovative materials and packaging

Finally, innovative materials and packaging represent another promising field, where agricultural and food waste can be exploited. Biodegradable biomaterials have been produced from lignin, cellulose, and hemicellulose found in wastes like shells, straw, or bagasse.

Bioaerogels from corn straw, rice husks and wheat have been developed for oil absorption and pharmacological applications (Rajanna *et al.*, 2015; S.-W. Liu *et al.*, 2016). Biofilms from bagasse and maize leaves were found to be flexible and biodegradable, with potential to replace conventional plastics (Bhardwaj *et al.*, 2020). Bionanocomposites from gelatine and cellulose and composites with nanofibers from pineapple or rice straw have been used for food packaging or drug delivery (Xu *et al.*, 2018; Mahardika *et al.*, 2019).

Overall, these recent studies show that agro-industrial and pharmaceutical waste can be valuable sources of high-value compounds exploitable in different industrial sectors and reintroduced in new production cycles.

However, more work is necessary to improve recovery technologies and make them scalable.

1.3 Bioactive compounds

Bioactive compounds are substances naturally present in plants and foods that can interact with biological systems, influencing physiological processes and contributing to the maintenance or improvement of human health (Biesalski *et al.*, 2009). Their remarkable structural diversity stems from the wide range of functional groups, such as hydroxyl, carbonyl, alkyl, and aromatic moieties, that characterize plant metabolites and determine their specific physicochemical and biological properties (Roessner and Beckles, 2008). Among these, phenolic compounds represent one of the most abundant and widely distributed classes, with over 8,000 structures identified in the plant kingdom (Durazzo *et al.*, 2019). In addition to polyphenols, there are also carotenoids, which constitute a large group of isoprenoid pigments synthesized by plants, algae, and certain species of fungi, bacteria, and insects. They play an important role in photosynthesis and photoprotection, contributing to the characteristic colouration of many fruits and vegetables (Maoka, 2020). Glucosinolates, on the other hand, are sulphur-containing compounds found mainly in *Brassicaceae* species. Once hydrolyzed, they release biologically active substances such as isothiocyanates and indoles,

known for their antioxidant, anti-inflammatory, and chemoprotective properties (Miękus *et al.*, 2020).

Furthermore, phytochemicals are not only found in edible plant tissues, but are also present in agro-industrial by-products, which represent an important but often overlooked reservoir of bioactive molecules.

1.3.1. Chemical insights into phytochemicals: polyphenols

Phenolic compounds are one of the most significant classes of phytochemicals with recognized antioxidant activity. The presence of one or more hydroxyl groups and at least one aromatic ring characterizes their chemical structure, which can range from simple molecules to complex polymers with larger molecular weights. These molecules are the main secondary metabolites of plants, which are synthesised via the shikimic acid pathway (Figure 1), with the enzyme phenylalanine ammonia-lyase facilitating the conversion of the aromatic amino acid phenylalanine into phenolic compounds (Santos-Sánchez *et al.*, 2019). Other pathways involved in the biosynthesis of polyphenols include the pentose phosphate pathway and the phenylpropanoid pathway.

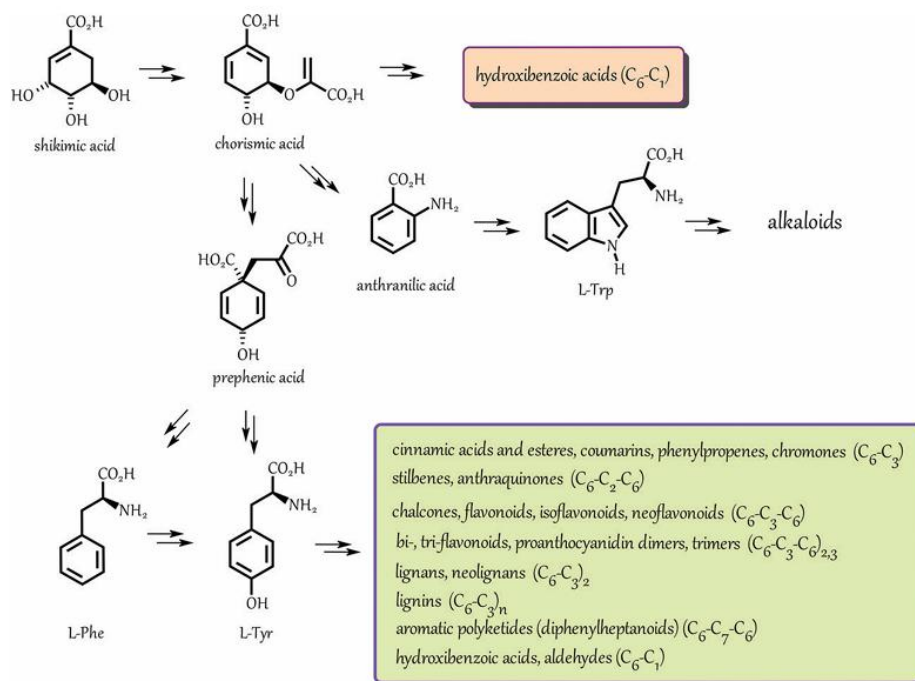


Figure 1. Shikimic and chorismic acids are precursors for the synthesis of amino acids L-Phe, L-Tyr, and L-Trp and different phenolic compounds – from Ref. (Santos-Sánchez *et al.*, 2019).

Polyphenols comprise several classes, can be categorized into two principal groups based on their chemical structure: flavonoids and non-flavonoids.

The flavonoid class comprises flavanols, flavones, anthocyanidins (often found in the form of glycosides), isoflavonoids, and neoflavonoids. Instead, the non-flavonoid class consists of phenolic acids (divided into hydroxybenzoic and hydroxycinnamic acids), lignans, tannins, and stilbenes (Sagar *et al.*, 2018).

Flavonoids are one of the most widespread classes of plant polyphenols (over 4,000 flavonoids identified to date) (Ciupei *et al.*, 2024). They are characterized by a common carbon skeleton consisting of two aromatic rings (A and B) connected by a three-carbon bridge that forms the central heterocyclic ring (C), constituting the characteristic (C₆-C₃-C₆) backbone, also known as the phenyl-benzopyran structure, shown in Figure 2 (Panche, Diwan and Chandra, 2016).

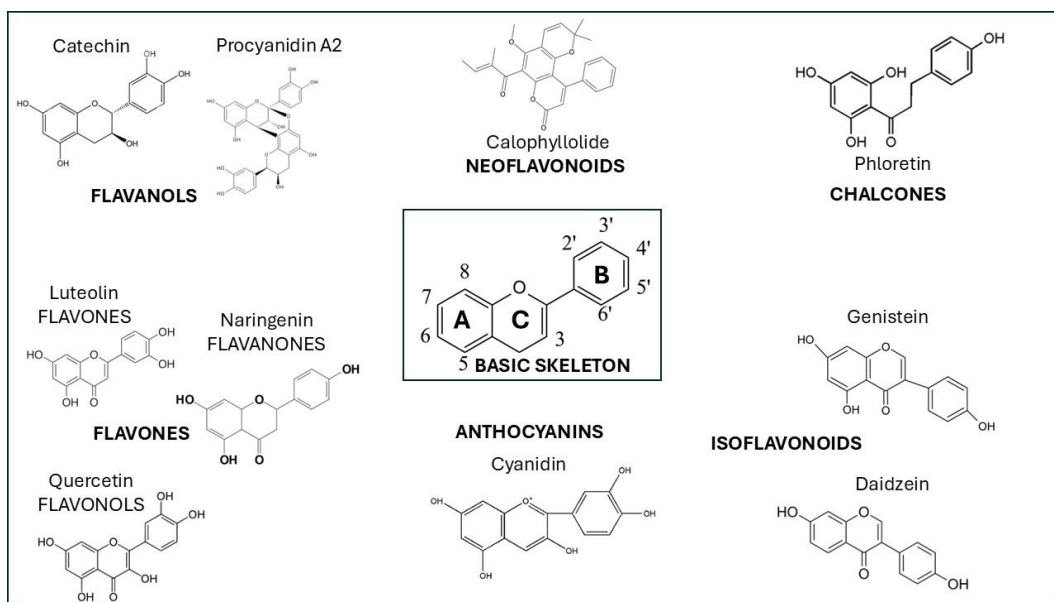


Figure 2. Basic chemical skeleton of flavonoids (C₆-C₃-C₆) and representative compounds of their main subclasses.

From a structural perspective, flavonoids can be categorized based on the location of ring B attachment as well as the degree of oxidation and unsaturation of the central heterocyclic ring. They are frequently divided into a number of subclasses, including flavanols, flavones, anthocyanins, isoflavonoids, and neoflavonoids, according to these characteristics. Some of these categories comprise other subclasses. For example, flavones include similar substances like flavanones and flavonols. According to Durazzo *et al.*, (2019), each subclass has unique structural characteristics that dictate its existence in plant tissues, physicochemical behaviour, and particular biological activities.

Flavanols, flavan-3-ols or catechins

Flavanols, also known as flavan-3-ols or catechins, are a subgroup characterized by the presence of a hydroxyl group at position 3 of the C ring and the absence of a double bond between positions 2 and 3. Their basic structure derives from 3,3',4,5,7-pentahydroxyflavone, which exists in several stereoisomeric forms, such as (+)-catechin and (–)-epicatechin. These compounds can also form conjugates with gallic acid, giving rise to derivatives such as epigallocatechin and epicatechin gallate.

Flavanols are widely distributed in nature and are particularly abundant in tea leaves, cocoa, grapes, apples, and berries, as well as in nuts, legumes, and red wine. In plant tissues, they often occur in monomeric or polymeric form, consisting of the condensation of flavan-3-ol units. In this case, they are

known as proanthocyanidins, which contribute to pigmentation and defence mechanisms (Panche, Diwan and Chandra, 2016; Durazzo *et al.*, 2019).

Flavones (Flavonols, Flavanones, Flavones)

These three subclasses share the characteristic flavonoid backbone but differ in the degree of oxidation and saturation of the heterocyclic C ring, as well as in the number and position of hydroxyl and methoxy substituents.

Flavones are characterized by a double bond between positions 2 and 3 and a ketone at position 4 of the C ring. They are commonly found as glycosides and are abundant in leaves, flowers, and fruits. Typical dietary sources include celery, parsley, red peppers, chamomile, and citrus peels, which are rich in polymethoxylated flavones such as tangeretin, nobiletin, and sinensetin. Among the most representative flavones are luteolin and apigenin, widely distributed across vegetal foods (Manach *et al.*, 2004).

Flavonols, structurally similar to flavones, differ by the presence of a hydroxyl group at position 3 of the C ring, which can be further glycosylated. They are among the most ubiquitous flavonoids in the diet and occur in onions, apples, grapes, berries, kale, tomatoes, tea, and red wine. The most studied include quercetin, kaempferol, myricetin, and fisetin (Panche, Diwan and Chandra, 2016).

Flavanones, also referred to as dihydroflavones, are structurally distinguished by the absence of a double bond between positions 2 and 3, which makes the C ring saturated. They occur mainly in citrus fruits, where

they contribute to the bitter flavour of peels and juices. Major compounds of this class are hesperetin, naringenin, and eriodictyol, which are often present as glycosidic derivatives in plant matrices (Durazzo *et al.*, 2019).

Anthocyanins

Anthocyanins are pigments, readily soluble in aqueous media, mainly localized in the outer cell layers and responsible for the vivid red, purple, and blue shades of many fruits, flowers, and vegetables. Structurally, they are derived from the flavylium ion (2-phenylchromenylium) and often are in the form of glycosides or acylated, where organic acids such as p-coumaric, caffeic, or ferulic acid are linked through ester bonds. The most common anthocyanidins, the aglycone forms, include cyanidin, delphinidin, malvidin, pelargonidin, and peonidin (Durazzo *et al.*, 2019).

Isoflavonoids

Isoflavonoids are characterized by the attachment of the B ring at position 3 of the heterocyclic C ring, rather than at position 2 as in other subclasses. This structural feature confers them unique biological roles in plants. Isoflavonoids have a restricted distribution in nature, occurring mainly in soybeans and other leguminous species, though small amounts have also been detected in some fruits, vegetables, and cereals (Panche, Diwan and Chandra, 2016). In the plant kingdom, these compounds serve essential functions, acting as precursors for phytoalexins, which are defensive molecules. The most studied

isoflavones, such as genistein, daidzein, biochanin A, and glycitein, are often present as glycosides and contribute to plant-microbe interactions and defence mechanisms (Wang *et al.*, 2022).

Chalcones

Chalcones represent open-chain flavonoids characterized by two aromatic rings connected by a three-carbon α,β -unsaturated carbonyl bridge. They act as key biosynthetic precursors of several flavonoid subclasses, such as flavanones and flavones, and occur naturally in various fruits, vegetables, and legumes (Panche, Diwan and Chandra, 2016).

Neoflavonoids

Neoflavonoids, unlike other flavonoids that have a molecular structure based on 2-phenylchromen-4-one, have a molecular structure based on 4-phenylchromen without a hydroxyl group in position 2. The first neoflavone isolated from natural sources was calophyllolide from the seeds of *Calophyllum inophyllum* (Garazd, Garazd and Khilya, 2003).

Phenolic acids - Hydroxycinnamic and hydroxybenzoic acids

Phenolic acids are non-flavonoid polyphenols characterized by a single aromatic ring functionalized with a carboxyl group and one or more hydroxyl substituents (Figure 3). They are often found in plants conjugated with amides, esters, or glycosides, which affect their solubility and bioavailability

(Ciupei *et al.*, 2024). From a structural point of view, they are classified into two main subgroups: hydroxybenzoic acids, derived from benzoic acid with a C₆–C₁ skeleton, and hydroxycinnamic acids, derived from cinnamic acid with a C₆–C₃ skeleton.

Hydroxybenzoic acids include gallic, vanillic, syringic, and p-hydroxybenzoic acids, while the main hydroxycinnamic acids are caffeic, ferulic, sinapic, and p-coumaric acids. These compounds are widely distributed in the plant kingdom and are found in fruits, vegetables, cereals, legumes, herbs, coffee, and other foods (El-Seedi *et al.*, 2012; Călinoiu and Vodnar, 2018). In plant tissues, hydroxybenzoic acids are generally found in free form, especially in fruits and vegetables, while hydroxycinnamic acids usually occur in bound form, as esters with glucose or quinic acid, or conjugated with amino acids and peptides (Tsimogiannis and Oreopoulou, 2019).

Among hydroxycinnamic acids, chlorogenic acids (CGAs) (esters of caffeic and quinic acids) are particularly abundant in coffee beans, potatoes, and certain fruits, making coffee one of the main dietary sources of CGAs worldwide (Clifford *et al.*, 2017). Other phenolic derivatives that share the same C₆–C₃ phenylpropanoid skeleton include curcuminoids, coumarins, and chromones. Curcumin, the main component of *Curcuma longa* (turmeric), is the best-known curcuminoid; coumarins occur naturally in plants such as cinnamon and sweet clover, where they act as phytoalexins; while chromones, found for example in *Ammi visnaga*, represent the structural core of numerous biologically active molecules (Venkata Sairam *et al.*, 2016; Durazzo *et al.*, 2019).

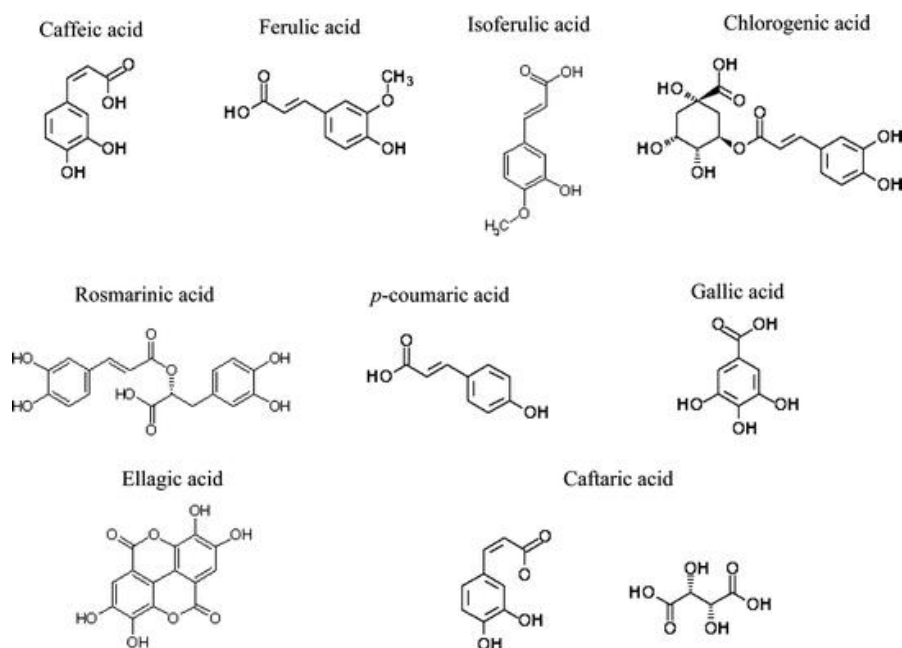


Figure 3. Principal structures of phenolic acids – from Ref. (Lafay and Gil-Izquierdo, 2008).

Xanthoness, stilbens, lignans, and tannins

Xanthoness, stilbenes, lignans, and tannins are among the non-flavonoid polyphenols (Kabera *et al.*, 2014; Durazzo *et al.*, 2019). Xanthoness have a very stable and symmetrical dibenzo- γ -pyrone (xanthene-9-one) scaffold. They are typical of mangosteen (*Garcinia mangostana*) and mango (*Mangifera indica*), as well as other tropical species (their interaction with multiple targets is favoured by their tricyclic aromatic system) (Gutierrez-Orozco and Failla, 2013).

Stilbenes have a $C_6-C_2-C_6$ skeleton with a central double bond, mostly in the E configuration. They are widely distributed in grapes and grape derivatives (grapes, wine), peanuts, and small red and blue fruits. Their diversity of substitutions and polymerization affects absorption and metabolism (El Khawand *et al.*, 2018; González-Laredo *et al.*, 2018).

Lignans derive from the union of two phenylpropanoid units (C_6-C_3) with a $\beta-\beta'$ bond ($C8-C8'$). The main dietary sources of lignans include seeds (flax, sesame), whole grains, legumes, berries and, to a lesser extent, beverages such as coffee, tea and wine (Durazzo *et al.*, 2018). After ingestion, plant lignans are converted by the microbiota into enterodiols and enterolactones (bioactive enterolignans), with the yield influenced by the microbial composition and the presence of fiber (Li *et al.*, 2018; Sawane *et al.*, 2022; Berenshtein, Okun and Shpigelman, 2024).

Tannins include both hydrolyzable forms (such as gallotannins and ellagitannins) and condensed forms, also known as proanthocyanidins, already described among flavan-3-ols. They are widespread in legumes, fruit (especially berries), nuts, and extracts of grape seeds and pine bark; phlorotannins (oligo/polymers of phloroglucinol) appear in brown algae (Serrano *et al.*, 2009; Arapitsas, 2012).

1.3.2. Biological activities and health benefits associated with polyphenol intake

Polyphenols constitute a broad and diverse class of bioactive compounds of plant origin, widely studied for their role in the prevention and therapeutic support of numerous chronic degenerative diseases. Their biological activities derive from a combination of structural characteristics, bioavailability, and metabolic transformations that modulate their systemic efficacy (Luca *et al.*, 2020; Ciupei *et al.*, 2024).

Antioxidant and anti-inflammatory role

The antioxidant role is one of the main mechanisms of action of polyphenols. These compounds can convert reactive oxygen species (ROS) into non-radical species (Figure 4). In order to reduce oxidative stress in cells, they interact synergistically with endogenous antioxidants such as glutathione, glutathione peroxidase, superoxide dismutase, catalase, and dietary vitamins (Engwa, Nweke and Nkeh-Chungag, 2022).

The polyphenols also have anti-inflammatory effects by modulating signalling pathways involved in the immune response, such as suppressing NF- κ B activation and reducing the synthesis of pro-inflammatory cytokines and lipid mediators (Ciupei *et al.*, 2024).

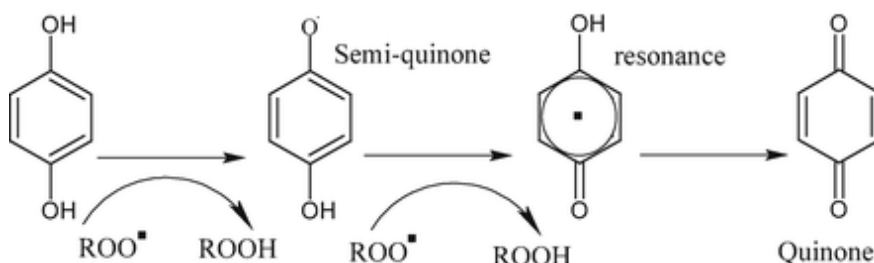


Figure 4. Antioxidant action mechanism of polyphenols – from Ref. (Smetanska, 2018).

Prevention of cardiovascular and metabolic diseases

Numerous epidemiological and clinical studies indicate a correlation between high polyphenol intake and a reduced risk of cardiovascular disease. The protective effect is attributed to improved endothelial function, blood pressure modulation, and improved lipid profile, partially mediated by increased nitric oxide bioavailability and reduced lipoprotein oxidation (Tresserra-Rimbau *et al.*, 2014; Medina-Remón *et al.*, 2017). In particular, flavanols, stilbenes, and lignans are associated with a reduction in cardiovascular mortality and hypertensive risk, while hydroxycinnamic acids and flavones exhibit vasoprotective actions (Grosso *et al.*, 2018).

With regard to metabolic disorders, it has been shown that various polyphenols contribute to improving insulin sensitivity and glycaemic control. Flavanones, isoflavones, and stilbenes, including resveratrol and genistein, have been shown to have beneficial effects in reducing the incidence

of type 2 diabetes by activating insulin-dependent signalling pathways and reducing oxidative and inflammatory stress (Rienks *et al.*, 2018). Regular consumption of foods rich in polyphenols is also associated with a lower risk of metabolic syndrome and obesity, thanks to their synergistic action on lipid metabolism and appetite regulation (Adriouch *et al.*, 2018).

Antidiabetic effects and effects on the gut microbiota

Polyphenols have an antidiabetic effect, thanks to their role in glycaemic regulation by reducing glucose absorption, inhibiting α -glucosidase and α -amylase enzymes, and modulating insulin secretion (Bolat *et al.*, 2024). In addition, they act as substrates for intestinal microbial fermentation, promoting the growth of beneficial bacteria and the production of bioactive metabolites (e.g. short-chain fatty acids) with systemic effects on metabolism and inflammation.

Neuroprotective effect

Several polyphenols, including curcumin, quercetin, epigallocatechin gallate and anthocyanins, exhibit neuroprotective activity due to their ability to contrast oxidative stress, modulate neuronal inflammation and improve synaptic plasticity. Epidemiological evidence suggests that diets rich in phenolic compounds are associated with a lower risk of cognitive decline and a reduced incidence of neurodegenerative diseases such as Alzheimer's and Parkinson's (Caruso *et al.*, 2022).

Anticancer properties

In clinical trials, several polyphenols, including genistein, resveratrol, quercetin, and epigallocatechin gallate, have demonstrated chemopreventive and anticancer properties (Rothwell, Knaze and Zamora-Ros, 2017). By causing apoptosis, preventing angiogenesis, and altering the gene expression of enzymes and kinases involved in cell proliferation, these substances can impede the development and spread of tumors. Specifically, consumption of isoflavones has been linked to a lower incidence of hormone-dependent cancers, including prostate and breast cancer (Grosso *et al.*, 2017; Durazzo *et al.*, 2019).

Skin health and anti-ageing effect

Polyphenols contribute to maintaining skin health thanks to their antioxidant and anti-inflammatory actions. Compounds such as green tea catechins, curcumin and grape seed extracts can reduce UV-induced damage, stimulate collagen synthesis and improve skin hydration, proving effective in counteracting ageing processes and oxidative stress in the skin (Bolat *et al.*, 2024).

In conclusion, polyphenols have a broad spectrum of biological activities that are useful in the prevention and management of numerous chronic diseases. In addition to a diet rich in plant-based foods, the use of nutraceutical formulations can allow for the intake of higher and more standardised

concentrations of specific phenolic compounds compared to individual foods, potentially optimising their effects.

1.3.3. Properties of Chemical Compounds in Waste

Plant-derived matrices contain both macronutrients and micronutrients that collectively define their nutritional and functional characteristics. Macronutrients, mainly carbohydrates, proteins, and lipids, are generally found in higher concentrations and provide essential structural and energetic functions. Conversely, micronutrients, including vitamins, minerals, and a variety of phytochemicals such as polyphenols, carotenoids, and alkaloids, are present in smaller amounts but exert remarkable biological and physiological effects (Tiwari and Shukla, 2020).

The chemical compounds present in agro-industrial wastes, therefore, exhibit a wide range of properties (Table 1). Pomace, peels, and seeds, in particular, contain variable levels of simple sugars, dietary fibers, crude proteins, minerals, organic acids, vitamins, and phenolic compounds, depending on the plant source and processing method (Espro et al., 2021).

In the following section, the major chemical classes are summarized together with representative matrices and indicative quantitative ranges.

Dietary fibers

Dietary fiber (DF) is a fundamental component of a healthy diet for humans (Khanpit, Tajane and Mandavgane, 2025). DF are plant-derived carbohydrate polymers not digested in the human small intestine, but partially or completely fermented in the colon. They include cellulose, hemicelluloses, lignin, pectins, β -glucans, gums, mucilages, resistant starches and oligosaccharides (Narayanan and Pitchumoni, 2021). According to their solubility, DF can be divided into two categories: soluble dietary fiber (SDF), which includes pectin and gums and is more fermentable by gut flora, and insoluble dietary fiber (IDF), which primarily consists of lignocellulosic components (Radha *et al.*, 2024). The primary sources of dietary fiber are fruits and vegetables, but their direct use is restricted due to significant losses throughout the production process. A novel and successful way to address the rising demand for nutritional fiber while assisting in the reduction of environmental impact is to extract and purify fiber from fruit and vegetable waste (FVW).

About vegetable waste, the amount varies in the different parts of the vegetable. Onion peels contain up to 68% DM of dietary fibers, expressed as % of dry matter (DM), while the edible pulp has only ~12% DM (Jaime *et al.*, 2002). Potato peels provide 61–125 g/kg crude fiber, and potato pulp from starch factories is also rich in rhamnogalacturonan I (Byg *et al.*, 2012; Ncobela *et al.*, 2017). Tomato pomace, a waste generated during juice and paste production, may contain up to 50% DM, with peel-derived fractions

exceeding 80% DM (Del Valle, Cámara and Torija, 2006; García Herrera, Sánchez-Mata and Cámara, 2010). While carrot pomace contains 63% DM (50% insoluble, 13% soluble) (Chau, Chen and Lee, 2004).

In fruit waste, grape by-products, especially pomace, are consistently rich in dietary fiber in all cultivars, with a total content that is often comparable (around 70-77% of DM), but the balance between soluble and insoluble fractions varies (Llobera and Cañellas, 2008). Red pomace, like Manto Negro or Tempranillo, has higher fiber levels (up to 77% DM or 36.9 g/100 g fresh weight), while white pomace provides only about half that amount (17-28% total dietary fibers TDFs), although in both cases insoluble fiber prevails (>97%) (González-Centeno *et al.*, 2010; Deng, Penner and Zhao, 2011). Mango peels contain 40–73% DF depending on cultivar, including glucose, galactose, and arabinose (Ajila *et al.*, 2010).

Lemon waste is a source of DF, with values of 53 % for peel and higher values for pulp 77% with balanced soluble and insoluble fractions (Russo *et al.*, 2014). In contrast to lemons, apple peel contains more dietary fiber (0.91% fresh weight) than the pulp, with similar proportions of insoluble fiber (0.46% fresh weight) and soluble fiber (0.43% fresh weight) (Sagar *et al.*, 2018).

Proteins, bioactive peptides and amino acids

Proteins are essential macronutrients formed by amino acids and indispensable for growth, development and maintenance of the body.

Agri-food residues are also valuable protein sources, especially when derived from legumes, oilseeds, fruit kernels and peels, vegetable leaves, as well as fish and meat processing by-products. These proteins can be enzymatically or microbially hydrolysed to obtain bioactive peptides with antioxidant, antihypertensive (ACE-inhibitory), immunomodulatory, antimicrobial and antidiabetic functions (Chourasia *et al.*, 2023).

The peel of fruits contains protein in different percentages. Citrus peel contains 2.5–9% protein, while kiwi, avocado and papaya have lower values (1.5–1.8%) (Chitturi, Talatam and Vuppu, 2013; Mamma and Christakopoulos, 2014). Other fruit by-products include, for example, mango and pineapple (~9%), banana (~6%) and apple pomace (4–5%). Vegetable waste is generally richer than fruits, carrot pulp (10%), tomato residues (17–22%), cabbage leaves (20%), cauliflower (16%) and pea pods (20%) (Sharma, Oberoi and Dhillon, 2016).

In addition to crude protein, protein-based functional compounds have been isolated from various wastes, such as actinidin from kiwi seeds, leptin-like proteins from jackfruit and vicilin-like proteins from watermelon seeds (Wani *et al.*, 2008; Devalaraja, Jain and Yadav, 2011; Boland, 2013).

Cauliflower leaves and residues contain bioactive peptide hydrolysates, including ACE-inhibiting peptides and calcium-binding phosphopeptides, with benefits for blood pressure regulation and bone health, respectively (Montone *et al.*, 2018). In broccoli by-products, proteins and peptides constitute about 23% of bioactive compounds; enzymatic hydrolysis improves

their solubility and bioactivity, generating peptides with antioxidant and lipid-lowering effects (H. Chen *et al.*, 2020).

Lipids and fatty acids

Lipids are one of the main classes of compounds found in agri-food by-products, including triglycerides, phospholipids, and free fatty acids. Chemically, fatty acids can be classified by their degree of saturation as saturated (e.g., capric acid), monounsaturated (e.g., oleic acid), or polyunsaturated (e.g., linoleic acid). Polyunsaturated fatty acids (PUFAs) are characterised by multiple double bonds along their hydrocarbon chain. Within this group, the omega-3 series—including α -linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)—and the omega-6 series are of particular biological importance. These essential fatty acids are vital for maintaining cellular membrane integrity and supporting the development and proper functioning of the nervous system (Djuricic and Calder, 2021).

Fish processing wastes (viscera, heads and skeletons) are one of the principal sources of EPA and DHA, while oilseed by-products, such as linseed, soybean and rapeseed meal, are rich in α -linolenic acid (Rai, Bhaskar and Baskaran, 2015; Yamagata, 2017). Starchy residues from cassava, corn, potatoes and rice have also been used as fermentation substrates for the production of PUFA-enriched microbial biomass (Pandey *et al.*, 2000).

Additional unconventional matrices, such as dairy waste, spent brewer's yeast, and exhausted cooking oils, have been used for DHA biosynthesis by oil-producing microorganisms, particularly tustochytrid protists such as *Aurantiochytrium mangrovei* (Russo *et al.*, 2021; Patel *et al.*, 2022).

Vegetable oils from olives, palm, sunflower seeds, corn, walnuts and peanuts are the main sources of lipids, but fruits and vegetables such as avocados, aubergines, Brussels sprouts and parsley also have a significant fatty acid content (Silva *et al.*, 2021).

Carotenoids

Carotenoids, in plants, play essential roles in photosynthesis and photoprotection, preserving the photosynthetic apparatus against photooxidative stress (Beltrán and Wurtzel, 2025). Several carotenoids (e.g., β -carotene, α -carotene, β -cryptoxanthin) act as provitamin A precursors, thereby contributing to the immune system, epithelial integrity and vision (reducing risk of ocular disorders such as age-related macular degeneration and cataracts (Amengual, 2019)).

Food by-products represent an alternative source of carotenoids. Carrot by-products are particularly abundant in β -carotene, with reported concentrations ranging between 200–1000 mg/kg dry matter (Sharma *et al.*, 2012). Tomato pomace and peels are notable sources of lycopene, while mango peels provide 37–395 mg/100 g carotenoids depending on cultivar and ripening stage (Saini, Panesar and Bera, 2019). Citrus peels also contribute

significant amounts of carotenoids, including lutein, β -cryptoxanthin, and neochrome (Trigo *et al.*, 2022). Pineapple by-products further highlight the potential of fruit residues as carotenoid sources: β -carotene is the main carotenoid in pineapple core and peel, with concentrations of 960 and 2500 $\mu\text{g}/100\text{ g}$ dry weight, respectively (Freitas *et al.*, 2015). Lutein is also abundant in pineapple peel, ranging from 300 to 1120 $\mu\text{g}/100\text{ g}$ dry weight, alongside smaller amounts of α -carotene (89–126 $\mu\text{g}/100\text{ g}$ dry weight) (de Moraes Crizel *et al.*, 2016). Although the carotenoid profile may vary with different factors, i.e. cultivar, ripening stage, and environmental factors, pineapple residues consistently represent an important source of carotenoids (Meléndez-Martínez *et al.*, 2022).

Polyphenols and phenolic compounds

Polyphenols are abundant in fruit and vegetable residues, concentrated in the peel, rind and seeds. For example, potato peel contains higher amounts of chlorogenic and caffeic acid than the pulp, while cucumber peel is an economic source of flavonoids (Madhu Agarwal *et al.*, 2012). In citrus residues, the peel and seeds contain flavanones and phenolic acids; in the peel, ferulic, caffeic, vanillic, gallic, p-coumaric, sinapic and chlorogenic acids are reported (Rafiq *et al.*, 2018). In the peels of apples, peaches and bananas, the concentrations of polyphenols are higher than in the pulp, as well as grape seeds and skins from the wine industry, which are rich in proanthocyanidins and anthocyanins (González-Montelongo, Gloria Lobo and González, 2010;

Manfra *et al.*, 2011; Suleria, Barrow and Dunshea, 2020). Olive residues, such as leaves, vegetation water and pomace, are also valuable sources of bioactive compounds, including hydroxytyrosol, tyrosol, coumaric and ferulic acids. Oleuropein is particularly relevant; during processing, it is converted into hydroxytyrosol, a powerful free-radical scavenger (Talhaoui *et al.*, 2015).

Other phytochemicals

In addition to the main chemical constituents mentioned above, agro-wastes contain other phytochemicals. Among these, saponins (triterpenoid or steroidal glycosides), found for example in some vegetables and legumes, are particularly noteworthy for their cytotoxic, antifungal, and antibacterial properties, as well as their capacity to lower cholesterol (Wiesner *et al.*, 2017). Alkaloids, nitrogen-containing metabolites derived from amino acids, represent another significant group; one example is solanine, found in potato skins, which exhibits both pharmacological effects and potential toxicity (Vuong, 2017). Glucosinolates, sulphur-containing glycosides abundant in *Brassicaceae* by-products such as broccoli, cabbage and cauliflower, can release isothiocyanates through hydrolysis, molecules known for their anti-carcinogenic properties and for their use as biopesticides (Shinali *et al.*, 2024). Finally, phytosterols, cholesterol analogues found in fruit seeds and oilseed cakes, help reduce intestinal cholesterol absorption and are widely used in functional foods (Gachumi *et al.*, 2021).

Table 1. Main waste reported, with bioactive compounds and related health benefits.

Matrix	Waste	Bioactive compounds	Health Benefit	References
Onion	Peel	Dietary fiber	digestion, cardiovascular health	Jaime et al., 2002
Potato	Peel, pulp, starch residues	Dietary fiber, polyphenols, alkaloids (solanine)	antioxidant, gut health, pharmacological but potential toxicity	Byg et al., 2012; Ncobela et al., 2017; Madhu Agarwal et al., 2012; Vuong, 2017
Tomato	Pomace, peel	Dietary fiber, carotenoids, saponins	antioxidant, cholesterol- lowering	Del Valle et al., 2006; García Herrera et al., 2010; Sharma et al., 2012; Wiesner et al., 2017
Carrot	Pomace, pulp, by- products	Dietary fiber, β - carotene, proteins	antioxidant	Chau et al., 2004; Sharma et al., 2012; Sharma et al., 2016
Grape	Pomace, seeds, skins	Dietary fiber, polyphenols (proanthocyanidins, anthocyanins)	antioxidant, cardioprotective	Llobera and Cañellas, 2008; González- Centeno et al., 2010; Deng et al., 2011; Suleria et al., 2020
Mango	Peel	Dietary fiber, carotenoids, proteins	antioxidant	Ajila et al., 2010; Saini et al., 2019
Lemon	Peel, pulp	Dietary fiber, pectin, carotenoids	gut health, antioxidant, improved bioavailability of bioactives	Russo et al., 2014; Panwar et al., 2021; Trigo et al., 2022
Apple	Peel, pomace	Dietary fiber, polyphenols	antioxidant, gut health	Sagar et al., 2018; González- Montelongo et al., 2010
Kiwi	Seeds, peel	Actinidin enzyme, proteins	antioxidant	Boland et al., 2013
Avocado	Peel	Proteins, fatty acids	cardiovascular benefit	Mamma and Christakopoulos, 2014; Silva et al., 2021

Pineapple	Peel, core	Proteins, carotenoids	antioxidant, provitamin A	Freitas et al., 2015; de Moraes Crizel et al., 2016
Banana	Peel	Proteins, polyphenols	antioxidant	Manfra et al., 2011
Cabbage	Leaves	Proteins, bioactive peptides, glucosinolates	antihypertensive, anticancer	Sharma et al., 2016; Montone et al., 2018; Shinali et al., 2024
Cauliflower	Leaves, residues	Proteins, ACE-inhibiting peptides, glucosinolates	blood pressure regulation, bone health, anticancer	Sharma et al., 2016; Montone et al., 2018
Broccoli	By-products	Proteins, peptides, glucosinolates	antioxidant, lipid-lowering, anticancer	Chen et al., 2020; Shinali et al., 2024
Olive	Leaves, vegetation water, pomace	Polyphenols (oleuropein, hydroxytyrosol)	antioxidant, antimicrobial, cardioprotective, neuroprotective	Talhaoui et al., 2015
Red orange	By-products	Anthocyanins, flavanones	antioxidant, hepatoprotective (anti-steatosis)	Chiechio et al., 2021; Burò et al., 2022
Cucumber	Peel	Flavonoids	antioxidant	Madhu Agarwal et al., 2012
Fish	Processing waste (viscera, heads, skeletons)	EPA, DHA (PUFAs)	cardiovascular, neuroprotective	Rai et al., 2015; Yamagata, 2017
Oilseeds	Meal, residues	α -linolenic acid	cardiovascular, essential fatty acids	Rai et al., 2015
Cassava, corn, potato, rice	Starchy residues	PUFA-enriched microbial biomass	functional lipids	Pandey et al., 2000
Eggplant, Brussels sprouts, parsley	By-products	Fatty acids	cardiovascular benefit	Silva et al., 2021
Legumes	By-products	Saponins	cholesterol-lowering, antimicrobial	Wiesner et al., 2017

1.4 Wheat: an overview

Triticum aestivum, also named *Vulgare* and commonly known as soft wheat, is a member of the botanical tribe *Triticeae* in the grass family *Poaceae* (Snape and Pánková, 2013). Historically and geographically, its origins date back to between 7500 and 6500 BC (approximately 9000 years ago) in the Levant region of the Near East, where its domestication is attested in the area of the “Fertile Crescent” (mountainous regions around Mesopotamia and the Syrian desert) (Lim, 2013). In this scenario, south-eastern Turkey is one of the main centres of domestication identified by archaeological and genetic evidence (Heun *et al.*, 1997; Özkan *et al.*, 2002). It subsequently reached Europe via the south-eastern Balkans, following two main routes of expansion, one continental and one coastal (Feldman, 2001). Wheat crops are suited to a wide range of soil and climate conditions, but grow best at temperatures between 10 and 24 °C and with annual rainfall of 375-875 mm. About the botanical aspect, wheat is an annual grass that grows to an average height of 1–1.5 m, with hollow stems and linear leaves. The inflorescence (ear) is a terminal spike 4-18 cm long, and the fruit is an ellipsoidal caryopsis varying in colour from yellow to reddish-brown (Lim, 2013) (Figure 5 and Figure 6).



Figure 5. Wheat crop ready for harvest – from Ref. (Lim, 2013).



Figure 6. Detail of the ear, highlighting spikelets bearing elongated-awn lemmas –
from Ref. (Lim, 2013).

1.4.1. Economic and Industrial Relevance of Wheat

Wheat is the most widely grown crop in the world (Triennium Ending - TE2018), with approximately 217 million hectares cultivated, surpassing both corn and rice in terms of area. In terms of global production, amounting to 752 million tonnes, it is lower than that of rice and corn. Unlike these two cereals, the expansion of wheat production in recent decades has not been due to an increase in cultivated surface area, which has remained essentially stable or slightly decreased compared to the 1960s, but rather to a steady increase in yields, which rose from just over 1 t/ha in the 1960s to 3.5 t/ha in 2018, resulting in a nearly fourfold increase in global production (Erenstein *et al.*, 2022).

Wheat provides about 19% of total calorie consumption worldwide and is the main agricultural commodity traded internationally, as well as the most important food source and a strategic reserve against production shortages (Atchison, Head and Gates, 2010). According to FAO Stat, (2020), wheat cultivation is widespread in over 120 countries around the world and is particularly well-suited to temperate environments, with the ability to tolerate cold where other crops cannot survive. Geographically, Asia is the main production hub (44%), followed by Europe (34%) and North, Central and South America (15%), with marginal but significant shares in Africa and Oceania (Erenstein *et al.*, 2022). According to the OECD-FAO Agricultural Outlook 2024–2033, world trade in cereals is expected to grow by 16% by 2033, reaching 551 Mt. Wheat will contribute 35%, maize 52% and rice 7% of this

growth (OECD and Food and Agriculture Organization of the United Nations, 2024).

Industrially, wheat is exploited not only for food, for human and animal nutrition and feeding, but also in numerous other applications. In fact, wheat has various uses depending on the characteristics of its starch. Grains with a high amylose content, long chains of amylopectin and lipids produce resistant starches and slow-digesting sugars, which are useful for dietary and functional foods, capable of promoting cholesterol metabolism, managing obesity and reducing the risk of diabetes and colon cancer. On the other hand, waxy starches, which are easily hydrolysable, are ideal for the production of sweeteners and industrial alcohol, where rapid conversion to sugar is required (Shevkani *et al.*, 2017). Low-grade grain is used, for example, to make alcohol, adhesives, paper additives, soaps, rubbers, cosmetics, and varnishes (Peña-Bautista *et al.*, 2017). Grain application is reported in the beverage industry (spirits, beer) (Diekmann, 2009) but also in the ethanol industry to produce bioethanol (Rusănescu *et al.*, 2024). Wheat straw is also widely employed for the extraction of hemicellulose, which in turn has various industrial purposes. It can be converted into biofuels through hydrolysis, used in packaging materials due to its biodegradability, hydrophilicity, and low cost, and employed as an adsorbent in the environmental field. In addition, the latest research aims to develop hemicellulose-based nanomaterials, with potential uses in biomedicine, pharmaceuticals, food,

energy and environmental protection, thanks to their biocompatibility and low toxicity (Huang *et al.*, 2021).

Recent studies promote its use in the production of biopolymers, for example, in the sustainable production of polyhydroxybutyrate (PHB) (Xu *et al.*, 2010), or in the production of biomaterial from wheat bran, a relatively low-cost and easily accessible material that has been used effectively as an adsorbent for removing dyes from wastewater (Zhai *et al.*, 2021).

Wheat and other cereals, such as corn, are also used for starch extraction. Obtained starch is used in numerous sectors, from food (additives, baked goods, dairy products, baby food) to industry (bioethanol, bioplastics, paper, adhesives) and animal husbandry (feed and aquaculture). Its production generates gluten as a by-product, now considered the most economical “green” protein, used to improve the technological properties of flours, as a binder in food and feed, and in the development of sustainable biopolymers (Johansson *et al.*, 2013; Igrejas and Branlard, 2020).

1.4.2. Production of Wheat Extract

Wheat extracts can be obtained from different parts of the plant, including seeds, sprouts, bran and straw, each of which provides matrices with different characteristics and potential applications (Falcioni *et al.*, 2002; Kobayashi *et al.*, 2025). These extracts are mostly known for their antioxidant properties and are used in various industrial sectors. For example, in the cosmetics field, the

company Hitex has developed an extract rich in ceramides and digalactosyl diglycerides, in two types, in the form of wheat extract oil and wheat extract powder (Guillou *et al.*, 2011). Furthermore, wheat germ oil can be extracted through various processes, both traditional ones, such as mechanical pressing or solvent extraction, but also innovative approaches, such as extraction with supercritical carbon dioxide (Brandolini and Hidalgo, 2012).

In this context, Farmaceutici Damor, a historic Italian pharmaceutical company operating since 1943, fits perfectly, as its foundation is research into the aqueous extract of *Triticum Vulgare*, used in their products. As regards their aqueous wheat extraction process, it cannot be described in great detail as it is a patented procedure (US Patent No. 9,895,392) (Riccio, 2018). The process involves harvesting wheat seeds from the fields and subjecting them to a standardised germination process, carried out entirely within the company under specific monitored conditions of temperature, light and humidity. When the plants reach pre-established growth parameters, water extraction of the whole germinated plant is carried out through a repeated pressing process (under a 300 atmospheres pressure). Then, the crude extract is subjected to an ultrafiltration process using membrane filter with a cut-off below 30 kDa, and it is then sterilized to be used in their formulations.

The aqueous extract of *Triticum Vulgare* (TVE) was obtained through an extraction process without potentially toxic or harmful solvents. The products containing this extract are both pharmaceuticals and medical devices and are sold in Italy and abroad, and there are two available lines: pharma and

medical device, with a wide range of products, such as Fitostimolines® and Rigenase® (Sanguigno *et al.*, 2015).

This production process involves the generation of solid waste, consisting of pressing residues, which account for 7.5% of the production process in terms of quantity. On the other hand, the final ultrafiltration step produces liquid waste, which accounts for 5% of the production process in terms of quantity.

1.4.3. Food, Cosmetic, Nutraceutical and Pharmaceutical Applications of Wheat-Derived Extracts

Wheat and its extracts possess numerous biological properties that determine their applications in various sectors, including food, cosmetics, nutraceuticals, and pharmaceuticals. A by-product of milling wheat, wheat bran, is produced when the grain's outer layers are removed during the manufacture of refined flour. Compared to the central endosperm, which is utilized to make flour, it has less starch but is higher in fiber, protein, minerals, and other beneficial compounds. Wheat bran extract (WBE) is now recognised as a multifunctional ingredient of great interest in both the food and cosmetics industries (Kobayashi *et al.*, 2025). In the food industry, WBE is notable for its ability to improve the technological properties of baked products. For example, it has been observed that adding 7.5% aqueous extract increases the specific volume of bread and favourably modifies the rheological characteristics of dough (Šulniūtė *et al.*, 2016). In addition to this functional role, WBE shows

antimicrobial and preservative properties. For instance, a peptide (WBp-1) active against *Listeria monocytogenes* with a minimum inhibitory concentration (MIC) of 150 µg/mL has been isolated from wheat bran (Zou *et al.*, 2025), while other studies have shown that soluble bran extracts inhibit biofilm formation and modulate quorum-sensing mechanisms (González-Ortiz *et al.*, 2014). Specific bran compounds, such as phytic acid and phytate, have also shown marked antimicrobial activity and significant antioxidant capacity, with free radical scavenging activity of 36.16% (Sorour *et al.*, 2022). Therefore, WBE can be considered a potential natural preservative.

Another study evaluated the use of extracts from three different cultivars of hard winter wheat (Trego, Akron and Platte) for their antioxidant properties, particularly in the prevention of lipid peroxidation in fish oils, and therefore as natural antioxidants for food applications (Yu *et al.*, 2002).

Wheat sprout oil has been used in Iranian traditional medicine in the cosmetic field (Mikaili *et al.*, 2012). Even today, wheat extracts are widely used also in cosmetics due to their promising emulsifying properties, emulsion stability, skin-whitening properties, antimicrobial effects, anti-ageing and antioxidant capacities (Kobayashi *et al.*, 2025). Lipids extracted from bran have been shown to increase the stability of emulsions compared to those formulated with soybean oil, for example, as well as exhibiting a whitening effect on the skin (Han *et al.*, 2023; Manful, Ahmed and Barry-Ryan, 2024). The extract also contributes to improving hydration, softness and protection against UV damage, thanks to the presence of ferulic acid, which in destarched bran can

reach concentrations of up to 5157 ± 66 mg/kg (Pazo-Cepeda, Aspromonte and Alonso, 2021) and it is also applied in hair care products (NAKANO and Lourenco, 2023). The fractions of WBE containing xylo-oligosaccharides and protein hydrolysates showed a high capacity to inhibit enzymes associated with the skin ageing process, such as collagenase, elastase and hyaluronidase, and the phenolic component acts to inhibit hyaluronidase (C. Li *et al.*, 2024). As for industrial application, the extract produced by the company Hitex has been designed for use as a nutraceutical supplement to improve skin hydration and reduce signs of dry skin, thanks to the results demonstrated in clinical studies (Guillou *et al.*, 2011).

In addition to its effects on the skin, preliminary studies have highlighted the potential neuroprotective role of WBE, which is capable of counteracting β -amyloid-induced neuronal death and improving memory in animal models, suggesting potential applications in the management of neurodegenerative diseases such as Alzheimer's (Lee *et al.*, 2015).

The aqueous extract of *Triticum vulgare* (TVE), obtained from the whole germinated plant and rich in oligosaccharide components, is the active ingredient in numerous pharmaceutical formulations and medical devices (Farmaceutici Damor) (Sanguigno *et al.*, 2015). These formulations, available in various forms (creams, medicated gauze, ovules, vaginal washes and sprays), are widely used clinically in various therapeutic areas thanks to their wound healing (Sanguigno *et al.*, 2015), anti-inflammatory (Sanguigno *et al.*, 2018) and antioxidant properties (Antonucci *et al.*, 2018). In dermatology, TVE

is widely used to promote the healing of acute and chronic skin lesions, such as pressure ulcers, burns and delayed healing. Its mechanism of action has been associated with the stimulation of chemotaxis and fibroblast maturation, increasing the fibroblast index, considered a key parameter in the tissue repair process of phytostimulins (Viano and Santiano, 1978; Fiore *et al.*, 1993). It has also been observed that the extract promotes protein synthesis and the uptake of proline, an amino acid essential for collagen formation, thus confirming its ability to support the regeneration of the extracellular matrix (Vanden Berghe *et al.*, 1993; Midwood, Williams and Schwarzbauer, 2004). In gynaecology, vaginal formulations based on Fitostimoline have proven effective in the treatment of bacterial vaginosis and inflammatory and dystrophic processes of the female reproductive system, proving to be well tolerated and free from significant side effects (Boselli *et al.*, 2012).

In angiology, the topical application of creams, hydrogels and gauze containing TVE has been shown to significantly improve the healing of venous ulcers of the lower legs, reducing the area of the lesions and improving local symptoms, with no evidence of significant adverse events (Romanelli *et al.*, 2015). In dentistry, the oral spray based on *Triticum vulgare* has recently been evaluated as an adjunctive treatment for plaque gingivitis in children. The study by Bello *et al.* (2020) showed a significant improvement in gingival inflammation in treated children, suggesting a potential use of TVE as a safe and effective natural agent in the prevention of gingival diseases (Bello *et al.*, 2020). In addition to its established clinical applications, the extract has shown

a remarkable antioxidant effect. In particular, the Rigenase® has been shown to effectively neutralise free radicals, with a scavenger action superior to that of ascorbic acid (Antonucci *et al.*, 2018). This property is a key feature in the treatment of chronic wounds, where oxidative stress is a limiting factor during healing processes.

About the anti-inflammatory activity of TVE, the extract decreased the formation of pro-inflammatory mediators like nitric oxide (NO), IL-6, TNF- α , and PGE2 in cellular models of LPS-stimulated murine microglia. It also altered microglial activation and the expression of the p65 protein, which is a sign of the inflammatory response (Sanguigno *et al.*, 2018). Similar results were confirmed in BV-2 cells (murine microglial cells), paving the way for potential applications in neurology and the treatment of conditions associated with neuroinflammation (Funel *et al.*, 2020).

Finally, the study by D'Agostino *et al.*, (2020) investigated the molecular mechanisms responsible for the regenerative action of TVE on the skin. In HaCat human keratinocytes subjected to scratch tests, the extract stimulated cell proliferation and migration, modulating the activity of metalloproteinases (MMP-2 and MMP-9) and promoting the deposition of collagen, elastin and integrins (D'Agostino *et al.*, 2020). This activity was also confirmed by further studies, which highlighted the role of TVE in promoting actin polymerisation and the synthesis of fibronectin, hyaluronic acid and epidermal lipids, consolidating its value in tissue regeneration (Tito *et al.*, 2020; Morretta *et al.*, 2022). Overall, the aqueous extract of *Triticum vulgare*, thanks to its properties,

described above, is an active ingredient of great therapeutic interest, already widely used in clinical practice and with prospects for further development in even broader areas of regenerative medicine.

1.4.4. Physicochemical Properties and Main Bioactive Compounds of Wheat Waste

In the wheat industry, by-products are obtained in different forms through cultivation, processing and milling processes. Post-harvest residues, including straw, are the main agricultural by-product, while processes of milling produce bran, germ and semolina, which are rich in bioactive compounds and nutrients. Other by-products include dust and fine particles generated during equipment cleaning, wastewater with starches and proteins, and non-spec food products, e.g., broken pasta or unsold bread (Danciu *et al.*, 2023). These by-products, generally considered as wastes, have high potential for recovery in animal feed, biotechnology, bioenergy production, and in the manufacture of nutraceutical or cosmetic ingredients.

Among the by-products of wheat processing, bran and germ are the main sources of bioactive compounds with high nutritional and functional value. Wheat bran, obtained as a residue from milling, is characterised by a complex composition that includes structural polysaccharides, lignin and a wide spectrum of phytochemicals. Among the phytochemicals of bran, the main bioactive compounds are phenolic acids, including ferulic, gallic, syringic, vanillic and p-coumaric acids, which are known for their antioxidant activity

(Mencin, 2024; Saroj *et al.*, 2024). In addition to phenolic acids, bran also contains flavonoids and carotenoids (including lutein and β -carotene) (Brewer *et al.*, 2014; Talawar *et al.*, 2021), as well as tocopherols, compounds that are particularly sensitive to light and heat. In fact, heat treatments such as baking significantly reduce β -carotene levels, causing a decrease in carotenoids and tocopherols in baked products (Hidalgo *et al.*, 2006; Luthria, Lu and John, 2015). Other compounds commonly detected in bran are also alkylresorcinols (ARs), lipophilic molecules with antioxidant activity and the ability to protect LDL from oxidation (Gunenc *et al.*, 2013). Bran is also a good source of bioactive compounds named phytosterols, phytic acid, and saponins. These substances have complementary health-promoting qualities, such as lowering cholesterol (primarily from phytosterols and saponins), controlling metabolism, and having anticancer effects (Zhu, Soroka and Sang, 2015; López-Perea *et al.*, 2019). Additional significant constituents include vitamins (vitamin E and B vitamins) and essential minerals such as iron, zinc, manganese, and selenium (López-Perea *et al.*, 2019). In terms of polysaccharide content, it contains arabinoxylans, which are linked to prebiotic effects, cholesterol reduction and improve postprandial glycaemic response, reducing glucose and insulin peaks, thanks to their viscous and modulating effect on nutrient absorption (Broekaert *et al.*, 2011). There is also a small proportion of approximately 1% β -glucans in bran, located mainly in the aleurone layer, endosperm and bran itself (Saini *et al.*, 2019).

Wheat germ, which accounts for about 2.5-3.8% of the seed's weight, is also a high-value-added by-product. It contains 10-15% lipids, 26-35% proteins, 17% sugars, up to 4.5% fiber and about 4% minerals (Brandolini and Hidalgo, 2012). It is particularly rich in tocopherols (300-740 mg/kg DM), phytosterols (24-50 mg/kg), policosanols (10 mg/kg), carotenoids (4-38 mg/kg), as well as thiamine and riboflavin. Wheat germ oil is mainly composed of triglycerides, especially linoleic, palmitic and oleic acids, with a proportion of unsaturated fatty acids close to 80% (Hidalgo, Brandolini and Ratti, 2009). It is also an important source of lipophilic antioxidants such as tocopherols (1300-2700 mg/kg) and carotenoids (56 mg/kg), as well as phytosterols, mainly sitosterol and campesterol, with cholesterol-lowering effects (Ghafoor *et al.*, 2017). The extraction of wheat germ oil varies in efficiency and quality depending on the technique used. In fact, mechanical pressing recovers about 50% of the lipids, while solvent extraction can recover over 90% (Brandolini and Hidalgo, 2012). While innovative methods such as supercritical CO₂ extraction allow high yields without leaving toxic residues (Panfili *et al.*, 2003).

The defatted germ, a by-product of lipid (oil) extraction, retains a high protein content (30–35%), with a balanced amino acid profile rich in lysine (Zhu, Zhou and Qian, 2006b). It is also a source of carbohydrates, fiber, carotenoids, flavonoids and minerals such as potassium, magnesium, calcium, zinc and manganese (Panfili *et al.*, 2003; Zhu, Zhou and Qian, 2006a). Its marked antioxidant activity, attributed to phenolic compounds, supports its use in nutraceuticals and functional foods against oxidative stress (Zhu, Zhou and

Qian, 2006b). In addition, defatted germ proteins have emulsifying, foaming and water retention properties comparable or even superior to those of some animal proteins, making them of great interest for technological and food applications (Ge *et al.*, 2000).

Therefore, the valorization of wheat waste represents an opportunity to enrich the nutritional quality of foods and to develop functional and nutraceutical ingredients with high added value.

1.5 Techniques for Extraction and Characterization of Bioactive Compounds

The extraction and the analysis of bioactive compounds need to be carried out using established analytical methods to ensure proper identification, quantification and optimization of extraction. Currently, several extraction techniques are reported in the literature, each with specific advantages and disadvantages (Geow *et al.*, 2021; Fărcaș *et al.*, 2022) (Table 2). Choosing the most appropriate method is therefore a critical and crucial step, and depends on multiple factors that must be taken into consideration, including the properties of the matrix, the physical-chemical characteristics of the compounds of interest, the required efficiency of the process, economic aspects, and the analytical resources available in the laboratory. Among the multitude of techniques, solid-liquid extraction is still the most widely used to date, recognised for its versatility and simplicity (Guido and Moreira, 2017).

Over time, other conventional and non-conventional methods have been developed and optimised, offering various advantages in terms of selectivity, efficiency, and scalability. In addition to extraction, characterization is a crucial step in assessing the molecular nature and biological function of the recovered compounds. Chromatographic, spectroscopic and spectrometric techniques are widely used to generate detailed, reproducible and accurate data on complex matrices such as plant materials. More recently, the combination of these classic analytical tools with omics sciences and high-throughput technologies has opened the way to a more comprehensive understanding of the molecular composition and bioactivity of extracts from agri-food by-products.

1.5.1. Conventional Extraction Techniques

Solid-liquid extraction

Solid-liquid extraction (SLE), also known as leaching, is one of the oldest and most widely applied techniques for the extraction of bioactive compounds from plant matrices (Guido and Moreira, 2017; Bonifácio-Lopes, Teixeira and Pintado, 2020). The method is based on the transfer of soluble molecules from a solid matrix to a liquid solvent, driven by concentration gradients and enhanced by diffusion processes (Corrales *et al.*, 2009; Sagar *et al.*, 2018). Its simplicity and suitability are the reasons why it has remained a major method in both analytical chemistry and industrial applications. The efficiency of SLE

is strongly affected by several parameters, including solvent polarity, temperature, solid material particle size, solid-liquid ratio, and extraction time, with the last two being the most crucial (Hayouni *et al.*, 2007). Certain pre-treatments, such as grinding, milling or drying, can significantly improve the process as they increase the contact surface between the solvent and the solid matrix (Guido and Moreira, 2017). The solvent plays an important role. Indeed, polar solvents such as ethanol, methanol or acetone, often mixed with water, are generally preferred for phenolic compounds, while less polar solvents such as hexane are more suitable for lipids and hydrophobic metabolites (Fărcaș *et al.*, 2022). At the end of this type of extraction process, the samples are commonly centrifuged and filtered to remove solid residues, and the extract can then be exploited as an additive, food supplement or for the preparation of functional foods (Zulkifli *et al.*, 2012).

Among the applications of cereal by-products, there are several examples that highlight both the potential and the limitations of this approach. A study on brewers' spent grain (BSG) has shown that aqueous mixtures of acetone or ethanol maximise the recovery of total phenols, flavonoids and tannins (Guido and Moreira, 2017).

However, this extraction technique often requires long extraction times and the consumption of large amounts of solvents, raising concerns about sustainability and the potential thermal degradation of heat-sensitive compounds (when extraction comprises steps as thermal baths with higher temperatures) (Kumar *et al.*, 2017). From a practical point of view, the main

advantages of SLE are its low cost, wide applicability and user-friendly operation even without specialised equipment. On the other hand, its disadvantages include low selectivity, the use of toxic solvents (unless replaced by more environmentally friendly alternatives) and the need for time-consuming post-treatment steps, such as evaporation or solvent concentration (Kumar *et al.*, 2017).

In conclusion, solid-liquid extraction is the most accessible and versatile classical method for the recovery of bioactive compounds, but its limitations in terms of sustainability and efficiency have motivated the development of alternative and innovative extraction techniques, which are discussed in the following sections.

Acid and Alkaline Hydrolysis

Acid and alkaline hydrolysis are two conventional methods widely utilized, for example, to release phenolic acids bound to lignin and polysaccharides in plant cell walls (Fărcaș *et al.*, 2022). It can be carried out at high temperatures and high pressures in cylindrical stainless-steel reactors or auto-pressurized tubes (Ideia, Sousa-Ferreira and Castilho, 2020). Alkaline hydrolysis is particularly efficacious for the extraction of ferulic and p-coumaric acids from wheat bran, maize germ and BSG (Bonifácio-Lopes, Teixeira and Pintado, 2020). The main advantage is the effective recovery of otherwise inaccessible bioactive compounds; however, disadvantages include oxidation, partial degradation and loss of antioxidant synergy. Although hydrolysis can also

depolymerise cell wall structures, it often offers lower yields and can cause further phenolic structural changes. Despite these drawbacks, acid and alkaline hydrolysis continue to be economical and simple alternatives for the extraction of bound phenols from different by-products, including cereal by-products.

Soxhlet extraction

Soxhlet extraction (SE) consists of a continuous flow of hot organic solvent through the solid matrix, allowing for almost complete extraction. It is a reference technique that is reproducible and well-established (Soxhlet, 1879; Fărcaș *et al.*, 2022). However, it requires large quantities of solvent, takes a long time and causes degradation of thermolabile compounds (Seidel, 2012). In fact, in bran and BSG, the ferulic acid yields obtained with Soxhlet are much lower compared to other methods (Moreira *et al.*, 2012).

Maceration

Maceration is based on static or agitated contact between the solvent and the plant matrix at room temperature or slightly above. It is a simple and inexpensive method, suitable for heat-sensitive compounds, and useful for lab-scale applications (Sagar *et al.*, 2018). However, it usually provides low yields and requires long extraction times, and its efficiency strongly depends on the solvent used, with hydroalcoholic mixtures frequently chosen to improve recovery.

Hydro-distillation

Hydro-distillation consists of extracting essential oils and volatile compounds using water or steam (vapour). It is widely used for aromatic and lipophilic metabolites, but is not appropriate for thermolabile compounds, which are subject to degradation due to high temperatures. It remains in use for the extraction of essential oils at an industrial scale (Vankar, 2004; Silva *et al.*, 2005).

1.5.2. Innovative Extraction Techniques

In the last decades, a variety of physicochemical methods have been developed both as a result of technological progress and to meet specific requirements related to the nature of different matrices, but also to overcome some of the limitations of classic SLE and increase yields. These methods are often regarded as more environmentally friendly than traditional ones, because thanks to the exploitation of membrane systems, pressurized fluids, enzymes, or alternative energy sources, they are able to recover bioactive compounds from complex matrices, reducing solvent use, waste, and energy demand.

Ultrasound-Assisted Extraction

Ultrasound-Assisted Extraction (UAE) employs ultrasound waves characterized by extremely high frequencies (not detectable by humans),

which, through cavitation, increase cell permeability and speed up mass transfer. It provides quick extraction times and uses less solvent and energy, so it's seen as greener than other techniques and is good for heat-sensitive compounds (Gallo, Ferrara and Naviglio, 2018; Bhargava *et al.*, 2021). UAE has been used on a variety of plant wastes and cereal residues. Examples include its application to grape pomace and BSG for the recovery of polyphenols and anthocyanins, achieving higher yields than maceration and other classic techniques (Ghafoor, Hui and Choi, 2011; Alonso-Riaño *et al.*, 2020; Osorio, Flórez-López and Grande-Tovar, 2021). Extraction efficiency depends on different parameters such as frequency, power and intensity (ultrasound parameters); solvent properties (viscosity, surface tension, vapour pressure, and solubility of target compounds), temperature, and matrix characteristics like particle size. Although the use of UAE is generally considered a green technology, its main disadvantages are its possible non-selective extraction and the fact that it can induce partial degradation if not adequately controlled (Rutkowska, Namieśnik and Konieczka, 2017).

Microwave-Assisted Extraction

Microwave-Assisted Extraction (MAE) uses electromagnetic radiation (300 MHz-300 GHz) to obtain rapid heating within the matrix and solvent through dipole rotation and ionic conduction mechanisms, thus promoting the release of bioactive compounds (Vinatoru, Mason and Calinescu, 2017; Ulloa *et al.*, 2019). It is considered a more green and sustainable technique compared to

conventional methods, as it guarantees high extraction efficiency, reduced solvent consumption and reduced extraction times (Cravotto *et al.*, 2008; Alupului, Călinescu and Lavric, 2012). The extraction yield of MAE depends on various parameters such as solvent nature, temperature, pressure and extraction time, which need optimization to maximise yields and avoid degradation (Angiolillo, Del Nobile and Conte, 2015; Zuorro, Iannone and Lavecchia, 2019). Polar solvents, such as mixtures of water with ethanol, methanol or acetone, are the most suitable for this technique. The systems of MAE can be of two types: closed or open. The close ones allow higher yields but pose risks of volatile compound losses, while the open systems are more suitable for thermolabile and volatile molecules (Chan *et al.*, 2011). It has been widely used for cereal by-products such as bran, germ and husks, allowing the recovery of polyphenols and polysaccharides (Görgüç, Özer and Yılmaz, 2020). Furthermore, combining it with other technologies (e.g. ultrasound, sub/supercritical fluids, enzymatic hydrolysis) has further improved yields and reduced energy consumption (Ekezie, Sun and Cheng, 2017).

Mechanochemical extraction method

The mechanochemical method is a solvent-free extraction technique that uses mechanical forces to induce chemical reactions and release intracellular metabolites. It is a rapid and sustainable process with promising applications for agro-industrial residues rich in polysaccharides. Despite its potential, mechanochemistry has disadvantages such as low selectivity, possible

degradation of compounds, and difficulties in transitioning to industrial applications (Espro *et al.*, 2021).

Pressurised Fluid-Based Techniques

The main extraction techniques based on the use of pressurized fluids are Pressurized Liquid Extraction (PLE), Subcritical Water Extraction (SWE), and Supercritical Fluid Extraction (SFE). PLE and SFE are also known as accelerated solvent extraction (ASE).

PLE uses solvents at high temperature and pressure, but below the critical point, thus keeping them liquid throughout the extraction process (Alvarez-Rivera *et al.*, 2020). It allows the obtaining of high yields, with reduced solvent consumption and easy automation (Pazo-Cepeda *et al.*, 2020). Some of its applications include the recovery of ferulic acid from wheat bran and wheat germ oil (Dunford and Zhang, 2003; Pazo-Cepeda *et al.*, 2020). This method improves mass transfer by reducing viscosity and surface tension and increases the solubility of compounds (Belwal *et al.*, 2020). It is also possible to use environmentally friendly solvents such as ethanol or D-limonene (Alvarez-Rivera *et al.*, 2020). However, the limitations are the cost of equipment and the possible degradation of bioactive compounds at high temperatures (100-300 °C) (Antony and Farid, 2022).

In SWE, water is maintained at a temperature between 100 and 374 °C and under pressure, thus acquiring solvent properties similar to those of organic solvents. And with these conditions, the polarity of water decreases, giving

the possibility to extract compounds of medium and low polarity (Zhang *et al.*, 2020). It is an environmentally friendly, economical technique that leaves no toxic residues (Herrero *et al.*, 2006; Freitas *et al.*, 2021). SWE has been used, for example, to extract ferulic arabinoxylans and phenols from wheat bran (Pourali, Asghari and Yoshida, 2010; Rudjito *et al.*, 2019) and, more in general, to extract phenols without toxic residues (Baiano, 2014). It is extremely versatile as the polarity of water decreases with temperature, allowing the extraction of both polar and less polar compounds. The main drawbacks are the requirement for sophisticated equipment and the possible thermal degradation of thermolabile compounds (Freitas *et al.*, 2021).

SFE relies on the use of fluids above their critical point, most commonly supercritical CO₂, which combines the properties of gases and liquids (Ahmad *et al.*, 2019). It is specific for lipophilic compounds, like oil and carotenoids, and it generates solvent-free extracts and preserves the integrity of the compounds (Khosravi-Darani and Vasheghani-Farahani, 2005; Wang and Weller, 2006; Freitas *et al.*, 2021). Co-solvents such as ethanol are necessary for the extraction of polar compounds (Carpentieri *et al.*, 2021). Applications of vegetal by-products include the extraction of oils rich in tocopherols and phytosterols from wheat bran or various plants (Liza *et al.*, 2010; Kumar *et al.*, 2017). In addition to laboratory scale, SFE is also commonly used on an industrial scale, for example, in the decaffeination of coffee (Mediani *et al.*, 2023) and also for the extraction of essential oils, guaranteeing its thermal integrity (Dauber *et al.*, 2023). Limitations include the high cost of the

equipment and the requirement for temperature and pressure control (Chemat *et al.*, 2020).

Enzyme-Assisted Extraction

Enzyme-Assisted Extraction (EAE) is a technique that utilises various types of enzymes such as cellulase, xylanase and pectinase with the aim of degrading plant cell walls and releasing the bioactive compounds bound within them (Nadar, Rao and Rathod, 2018). It is extremely selective, environmentally friendly and operates under mild aqueous conditions (Puri, Sharma and Barrow, 2012). However, the cost of enzymes and the need to optimise the process (pH, temperature, enzyme selection) remain critical issues for this technique (Kaur *et al.*, 2020). In cereal by-products, EAE has been successfully applied to increase protein recovery and improve polyphenol extraction from BSG and wheat bran (Rommi *et al.*, 2018).

Steam Explosion

Steam Explosion (STE) is a three-step process that combines thermal, mechanical and chemical effects. The material to be extracted is heated with high-pressure saturated steam, hydrated and then subjected to rapid decompression, resulting in structural breakdown and hydrolysis of glycosidic bonds. This technology is widely used as a pre-treatment for the valorisation of lignocellulosic biomass from agricultural by-products such as cereal straw, sugar cane bagasse or pineapple leaves (Raud *et al.*, 2019;

Tanpichai, Witayakran and Boonmahitthisud, 2019). In the case of wheat bran, STE has been shown to promote the release of bound phenolic acids, particularly ferulic acid, improving its use as an antioxidant ingredient (L. Liu *et al.*, 2016). Furthermore, according to Kong *et al.*, (2020) the treatment enriches the bran with bioactive compounds (flavonoids, soluble fibers, phenols) and increases its antioxidant activity, while reducing phytic acid and rancidity, thereby extending its shelf life (Kong *et al.*, 2020). Thanks to the unique use of steam at high pressure, STE is a sustainable and cost-effective technique; however, it has a limitation in that it can cause undesirable changes in the fiber matrix, which must be considered in terms of technological and nutritional quality.

Pulsed Electric Field

Pulsed Electric Field (PEF) is based on the use of high-voltage electrical pulses to permeabilise cell membranes (electroporation), facilitating the release of metabolites (Buchmann *et al.*, 2019). It is an energy-efficient and non-thermal process, making it particularly suitable for thermolabile compounds (Picart-Palmade *et al.*, 2019). It has been applied to pomace and other by-products, demonstrating an increase in the yield of polyphenols, pigments and other metabolites (Boussetta *et al.*, 2014). At an industrial level, PEF is already applied to fruit juice processing, demonstrating good scalability (Jaeger *et al.*, 2012; Barba *et al.*, 2015).

High-voltage electrical discharge

High-voltage electrical discharge (HVED) employs electrical discharges in liquids to generate plasma, cavitation and turbulence, leading to cell disruption (Sarkis et al., 2015). Compared to PEF, HVED offers greater cell disruption, but is currently limited to laboratory scale (Fărcaș *et al.*, 2022). Studies demonstrated that this technique notably enhances protein and polyphenol recovery from oilseeds and cereal by-products (Boussetta *et al.*, 2014).

Membrane Separation Techniques

Compounds can be selectively separated according to their molecular size using membrane-based processes (microfiltration, ultrafiltration, and nanofiltration) (Vollet Marson *et al.*, 2021). They are widely spread for food, pharmaceutical and biotechnological applications and are characterised by high purity and low solvent consumption (Nazir *et al.*, 2019). In addition to their use for concentrate extracts, they allow the selective isolation of specific classes of bioactive compounds, such as the separation of proteins with different molecular weights. In the work of Jaeger *et al.*, (2021), ultrafiltration with a cut-off of 5-30 kDa from BSG recovered over 90% of the proteins with a high-quality, thanks to the absence of additional heat treatment. Limitations of this technique include the high cost of the filters and possible fouling, which require regular maintenance. However, their potential for integration into

biorefinery processes makes them interesting for the sustainable valorization of agro-industrial residues (Cassano and Galanakis, 2018).

Overall, the variety of traditional and non-conventional extraction methods shows the progress made in improving the recovery yields and quality of extracts (Table 2). Still, the issues related to solvent use, process time, and selectivity highlight the need for more sustainable and innovative solutions.

Table 2. Comparative overview of all extraction techniques discussed.

Technique	Principle	Advantages	Disadvantages
Conventional Extraction Techniques			
SLE	Solvent–solid diffusion	Simple, versatile, low cost	High solvent consumption, long processing times, degradation of thermolabile compounds
Soxhlet	Continuous cycle of hot solvent	High reproducibility, exhaustive extraction	Excess solvent, high T°, degradation of thermolabile compounds
Maceration	Static solvent–matrix contact	Low cost, suitable for small scale	Low yields, long processing times
Hydrodistillation	Steam/water dragging of volatiles	Simple, suitable for essential oils	Not suitable for thermolabile compounds
Acid/Alkaline Hydrolysis	Cleavage of ester/ether linkages in lignocellulosic matrix	Efficient release of bound phenolic acids (e.g. ferulic, p-coumaric)	Risk of oxidation, degradation, environmental concerns with strong reagents
Innovative Extraction Techniques			
UAE	Ultrasonic cavitation	Short times, lower solvent and energy use, suitable for thermolabile compounds	Non-selective, possible degradation
MAE	Internal heating by microwaves	High efficiency, reduced time and solvent use	Risk of volatile losses or degradation if poorly controlled

Mechanochemical extraction	Mechanical forces	Rapid	Non-selective, degradation of compounds, problem for scale-up
PLE	Solvents at high T and P (below critical point)	High yields, reduced solvent, automation possible	High equipment cost, risk of degradation
SWE	Liquid water at high T and P	Non-toxic solvent, eco-friendly, low cost	Possible thermal degradation, complex setups
SFE	Supercritical CO ₂ or other fluids	Pure extracts, solvent-free residues, selective for lipophilic compounds	Expensive equipment, co-solvents needed for polar compounds
EAE	Enzymes degrading cell walls	Selective, eco-friendly, mild conditions	Enzyme cost, optimization required
STE	Steam under pressure + sudden decompression	Cost-effective, releases bound phenolics	Fiber modification, possible degradation
PEF	High-voltage electric pulses	Non-thermal, low energy, rapid	Dedicated equipment, process optimization needed
HVED	Electrical discharges in liquid generating plasma	High efficiency, multipurpose	Still at lab scale, costly setup
Membrane separation	Size-selective molecular filtration	High purity, low solvent use, industrially scalable	Membrane cost, fouling

1.5.4. Bioactivity: Antioxidant Activity and Total Polyphenol Content

Antioxidants are key components in biological systems as they can reduce oxidative stress, maintaining cellular homeostasis. The most significant compounds in this category include vitamins C and E, flavonoids, polyphenols, and essential enzymes such as superoxide dismutase and catalase. The main role of these compounds is to contrast the harmful effects

of reactive oxygen species (ROS) and reactive nitrogen species (RNS), natural by-products of cellular metabolism that can cause damage if not regulated (Corpas, 2017). The mechanism of action of antioxidants consists of donating electrons or hydrogen atoms, which stabilise free radicals by neutralising them, and they also act as chelating agents for metals, inhibiting the formation of free radicals (Parcheta *et al.*, 2021).

Antioxidant activity is assessed using various techniques; the most important and common of these include spectrophotometric techniques such as the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the ferric reducing antioxidant power (FRAP) assay.

In the ABTS and DPPH tests, the samples react with chromogenic radicals (ABTS^{•+} or DPPH[•]), which are neutralised by antioxidant compounds, causing a colour change. The intensity of this colour change is then quantified using a spectrophotometer (Re *et al.*, 1999) (Blois, 1958).

In the FRAP test, on the other hand, the reduction of the cation Fe³⁺ to Fe²⁺ is verified during a redox reaction with antioxidants, which, if present in the sample, will also determine a colour change that can be measured with a spectrophotometer (Benzie and Strain, 1996). These three methods are now well-established and widely used techniques (Munteanu and Apetrei, 2021). In the three tests mentioned above, antioxidant capacity is therefore expressed in Trolox equivalents. Trolox is a powerful antioxidant and is therefore used as a reference standard. Another crucial spectrophotometric test is the Folin-

Ciocalteau assay, which quantifies the total phenolic content (TPC). The test measures the ability of polyphenols to reduce the Folin-Ciocalteau reagent, formed by phosphomolybdic and phosphotungstic acid within an alkaline medium. A blue chromophore is then generated through a resulting reaction, and this chromophore possesses the ability to absorb light at 760 nm. The result of this test is expressed as gallic acid equivalents, used as a reference standard for constructing a calibration curve. The curve converts absorbance values into concentration units, enabling the quantification of the total polyphenol content in the sample (Singleton and Rossi, 1965).

1.5.5. Evaluation of biological effects and biocompatibility profile

The assessment of biological effects and biocompatibility profile is a crucial step in the analysis of the cellular response of a potential nutraceutical. It is therefore important to test it in order to identify any undesirable cellular responses, validate its safety profile and verify its complete biocompatibility (Piccolo, Ferraro, *et al.*, 2019). The term biocompatibility refers to the compatibility between a material and the biological system (Peters, Unger and Kirkpatrick, 2021).

Various bioscreens can be used for *in vitro* assessment. Commonly used assays are MTT tests, which measure mitochondrial activity (Supino, 1995) and the trypan blue exclusion test, which assesses cell membrane integrity for cell counting (Strober, 2015). The MTT assay was introduced in the 1980s and

is based on the redox capacity of viable mitochondria to convert the yellow substrate 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) into insoluble purple formazan crystals (Supino, 1995).

The cell survival index is calculated as the arithmetic mean of the percentage values obtained from the MTT assay and the automatic cell count, thus providing a reliable parameter for describing cellular responses (Piccolo, Misso, *et al.*, 2019).

Finally, cytomorphological analysis can be performed, which is generally carried out at the end of treatment using phase contrast microscopy in order to monitor any morphological changes in the cells (Ferraro *et al.*, 2023).

1.5.6. Instrumental Techniques for Bioactive Compounds Characterization

Several techniques are used for the separation, identification and complete characterization of bioactive compounds from complex samples such as plant matrices, including chromatographic, spectrometric and spectroscopic approaches.

Among chromatographic systems, high-performance liquid chromatography (HPLC), developed between the 1960s and 1970s, is one of the most accurate techniques and is now widely used in the pharmaceutical, biotechnology, environmental, food and polymer industries (Ali, 2022). The principle is based on injecting the sample into a column with a stationary phase crossed by a

high-pressure mobile phase, with distinct retention times based on solute-stationary phase and solute-mobile phase affinity and interactions (Gerber *et al.*, 2004). It's really employed and appreciated for its versatility, high resolution, sensitivity and various operating modes, offering the possibility of using different mobile and stationary phases (Vidushi, Meenakshi and Bharkatiya, 2017).

Gas chromatography (GC), first introduced by James and Martin in 1952, is, on the other hand, more specific for volatile and heat-stable compounds and is used in the petrochemical, environmental, food, pharmaceutical and forensic sectors (James and Martin, 1952; Bartle and Myers, 2002). It is recognised for its sensitivity and speed, but is less suitable for thermolabile compounds such as polyphenols, which can be degraded during the vaporisation required before injection.

Among other techniques, capillary electrophoresis, developed by Jorgenson and Lukacs in 1981, represents an advanced tool, capable of separating ions and complex biomolecules with high efficiency and reduced sample consumption, although it has limitations related to low sensitivity and instrumental complexity (Voeten *et al.*, 2018). In contrast, thin-layer chromatography (TLC) is considered a minor technique, used mainly for rapid preliminary analyses due to its simplicity and low cost, but penalised by low sensitivity, poor reproducibility and limited quantification capacity (Cheng, Huang and Shiea, 2011).

In the last years, HPLC has been gradually replaced by ultra-high-performance liquid chromatography (UHPLC), which, thanks to columns packed with particles $<2\ \mu\text{m}$ and pressures greatly exceeding 400 bar (HPLC limit), allows for faster separations and higher resolution (Broeckhoven and Desmet, 2014).

For the detection and identification of compounds through their mass spectrum, chromatographic techniques are coupled with mass spectrometry (MS), as in LC-MS and GC-MS systems. Mass spectrometry provides data on the molecular weight and formula of analytes through ionisation and separation based on the m/z ratio. Spectrometry techniques can be divided into low-resolution techniques, such as triple quadrupole systems used mainly for targeted quantitative analysis, and high-resolution techniques (HRMS), including Orbitrap and Q-TOF, which offer greater accuracy in mass measurement and improved ability to distinguish ions with very similar m/z values (Ferrer and Thurman, 2012; Wood, 2019).

Among the most sophisticated instruments, the Q-Orbitrap enables precise analysis even at concentrations in the ppb ($\mu\text{g/kg}$) range and allows both targeted and untargeted approaches, with the option of full scanning and analysis of previously collected data (retrospective) without repeating experiments (Dong *et al.*, 2022). Actually, HPLC-MS combines the separation capacity of liquid chromatography with the precision of mass spectrometry, making it highly sensitive and selective. It is widely employed in the food, pharmaceutical and agrochemical industries for the purification of natural

extracts and the quantification of organic compounds of different polarities and obtained from solid or liquid matrices (Di Stefano *et al.*, 2012).

With regard to untargeted plant metabolomics analysis, comparative studies have shown that Orbitrap and Q-TOF achieve equivalent performance (Glauser *et al.*, 2013; Senyuva, Gökmen and Sarikaya, 2015). In conclusion, target analysis does not necessarily require high-resolution instruments; low-resolution systems, such as triple quadrupoles in MRM/dMRM mode (Gómez-Mejía *et al.*, 2019), are suitable for quantifying known analytes using reference standards (target analysis). High-resolution instruments (Orbitrap, QTOF) in full-scan + MS/MS mode are suitable for both advanced quantitative analysis and non-targeted identification of compounds in complex matrices (Gavage, Delahaut and Gillard, 2021). Therefore, the choice between the two approaches depends on the compromise between accuracy and range of information, as well as the availability of preliminary knowledge about the metabolites of interest (Ribbenstedt, Ziarrusta and Benskin, 2018).

Spectroscopic techniques, such as UV-Vis, IR and NMR, have a mainly descriptive and qualitative role, used for preliminary screening and to obtain structural information. In particular, nuclear magnetic resonance (NMR) provides valuable details on molecular structure, although in the case of thermolabile compounds such as polyphenols, alternative methods such as LC-MS are preferable as they better maintain chemical stability (Hatzakis, 2019; López-Fernández *et al.*, 2020). UV-Vis spectroscopy is mainly applied to evaluate the concentration of compounds containing chromophores (Ghosh

and Nandi, 2024), while IR spectroscopy is employed to identify functional groups within molecules (Dutta, 2017).

In conclusion, the characterization of bioactive compounds requires the complementary use of multiple techniques, as only their integration provides accuracy, reproducibility and in-depth knowledge of the bioactive profile of complex matrices.

1.5.7. Omics Sciences

Omics sciences are analytical approaches that, through a holistic and large-scale perspective, analyze complete sets of biological molecules (genomes, transcriptomes, proteomes, and metabolomes) to understand the molecular mechanisms and interactions within an organism (Misra *et al.*, 2019).

Taking the study of polyphenols as an example, the progress and establishment of omic sciences are evident. In fact, we have moved from paper chromatography and colorimetric assays (1950s–1960s) (still in use for total phenolic content determinations), through HPLC (1970s) and LC-MS (1990s), to today's high-throughput metabolomic platforms (Măchițescu *et al.*, 2024).

Moreover, the concept of foodomics has also been proposed, combining metabolomics, proteomics, and transcriptomics, allowing the investigation of the complex interactions and the relationship between food and human health, providing insights into food composition, bioactivity, safety and

traceability, as well as into the molecular responses obtained in the organism (Capozzi and Bordoni, 2013; Mahato *et al.*, 2024).

In this context, non-targeted approaches based on HRMS acquisitions allow the investigation of the entire metabolic spectrum of a matrix, generating hypotheses on hundreds or thousands of compounds in parallel, unlike targeted strategies that focus on selected metabolites. In addition to the methodological aspect, numerous recent studies have shown that non-targeted approaches can be successfully applied to the characterization of agri-food by-products. For example, untargeted analysis has been used to describe in detail the phenolic and flavonoid profiles of plum seed residues (Rodríguez-Blázquez *et al.*, 2024) and to identify antioxidant compounds in annatto seeds (Arruda-Peixoto *et al.*, 2025).

In this same perspective, non-targeted approaches have also been applied to different cereal crops, including rice, wheat, maize, barley, sorghum, oat (Huang *et al.*, 2025) and in wheat by-products at different times of fermentation (Li *et al.*, 2025).

These examples highlight how the metabolomic approach can be beneficial, not only allowing the chemical diversity of these matrices to be explored, but also representing a key tool for their exploitation in the nutraceutical and cosmetic fields, facilitating the recovery and sustainable reuse of by-products (D'Urso *et al.*, 2025).

Therefore, metabolomics is the large-scale analysis of metabolites in a specific biological state, and among omics strategies, it has gained popularity thanks

to its wide range of applications (Adebo *et al.*, 2021). Since the mid-2000s, the adoption of HRMS has improved identification coverage and quality, with applications gradually expanding to include lipidomics and glycomics (Junot *et al.*, 2014).

A typical LC/HRMS workflow consists of robust experimental design with QC (pooled samples) and randomisation; matrix-targeted preparation; acquisition with complementary chromatographic strategies (for example reverse phase RP for less-polar or moderately polar compounds, and HILIC or ion-pairing approaches for highly hydrophilic or strongly ionic analytes); pre-processing (peak picking/alignment), intra/inter-batch corrections; accurate mass annotation using databases; confirmation with MS/MS; statistical data mining (univariate and multivariate analysis) and biological interpretation (Guy *et al.*, 2008; Dunn *et al.*, 2011; Junot *et al.*, 2014; Zhou and Yin, 2016).

In order to clearly communicate the degree of confidence of tentatively identified compounds, the system proposed by Schymanski *et al.*, (2014) is widely adopted. Briefly, based on the data obtained, the following 5 confidence levels are attributed: Level 1: structure confirmed with standard (corresponding MS/MS and retention time). Level 2 (probable): 2a Library (unambiguous spectral match, with attention to collision energy/resolution/ionisation), 2b Diagnostic (the only structure compatible with the set of diagnostic evidence and the experimental context). Level 3: candidate/attempts (e.g. unresolvable isomers). Level 4: unambiguous

molecular formula but no proposed structure. Level 5: exact mass of interest without formula. This scheme was created to clarify the “grey areas” between Metabolomics Standards Initiative (MSI) guideline levels (previously proposed by Sumner *et al.*, (2007)) and to standardise reporting in suspect/nontarget HRMS analysis (Schymanski *et al.*, 2014).

Within the analytical workflow, data processing is a crucial and complex step, made possible by a series of bioinformatic software tools developed specifically for metabolomics. Among these, MS-DIAL is widely used for peak picking, peak filling, alignment and migration time correction (Tsugawa *et al.*, 2015). In parallel, programmes such as SIRIUS, which combine isotopic information and fragmentation trees, allow the determination of structural hypotheses even in the absence of reference spectra. At the same time, MS-FINDER stands out for its in silico fragmentation and integration of multiple scores, showing high performance in the identification of phenolic compounds. However, like SIRIUS, it is unable to distinguish isomers without further experimental evidence (Mallmann, O Rios and Rodrigues, 2023).

Dealing with the processing and interpretation of large amounts of complex data, omics sciences often use chemometrics. This discipline applies advanced mathematical and statistical tools to model and analyse high-dimensional chemical and biological data sets, producing interpretable and robust models even in the presence of incomplete, noisy or collinear information (Trygg, Holmes and Lundstedt, 2007). Software such as SIMCA allows metabolomic data to be processed using multivariate models, such as PCA, PLS-DA and

OPLS-DA, enabling both model validation and the identification of Variable Importance in Projection (VIP), i.e. the discriminating variables that measure the importance of each variable (e.g. a metabolite) in contributing to the discrimination between sample groups. The robustness and reliability of the models are also verified through internal validation tests, including permutation tests and CV-ANOVA, which allow the distinction between truly predictive models and those resulting from random adaptations to the data (Narduzzi *et al.*, 2024).

1.6 Current trends in sustainable chemistry

Conventional extraction techniques, although highly efficient, present several disadvantages, such as high consumption of organic solvents, which are often toxic and difficult to dispose of, being time-consuming and energy-demanding, and posing the risk of degradation of bioactive compounds (Fărcaș *et al.*, 2022).

To overcome these limitations, and thanks to the gradual establishment of concepts such as Green Chemistry, with its 12 principles, the evolution towards innovative and sustainable extraction approaches has been encouraged (Anastas, 2007; Nadar, Rao and Rathod, 2018). These strategies aim to improve yield and selectivity while minimizing waste and energy consumption, thus consequently reducing environmental impact, supported

by the use of green metrics to measure greenness and compare their sustainability (Section 1.6.1.) (T.-L. Chen *et al.*, 2020; Jurisch *et al.*, 2024).

As a result, alternative extraction methods have been developed (Section 1.5.2.), which allow operations to be carried out without solvents or to significantly reduce their use and processing times, thus reducing the environmental impact (Duarte *et al.*, 2014). Emerging strategies combine low environmental impact solvents, alternative energy sources and biotechnological tools, reflecting a broader evolution in analytical and industrial chemistry (Chemat *et al.*, 2020). In this context, the application of NADES in sample preparation procedures aligns with new trends in Green Analytical Chemistry (GAC) (Meher and Zarouri, 2025) and particularly with the principles of green sample preparation (GSP) (López-Lorente *et al.*, 2022), representing a concrete solution to make extraction processes more sustainable, safe, efficient and cost-effective (Benvenuti *et al.*, 2020).

In light of growing evidence of the impacts of environmental pollution, there is an increasing focus on environmental preservation and the promotion of sustainable practices. In this context, research is focused on developing methodologies capable of reducing the use of solvents and reagents, minimising waste and emissions, and limiting energy demand.

The concept of green chemistry and its 12 principles (green chemistry principles (GCP)) were first introduced by Paul Anastas and John Warner in 2000 (Anastas and Warner, 2000). These principles promote the design of safer chemicals, the use of catalysts instead of stoichiometric reagents and the

prevention of waste production (Zhang, 2017). The final goal is to reduce environmental impact by changing the processes and chemical products used upstream. These concepts have their historical roots in the United States Pollution Prevention Act (1990), which defines prevention as the reduction at origin of practices that avoid the release of hazardous substances into the environment and the resulting risks to public health (Nameroff, Garant and Albert, 2004; US EPA, 2017). Green Chemistry contributes not only to health and environmental protection but also to economic stability and is closely linked to the Sustainable Development Goals (SDGs). It is related, in particular, to reduce climate change (SDG 13), the availability of clean water (SDG 6) and the production and consumption of clean energy (SDG 7), but it also has connections with other goals, such as sustainable cities (SDG 11), life below water (SDG 14), industrial innovation (SDG 9) and responsible consumption and production (SDG 12) (T.-L. Chen *et al.*, 2020).

This scenario also includes the broader concept of sustainable chemistry, which aims to integrate the principles of green chemistry with a comprehensive assessment of environmental, economic and social aspects, defining long-term strategies for sustainable development (Marion *et al.*, 2017; Halpaap and Dittkrist, 2018). It also concerns the monitoring of water, energy, food, climate and population, confirming the central role of green chemistry in supporting the fundamental science behind the SDGs. In this context, green chemistry practices can serve as a key indicator of performance for measuring the effective achievement of global sustainability goals. In addition to green

chemistry, green analytical chemistry (GAC) has also been developed, representing the application of green chemistry principles to analytical processes. GAC, as well as green chemistry, is based on twelve fundamental principles (Gałaszka, Migaszewski and Namieśnik, 2013) that aim to reduce environmental impact and health risks through practices such as minimising the use of reagents and solvents, replacing toxic compounds with biodegradable alternatives, reducing waste, optimising energy consumption and promoting integrated, miniaturised and *in situ* techniques (Koel and Kaljurand, 2006; Armenta, Garrigues and de la Guardia, 2008). It should be noted that strict application of GAC criteria may have a drawback, i.e. it could compromise the practical functionality of the methods, raising the issue of combining environmental sustainability, analytical efficiency and economic feasibility. To address this challenge, the concept of White Analytical Chemistry (WAC) has been proposed (Nowak, Wietecha-Postuszny and Pawliszyn, 2021), which extends the principles of GAC by integrating analytical (validation criteria), ecological and practical-economic aspects. WAC therefore offers a more holistic view, closer to sustainable development goals, capable of guiding the choice and optimisation of methods not only in terms of their “greenness”, but also their actual applicability and usefulness (Nowak, Wietecha-Postuszny and Pawliszyn, 2021).

A further extension of the principles of Green Analytical Chemistry is represented by green sample preparation (GSP), which focuses more on the crucial step of sample preparation, traditionally recognised as one of the most

critical and environmentally impactful stage. Conventional methods, in fact, are often wasteful in terms of toxic solvents, disposable materials, energy and time, significantly contributing to the generation of laboratory waste (López-Lorente *et al.*, 2022). To address these issues, ten GSP principles have been proposed that aim to reduce environmental impact while maintaining analytical performance. Key points include the use of *in situ* and miniaturised preparations, the use of safer or alternative solvents and reagents (e.g. supercritical fluids, DES, NADES), the adoption of sustainable, renewable and reusable materials for extraction processes, and the systematic reduction of waste production and energy consumption. In addition, there is also an incentive to increase analytical throughput, simplify and automate procedures, and ensure operator safety (López-Lorente *et al.*, 2022). GSP is not a new sub-discipline, but rather a guiding principle aimed at promoting more sustainable sample preparation practices, in line with sustainable development goals and also adopting green metrics for an objective assessment of the “greenness” of each method (Pena-Pereira, Wojnowski and Tobiszewski, 2020).

A further advance is represented by Circular Analytical Chemistry (CAC), which aims to overcome the linear perspective typical of GAC and GSP and to promote the transition to a closed, cyclical, resource-efficient and waste-free analytical approach. CAC integrates the principles of the circular economy into analytical chemistry, considering the entire life cycle of materials, reagents and instruments, and promoting reuse, recycling and

recovery strategies in a closed-loop approach. This approach, also defined through twelve specific objectives, aims to ensure high analytical performance while minimising resource consumption and promoting a radical transformation of production, use and waste management processes (Psillakis and Pena-Pereira, 2024).

1.6.1. Green metrics

In recent years, to support the principles of green chemistry and, more specifically, green analytical chemistry, various metrics have been developed, i.e. informatics tools for the definition of the degree of “greenness” or sustainability of an analytical method according to objective criteria (Tobiszewski and Orłowski, 2015; Płotka-Wasyłka, 2018; Pena-Pereira, Wojnowski and Tobiszewski, 2020). The metrics are different, and each one is more suitable for a specific purpose, presenting advantages and disadvantages, with different levels of detail and applicability. Generally, at the end of the evaluation, they generate a score and, if the tool provides for it, a graphic pictogram (Table 3). Indeed, most Green Analytical Chemistry metrics provide a visual representation using pictograms or colour scales (e.g. NEMI, GAPI, AGREE, HEXAGON), while others return only a numerical value (e.g. Analytical Eco-Scale, AMGS, ChlorTox), which is still useful for quantitative comparisons but lacks graphical output.

In the field of GAC, it is essential to assess the sustainability of different analytical procedures and to minimise the negative effects of them on the

environment and human safety without compromising experimental validity and efficiency (Tobiszewski *et al.*, 2015). Traditional GAC metrics are often unsuitable for assessing the sustainability of analytical processes, as they may not focus on the analytical and methodological process in detail (Koel, 2016). Furthermore, some GAC metrics have been developed only for specific analytical procedures, while others are universally applicable and therefore more widely used (Shi *et al.*, 2023). According to a recent study by Yin *et al.*, (2024), the 15 most commonly utilized GAC metrics are the followings: National Environmental Methods Index (NEMI), Advanced NEMI, assessment of green profile (AGP), Chloroform-Oriented Toxicity Estimation Scale (ChlorTox Scale), Analytical Eco-scale, Green Certificate Modified Eco-scale, analytical method greenness score (AMGS), green analytical procedure index (GAPI), ComplexGAPI, red-green-blue (RGB) additive color model, RGB 12 algorithm, analytical greenness metric or calculator (AGREE), AGREE preparation (AGREEprep), HEXAGON, and blue applicability grade index (BAGI). These tools have proven to be versatile for a wide range of analytical methods (Kaya, Cetinkaya and Ozkan, 2022; Cetinkaya, Kaya and Ozkan, 2023).

One of the first proposed metrics was NEMI; its output is represented with a pictogram divided into four quadrants, providing an immediate qualitative indication of the use of persistent, bioaccumulative and toxic chemicals (PBT), hazardous solvents, pH conditions and waste (Keith, Gron and Young, 2007). Although it is simple and intuitive, it is not very detailed and does not allow

for quantitative assessments. Advanced NEMI introduced a colour scale (green-yellow-red), improving its comparative capacity but still maintaining a general approach (de la Guardia and Armenta, 2011).

The AGP (Analytical Greenness Profile) included in the evaluation five other areas (safety, health, waste, energy and environment) using colour coding based on National Fire Protection Association (NFPA) parameters, offering a more complete but still qualitative perspective (Tobiszewski, Mechlińska and Namieśnik, 2010). The Analytical Eco-Scale marked an important step forward, introducing a system that assigns an initial ideal score of 100 and then deducts penalty points to account for solvent and reagent use, energy demand, waste generation, and potential chemical hazards. Based on the final score obtained, the analyses are classified, with those scoring above 75 indicating excellent analysis, 50–75 acceptable, and <50 suggesting low sustainability (Wardencki, Curyło and Namieśnik, 2005). It allows for immediate quantitative and comparative assessment, although the final score does not provide detailed information on the causes of the impact and only a few analytical assays without sample processing are considered ideal green (Gałuszka *et al.*, 2012; Turner, 2013). This led to the Green Certificate Modified Eco-Scale, which translates the score into seven levels (A-G) with colour coding, facilitating communication and benchmarking (Gallart-Mateu *et al.*, 2015).

GAPI and ComplexGAPI are more oriented towards workflow and process steps, evaluating the various steps of the entire process. In detail, GAPI uses

a five-pentagon pictogram covering sampling, method type, preparation, consumption and waste, providing a semi-quantitative view of the various steps, although with broad categories that consequently reduce accuracy (Płotka-Wasyłka, 2018). The latter, ComplexGAPI, extends the assessment to the preliminary and final stages, increasing completeness but also complexity (Płotka-Wasyłka and Wojnowski, 2021). In addition to GAPI and ComplexGAPI, even more complete tools such as AGREE and AGREEprep have been introduced in recent years and are now very popular and spread. AGREE (Analytical GREENness Metric Approach), proposed by Pena-Pereira, Wojnowski and Tobiszewski, (2020), systematically converts the 12 principles of Green Analytical Chemistry into a quantitative assessment. Each principle is expressed as a normalised score (0–1) and represented in a clock-shaped pictogram, whose segments, coloured from green to red, reflect environmental performance, with the possibility of assigning different weights depending on the application context. This makes AGREE a comprehensive, flexible and easily interpretable metric, capable of highlighting strengths and weaknesses and supporting the comparison of analytical procedures. One of the main limitations of AGREE is its reduced ability to discriminate the impact of the sample preparation step, which is known to be the most critical phase from an environmental point of view (Pena-Pereira, Wojnowski and Tobiszewski, 2020). To fill this gap, AGREEprep was developed, which is the first tool entirely dedicated to sample preparation (Wojnowski *et al.*, 2022). It is based on the 10 principles of

Green Sample Preparation and evaluates parameters such as the use of renewable solvents and materials, waste minimisation, energy and sample quantity, the possibility of automation, analytical throughput and operator safety, without including costs and logistics (López-Lorente *et al.*, 2022). The output is a circular pictogram in which not only does the colour of the segments reflect sustainability, but their area also represents the different “weight” of the criteria in the final score, as in AGREE, although the difference is more evident in the AGREEprep pictogram. These weights, defined according to the relative importance of the principles considered, can also be modified by the user, provided that the choice is adequately justified. Compared to AGREE, AGREEprep is therefore more sensitive and discriminating, allowing for the distinction between similar procedures and the more accurate identification of areas for improvement (Wojnowski *et al.*, 2022). Another holistic approach is represented by the RGB model and the subsequent RGB 12 algorithm, which integrates three fundamental dimensions, i.e. analytical performance (in red), environmental sustainability (in green) and practical and economic aspects (in blue), resulting in an overall colour score (Nowak and Kościelniak, 2019; Nowak, Wietecha-Posłuszny and Pawliszyn, 2021). These two tools, which form the basis of White Analytical Chemistry (WAC), allow for a balanced assessment of methodologies, although they require a relatively complex calculation process. From this perspective, an analytical method can be defined as “white” when it achieves a balance between accuracy and precision (red), environmental sustainability

(green) and simplicity, cost-effectiveness and speed of use (blue) (Nowak, Wietecha-Postuszny and Pawliszyn, 2021). For practical applications in laboratories, AMGS is a very useful tool as it provides a single quantitative index calculated on the basis of solvent consumption, safety, health and environmental (SHE) attributes, cumulative energy demand and equipment requirements. Although it allows immediate numerical comparisons between different methods, the metric does not have a graphical interface and involves relatively complex calculations (Hicks *et al.*, 2019).

BAGI is more intuitive, combining scores, colours and pictograms on a scale of 25-100. It is an index of the transferability and applicability of the method, is quick and easy to communicate, but incomplete in terms of SHE and waste management. For BAGI, a method is practical, transferable and applicable if the score exceeds >60 (Manousi *et al.*, 2023). This tool is very important because, in some cases, even if a developed method is green although it can be complicated and not transferable.

Among the more specific metrics, the ChlorTox Scale quantifies chemical risk by expressing it in “equivalent masses of chloroform”, allowing direct comparisons of the impact of the substances used. However, it is a semi-quantitative measure and does not take into consideration the analytical performance (Nowak *et al.*, 2023). Finally, HEXAGON proposes a more complex and standardised model that integrates six domains (residues, carbon footprint, costs, toxicity/safety and two groups of analytical merit figures), with scores from 0 to 4. It is extremely comprehensive and suitable

for regulatory or industrial decisions, although it requires a large amount of data and is computationally intensive (Ballester-Caudet *et al.*, 2019).

In addition to the 15 techniques described by Yin *et al.*, (2024) as the most widely used, there is also the Sample Preparation Metric of Sustainability (SPMS), which has recently been proposed as a specific tool for assessing the sustainability of the sample preparation phase, traditionally considered the least “green” link in the entire analytical procedure (González-Martín *et al.*, 2023). Unlike previous metrics, such as GAPI or AGREEprep, which also include sampling and instrumental detection, SPMS focuses only on sample preparation, allowing for an objective comparison of different extraction techniques. The metric is based on the evaluation of nine parameters, each of which contributes a weighted score to the final value on a scale from 0 to 10 and the results are displayed in a clock-like diagram. This tool is particularly suitable for distinguishing between conventional and miniaturised methods, comparing microextraction strategies that differ in solvents or materials, and providing practical guidance on which aspects to optimise in order to improve the overall sustainability of sample preparation (González-Martín *et al.*, 2023).

Overall, these metrics show an evolution from a simple qualitative approach (NEMI) to increasingly quantitative, holistic and integrated systems (AGREE, RGB, HEXAGON), reflecting the growing interest in balancing analytical quality, environmental sustainability and practical feasibility in the field of sustainable analytical chemistry (Yin *et al.*, 2024).

HEXAGON		(Shishov and Mokhodoeva, 2024)
SPMS		(Rajput and Desai, 2024)

1.6.2. Sustainable Extraction Technologies and Green Solvents, with a Focus on NADES

Starting from all the limitations of traditional extraction methods discussed in the previous sections and considering how difficult it is to dispose of the solvents used for these extractions and how toxic they are to both humans and the environment, research is now focused on finding alternative solutions with less impact. The term GRAS solvents, or Generally Recognised as Safe solvents, has therefore been introduced, but in reality, few commonly used solvents belong to this group, and even those few solvents generally have low extraction yields (Burdock and Carabin, 2004). The fact that they are almost always polar limits their extraction to polar molecules, and they do not solve the problems of production and disposal of these solvents. A better strategy is therefore to use alternative and innovative solvents such as ionic liquid (ILs) solvents, deep eutectic solvents (DES), and natural deep eutectic solvents (NADES), usually referred to as green solvents.

Ionic liquids consist of molten salts that are in liquid form at room temperature. They are prepared by combining organic cations with organic or inorganic anions, and it is calculated that there are more than 10⁸ possible

combinations (Kunz and Häckl, 2016). Their main characteristic is that they have lower melting points than their constituents, and thanks to the different cation/anion combinations, their physical-chemical properties can be modulated to obtain desired conditions such as thermal stability, polar variability, high boiling points, good dissolution and extractability. Furthermore, as they are low in volatility and low in flammability, they are considered safer than conventional organic solvents. The disadvantage is that some anions and cations are toxic, and ILs have low biodegradability and are difficult to purify. Furthermore, the criticality has increased since they are not yet regulated, for example, by the Food and Drug Administration (FDA) or the European Food Safety Authority (EFSA), and their mechanism is not well elucidated (Benvenutti, Zielinski and Ferreira, 2019).

Recently, new solvents called (NA)DES, an acronym that stands for (Natural) Deep Eutectic Solvents, have emerged as a valid alternative. The name derives from the merging of two Greek words, “*εὖ*” (eu = good) and “*τηξίς*” (taxis = fusion) and refers to the main characteristic of these solvents, which is that once the components are mixed using a specific molar ratio, a homogeneous liquid is obtained with a melting point lower than that of the individual separate components (Liu *et al.*, 2018). DES therefore have the same characteristics as the ILs mentioned above, but also offers additional advantages. In particular, unlike ILs, they are not always formed through ionic interactions, but also through hydrogen bonds and other interactions. DES can consist of salts, but also carbohydrates, amino acids, polyols,

secondary metabolites and others. When they are composed of natural components derived from plant metabolites or their derivatives, they are called NADES. These systems are typically formed by combining hydrogen bond acceptors (HBAs), such as quaternary ammonium, and hydrogen bond donors (HBDs), such as urea, amines or carboxylic acids, and their structure is maintained by non-covalent interactions, such as hydrogen bonds, van der Waals forces and π - π interactions, which ensure their stability and unique properties. One of the main advantages over ILs is that DES are easily integrated and disposable, less toxic and more environmentally friendly. Since there are no chemical events involved in the development of the eutectic mixture's supramolecular structure, their interactions can be broken under the specific conditions without the need for laborious procedures. (NA)DES are typically made up of asymmetric components that lower the cross-linking energy, which decreases the stability of the ionic structure and, in turn, lowers the melting point of the system. They are also more viscous than water and other organic solvents (Jablonský *et al.*, 2018).

DES are commonly classified into five types based on the components involved (Type I-V). Type I systems are formed by combining a quaternary ammonium salt with metal halides (MCl_x), while Type II systems include hydrated metal halides, which reduce the crosslinking energy and promote the formation of eutectics at lower temperatures thanks to coordination water. Type III systems are obtained by combining a quaternary ammonium salt with an HBD, such as amides, carboxylic acids, or alcohols, representing the

most versatile and biodegradable formulations, and they have also been widely demonstrated to dissolve a variety of metal oxides when HBDs with complexing ability (e.g., urea, thiourea, oxalate) are employed. Type IV systems, instead, arise from the association of metal salts with HBDs, leading to the formation of complex species that expand the potential of DESs for metallurgical applications. Finally, Type V includes eutectics formed entirely from neutral molecular compounds, without the presence of quaternary salts, further expanding the application spectrum of these systems (Smith, Abbott and Ryder, 2014).

Another way of classifying (NA)DES was proposed by Espino *et al.*, (2016), classifying them into 4 groups based on HBD/HBA combination: i.e. Choline Chloride Derivatives; Organic Acid Derivatives (characterized by strong hydrogen bonding capacity); Sugar-Based Mixtures (hydroxyl-rich structures used for interactions with biomolecules) and Other Combinations (including water-containing NADES) (Fernández *et al.*, 2018). The most widely used HBA is choline chloride (ChCl), a widely used quaternary salt, but also betaine, a natural, biodegradable alternative considered safer than choline (Li *et al.*, 2016; Turck *et al.*, 2019). Finally, ternary deep eutectic solvents (TDES) have also been developed, in which the addition of an additional donor, such as a polyol, improves solubility and selectivity, for example, in the ChCl/oxalic acid/ethylene glycol mixture (Duarte *et al.*, 2017).

Alongside these traditional classifications, additional subclasses have been introduced in recent years. Among these, the subclass of magnetic DES

(MDES) is characterized by the incorporation of paramagnetic components within the eutectic mixture. This feature gives the solvents a strong response to external magnetic fields, which can be exploited to improve various extraction and industrial processes, for example, by simplifying phase separation without resorting to centrifugation or complex procedures. Despite their potential, the available information remains limited, especially regarding polarity, thermal stability, and toxicity (Makoś-Chełstowska *et al.*, 2022). Other systems include polymeric DES (polyDES), in which at least one component is a polymer, giving the system greater stability and distinct rheological properties compared to traditional DES. At the same time, polymerizable DES (PDES) have been developed, which contain polymerizable functional groups, such as acrylic or methacrylic units, capable of participating in radical reactions. DES therefore acts both as a reagent and as a reaction medium, and PDES are the precursors of polyDES, which constitute the final materials. These materials have modifiable properties, ranging from adhesives to elastomers, and can acquire bioactive functionalities if obtained from natural precursors. Recent studies highlight their potential in the development of antibacterial, thermoresponsive, or luminescent systems, confirming their versatility for applications in bioelectronics, packaging, and high-tech materials (L. Picchio *et al.*, 2024). In addition, another class of eutectic systems has recently emerged, eutectic molecular liquids (EMLs), characterised by a variety of non-covalent

interactions, which modulate their structural and functional properties (Yu *et al.*, 2019).

Since the 2000s, NADES has caught the attention of industry and scientific research (Abbott *et al.*, 2004). The principal constituents used to prepare these systems are choline chloride, citric acid, acetic acid, maleic acid, malic acid, fructose, glucose, trehalose, sucrose, terpenoids or water, among others. The concept of NADES has recently been extended to the pharmaceutical sector, giving rise to API-DES, designed to increase the solubility and skin permeability of drugs, and therapeutic deep eutectic solvents (THEDES), in which the active ingredient itself is an integral part of the eutectic system (Duarte *et al.*, 2017).

Although the fact that NADES cannot be purchased but must be prepared in the laboratory may seem like a limitation, on the other hand, it is well known that one of the advantages of these systems is that they can be prepared quickly and in an easy way. There are two preparation methods: vacuum evaporation and a heating method (Dai *et al.*, 2013). In the evaporating process, components that had been dissolved in water and evaporated using a rotary evaporator set at 50 °C. After that, the liquid was put in a desiccator using silica gel until its weight didn't change. In contrast, NADES can be prepared using a specified amount of water and the heating process. Water was added to the two-component mixture in a bottle with a cap and a stirring bar. Since water facilitates mass transfer, it is typically needed to lower the viscosity to help the extraction process. After that, it was heated and stirred

simultaneously in a water bath below 50 °C for 30-90 minutes, or until a clear homogeneous liquid formed. Using a ^1H NMR spectrometer, the viscous liquids were analyzed at 40 °C to verify the formation of the eutectic system. To obtain the highest extraction yield, it's possible to change the composition of the (NA)DES and to add or not the water to modify the viscosity and extraction properties, depending both on the matrix and on the extraction target.

NADES offer numerous advantages; they are easily biodegradable, have minimal or no toxicity, and can act as natural solvents (Benvenutti, Zielinski and Ferreira, 2019). They are also economically advantageous thanks to the low cost of their ingredients and availability (González-Laredo *et al.*, 2023). The fundamental properties of these systems include viscosity, density, surface tension, conductivity, volatility, and polarity, which depend on the nature of the HBAs and HBDs used, their molar ratio, and water content (Mohd Fuad, Mohd Nadzir and Harun@Kamaruddin, 2021). One of the main critical issues of these systems is their high viscosity, which can be modulated by adding water. Small additions (5-30%) reduce viscosity and improve solubility and mass transfer, while excessive dilutions can compromise the structure of the solvent and the extraction efficiency of hydrophobic compounds (Vilková, Płotka-Wasyłka and Andruch, 2020; Viñas-Ospino *et al.*, 2023).

After utilization, NADES can be recovered and reused multiple times, confirming good recycling performance (Grillo *et al.*, 2020). However, the

separation of the extracted compounds from the DES remains a critical step, as their low vapour point prevents separation by distillation (Wawoczny and Gillner, 2023). Alternative techniques have therefore been proposed, including solid-phase extraction (SPE), which guarantees yields of over 90%, the antisolvent method, suitable only for specific molecules, and back-extraction based on solvents that are immiscible with DES but soluble for the analyte (Xu *et al.*, 2019; Zhen *et al.*, 2022).

About toxicology, NADES generally exhibit low toxicity, especially when derived from primary metabolites, which allows for their use in pharmaceuticals and food (Paiva *et al.*, 2014; Bakirtzi, Triantafyllidou and Makris, 2016). However, their safety depends on their composition. In fact, terpene-based formulations or those containing organic acids have shown cytotoxicity and antimicrobial activity (Rodrigues *et al.*, 2020), while *in vivo* studies have reported adverse effects for betaine:glycerol (1:2) systems (Benlebna *et al.*, 2018). Their intrinsic bioactivity is also interesting, as some NADES protect extracted metabolites, as demonstrated for anthocyanins in acid-based NADES like the mixture choline chloride-citric acid-glucose (ChCaGlu), which are protected from light, pH, and temperature (Guo *et al.*, 2019). Furthermore, their biodegradability, with degradation rates exceeding 69% after 28 days (Zhao *et al.*, 2015), confirms the potential of NADES as green solvents.

Nowadays, NADES are used in various industrial sectors, from the production of ready-to-use extracts for food, drugs, and cosmetics to the

development of new functional formulations (Bragagnolo *et al.*, 2024). In cosmetics, for example, they have been used to obtain propolis extracts using the L-Proline/D,L-Lactic Acid/Water system (1:2:2.5 molar ratio), which are then added to cosmetic creams (Tzani *et al.*, 2023). In the field of biomedicine, they are emerging as cryoprotectants alternative to DMSO, thanks to their lower toxicity and ability to limit ice crystal formation and osmotic stress, thus increasing cell survival after freezing (Schuh *et al.*, 2023). They have also demonstrated remarkable effectiveness in the biorefinery sector, for example in the pretreatment of sugarcane (oilcane) bagasse for the production of bioethanol and biodiesel, a NADES based on choline chloride and lactic acid (1:2) solubilized over 66% of lignin and 73% of hemicellulose, with a 2.5-fold increase in lipid content and a post-hydrolysis glucose yield of up to 93%, while also demonstrating high solvent recyclability (95–98%) (Raj and Singh, 2024). In addition, they have also been used in the production of bioactive films, in a recent study by Campanella *et al.*, (2024), rosmarinic acid was extracted using a NADES based on lactic acid, ethylene glycol, and water (3:5:2) assisted with ultrasounds, which was then incorporated into PVA films with potential antioxidant and antimicrobial properties for food and pharmaceutical packaging. In addition to these applications, NADES are gaining importance in the valorization of agri-food waste, such as peels, pulp, leaves, and roots (González-Laredo *et al.*, 2023). From an operational point of view, to modulate polarity and viscosity, in addition to water content, it is also possible to act on temperature. In fact, moderate heating can improve

extraction efficiency, while excessive heating risks damaging thermolabile compounds (Wei *et al.*, 2015). To reduce time, energy and solvent consumption, classic solid-liquid extraction (SLE) approaches are complemented by assisted techniques such as ultrasound (UAE) and microwave (MAE), although the latter can sometimes compromise the stability of thermolabile metabolites (Wawoczny and Gillner, 2023). Among recent examples of bioactive compound extraction from waste using NADES, Rodríguez-Martínez *et al.*, (2022) used five different DES to extract phenolic compounds and flavonoids from dried avocado peels (DAP), obtaining the best results with ChCl-acetic acid and ChCl-lactic acid (92.03 ± 2.11 mg GAE/g DAP of TPC and 186.01 ± 3.27 mg RE/g DAP), with GAE standing for gallic acid equivalents, while RE stands for rutin equivalents. These results are superior to those of ethanol, with high antioxidant and antibacterial activity. Similarly, Cao *et al.*, (2023) developed a combined NADES-UAE approach for the extraction of polyphenols from broccoli leaves, identifying ChCl-1,2-propylene glycol (1:2) as the most effective formulation (4.91 mg/g TPC), rich in neo-chlorogenic acid and with biological activities superior to conventional methods.

Although NADES systems have numerous advantages, including increased extraction yield of certain classes of bioactive compounds, environmental sustainability, and the production of ready-to-use extracts with potential industrial applications, to date, due to the challenges inherent in scale-up, including high viscosity and pH control (Spaggiari, Hiemstra, *et al.*, 2025),

despite the numerous patents filed, only a few have actually found commercial application, with the cosmetics sector standing out as the most responsive (Caprin, Charton and Demarne, 2018; Charton, CAPRIN and Bechetoille, 2019).

Chapter 2

Research objectives

2.1. General objectives

The main objectives of this doctoral thesis are to study and enhance the value of wheat-based pharmaceutical waste generated during the production of the aqueous extract of *Triticum vulgare* (Damor Farmaceutici), by characterizing its chemical composition and evaluating its potential applications.

The research was conducted in two main phases. The first phase involved the characterization of a hydroalcoholic extract obtained from both the liquid (LW) and solid wastes (SW) and the evaluation of their biocompatibility. In the second phase, the hydroalcoholic extract from the solid waste was compared with an extract obtained using Natural Deep Eutectic Solvents (NADES).

Through a multidisciplinary approach, the study seeks to identify the bioactive compounds present in the production waste, evaluate their safety, and develop sustainable and innovative extraction strategies.

The overall goal is to change the perception of these by-products from simple waste, which entails disposal costs and environmental impact, to valuable resources that can be reintegrated into other production chains, in line with the principles of the circular economy. Such a transition would bring tangible benefits both for the environment and, consequently, also for human health.

2.2. Specific Objective 1

1. Chemical characterization of wheat-based wastes (LW and SW) by UHPLC-Q-Orbitrap HRMS, to establish both qualitative and quantitative profiles of bioactive compounds. To assess whether active and potentially exploitable compounds are still present.
2. Determination of bioactivity, with particular focus on polyphenols, through the evaluation of antioxidant capacity. The assessment includes spectrophotometric assays (ABTS and FRAP) as well as the quantification of the total phenolic content by the Folin-Ciocalteu method.
3. Determination of proximate composition of the solid waste fraction, including moisture (oven drying), crude fat (Folch), protein (Kjeldahl), and ash content.
4. Quantification of mineral content in both LW and SW, with particular focus on elements of nutritional and technological relevance (e.g., Ca, Fe, Zn, Cu, Mn, Se). Analyses are performed by GFAAS.
5. Assessment of *in vitro* biocompatibility to establish a reliable safety profile in preclinical models of human skin. Bioscreens are performed on HaCaT keratinocytes and HDFa fibroblasts, evaluating cell survival through the combined use of the MTT assay, automated cell counting, and cytomorphological analyses at multiple exposure times and concentrations.

2.3. Specific Objective 2

1. Screening of eight NADES systems for the extraction of ferulic acid from SW.
2. Development and optimization of an innovative extraction protocol for the solid waste, based on the use of NADES composed of betaine:1,3-butanediol:water (1:3:10.6, molar ratio). Optimization of ultrasound-assisted extraction (UAE) process parameters using Response Surface Methodology (RSM).
3. Non-targeted profiling of traditional hydroalcoholic and NADES-based extracts by UHPLC-Q-TOF-HRMS, combined with chemometric analysis using SIMCA software.
4. Quantitative evaluation of the greenness and applicability of the optimized NADES-based method using bioinformatic tools such as AGREEprep and BAGI.

Chapter 3

Specific Objective 1

3.1 Chemical characterization of wheat-based waste derived from a pharmaceutical process for its potential valorization

Materials and Methods

Chemicals and reagents

Methanol (MeOH), ethanol (EtOH), water (UHPLC-MS grade), acetonitrile (UHPLC-MS grade), chloroform, hydrochloric acid, glacial acetic acid, formic acid (FA) were purchased from Merk (Milan, Italy), whereas deionized water (<18 MX cm resistivity) was provided from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The standards of polyphenols (purity > 98%) were acquired from Merck group (Milan, Italy).

The compound of 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), ferrous chloride (FeCl_2), Folin–Ciocalteu reagent, Sodium carbonate anhydrous, potassium persulfate, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), ($\pm$)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid commonly called Trolox, gallic acid were acquired from Sigma Aldrich (Milan, Italy).

Sampling

Triticum vulgare was obtained through a standard manufacturing protocol of germination, under restricted conditions of temperature, light and humidity. The aqueous extract of *Triticum vulgare* (TVE) is produced using a newly developed extraction method patented by Farmaceutici Damor (Riccio, 2018), using a simple water-based extraction procedure with not potentially toxic or harmful solvents. Thus, it can be easily ascribed as a green approach for the recovery of bioactive compounds with a reduced environmental impact. In more detail, liquid extraction was performed from the germ wheat macerated and forced under 300 atm of pressure. The liquid sample was recovered through an ultrafiltration membrane filter with a cut-off below 30 kDa.

Wastes derived from the production process were then collected appropriately. Solid waste (SW) was generated from the exhausted solid-phase pressed while liquid waste (LW) was recovered after the ultrafiltration process. These wastes were provided by the company Farmaceutici Damor. SW was dried (to allow homogenization), ground, and subjected to analysis to investigate the profile of the polyphenolic component. Both solid and liquid wastes were analyzed for their phenolic components in different ways. LW was analyzed for evaluation of the qualitative-quantitative profile of soluble polyphenolic compounds, after filtration on 0.2-micron nylon filters and diluted with 1:10 (*v/v*) in methanol.

Polyphenolic extraction on solid waste

The polyphenolic compounds on solid waste were extracted following a procedure reported in the literature for the sprouted wheat, with little and opportune modifications (Hung, Hatcher and Barker, 2011). Free phenolics were extracted using 80% chilled ethanol as described in literature (Hung *et al.*, 2009). Shortly, 10 mL of 80% chilled ethanol were added to a 15 mL centrifuge tube containing 1.0 g of dried sample. The content was vortex mixed and tumbled for 20 min at a room temperature before centrifugation at 4000 g for 5 min. The supernatant (free phenolic extracts) was collected. The residue was re-extracted twice with 10 mL of 80% chilled ethanol and all supernatants were combined. The supernatant was dried using a rotary evaporator (Laborota 4000; Heidolph Instruments Italia Srl, Milan, Italy) and then reconstituted with 10 mL of methanol. The extracts were then stored at -18 °C under nitrogen until analyzed.

Determination of total phenolic content (TPC)

The Folin-Ciocalteu method was used for determining the total phenolic content in accordance with the procedure reported by Izzo *et al.*, (2020). Briefly, 500 µL of deionized water and 125 µL of the Folin-Ciocalteu reagent 2 N were added to 125 µL of extract. The tube was mixed and incubated for 6 min in dark conditions. Then, 1.25 mL of 7.5% of sodium carbonate solution and 1 mL of deionized water were added. The reaction mixture was maintained in dark conditions for 90 min. Finally, the absorbance at 760 nm

was measured through a spectrophotometer system. Results were expressed as mg of gallic acid equivalents (GAE)/g of dry weight sample for solid waste and as mg of gallic acid equivalents (GAE)/ mL of extract for liquid waste.

UHPLC-Q-orbitrap HRMS analysis

The qualitative and quantitative profile of bioactive compounds were performed by UHPLC (Dionex UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, United States) equipped with a degassing system and an autosampler device. Analysis chromatographic was carried out with a thermostat ($T = 30^{\circ}\text{C}$) using the Accucore aQ column (particle size $2.6\ \mu\text{m}$, $100 \times 2.1\ \text{mm}$), (Manufacturer: Thermo Fisher Scientific). The mobile phase consisted of 0.1% acetic acid glacial in water (A) and Acetonitrile (B). The injection volume was $5\ \mu\text{L}$. Metabolites of interest were eluted by setting a flow rate of $0.4\ \text{mL/min}$. The gradient elution program was as follows: 0–5 min-5% B phase, 25min-40% B phase, 25.1 min- 100% B phase, 27 min- 100% B phase, 27.1 min- 5% B phase, 35 min- 5% B phase. The total run time was 35 min, and the flow rate was $0.4\ \text{mL/min}$.

Mass spectrometry was performed on a Q-Exactive Orbitrap system equipped with an electrospray ionization (ESI) source operating in negative mode. The flow rates of the sheath gas, auxiliary gas and sweep gas were set at 35 arbitrary units (arb unit), 10 arb unit, respectively, with the spray voltage set to $-2.8\ \text{kV}$ under negative mode. The capillary temperature was set at 275°C , and the auxiliary gas heater temperature was 350°C . The S-lens RF level was

set at 50 V. Detection was achieved considering the exact mass with a mass error <5 ppm. Data analysis was performed using Xcalibur software 3.1.66.19. (Xcalibur, Thermo Fisher Scientific, Waltham, MA, United States).

Antioxidant activity

The antioxidant activity of the liquid waste and the extract of polyphenols obtained by the solid waste was assessed spectrophotometrically by using two different assays, namely, FRAP and ABTS. The obtained data were expressed as millimoles of Trolox Equivalents (TE)/kg of dry weight sample for solid waste and as millimoles of Trolox Equivalents (TE)/L for liquid waste.

ABTS assay

Free radical-scavenging activity was measured by using the method reported by Luz *et al.*, (2018). Briefly, 9.6 mg of 2,2 azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt was solubilized in 2.5 mL of deionized water (7 mM) and 44 μ L of solution of potassium persulfate ($K_2S_2O_8$; 2.45 mM) were added. The solution was kept in dark conditions at room temperature for 16 h prior to use. Afterwards, ABTS⁺ solution was diluted with ethanol to reach an absorbance value of 0.70 (± 0.02) at 734 nm. Then, to 1 mL of ABTS⁺ solution with an absorbance of 0.700 ± 0.050 , 0.1 mL of opportunely diluted sample was added. After 2.5 min wait, the absorbance was immediately measured at 734 nm. Results were expressed as mg of gallic acid equivalents

(GAE)/g of dry weight sample for solid waste and as mg of gallic acid equivalents (GAE)/mL of extract for liquid waste.

FRAP assay

The antioxidant capacity of samples was estimated spectrophotometrically following the procedure of Benzie and Strain, (1996). The ferric reducing/antioxidant power (FRAP) reagent was prepared by mixing acetate buffer (0.3 M; pH 3.6), TPTZ solution (10 mM), and ferric chloride solution (20 mM) in the proportion of 10:1:1 (*v/v/v*). Freshly prepared working FRAP reagent was used to perform the assay. Briefly, 0.150 mL of the appropriately diluted sample was added to 2,850 mL of FRAP reagent. The value of absorbance was recorded after 4 min at 593 nm. The method is based on the reduction of Fe^{3+} TPTZ complex (colorless complex) to Fe^{2+} -tripyridyltriazine (intense blue color complex) formed by the action of electron-donating antioxidants at low pH. Results were expressed as millimoles of Trolox Equivalents (TE)/kg of dry weight sample for solid waste and as millimoles of Trolox Equivalents (TE)/L for liquid waste.

Proximate composition analysis of solid waste

Water (moisture)

Water content was established via air oven drying (103°C) of samples of solid waste (5 g each per sample) until constant weight. Water values were calculated through the difference between the initial weight of the sample and

the constant weight of the sample, after drying process. The percent moisture was calculated by the following formula:

$$\%moisture = (w_1 - w_2)100/w_1$$

Where w_1 =initial weight of sample; w_2 = final weight of sample (after drying). The results are expressed as percentage of water (Stadlmayr *et al.*, 2020).

Fat content evaluation

Fat extraction was carried out following the method of Folch, Lees and Stanley, (1957) with some modifications. In brief, 1.0 g of sample was combined with 20.0 mL of a mixture chloroform/methanol (2:1 *v/v*) in a screw-top glass tube. The tubes were then shaken for 30 s before being shaken for 15 min in an orbital shaker at room temperature (~25°C). Next, the tubes were centrifuged at 1500 g for 15 min to then thoroughly decant the bulk of the chloroform/methanol solution, ensuring that all solid material remained at the bottom of the tube. The upper layer was covered, and the residue was extracted another time with 20.0 mL of mixture and the process was repeated. Finally, all the mixtures were collected (about 40 mL) and washed with 10 mL of water. The chloroform phase was dried, and the residue was weighed. The percent crude fat was determined by using the following formula:

$$\% crude fat = Wt\ of\ chloroformic\ extract \times 100\ Wt\ of\ sample$$

The fat content was then expressed as g/100 g sample.

Protein content evaluation

The quantitative determination of protein was carried out using the Kjeldahl, (1883), later modified by Gunning and Arnold (Pickel, 1915). In more detail, the procedure involved an initial step of mineralization of the total nitrogen in the sample, leading to the formation of ammonia, which was subsequently distilled and titrated. Concerning the volume of hydrochloric acid used, the nitrogen content is calculated. This value was then multiplied by a constant (5.7 for cereals), which allowed us to obtain the protein content value expressed as the percentage of protein per 100 g of dry sample.

Ash evaluation

The sample was calcined at 550°C for 2–4 h. The appearances of gray-white ash indicate complete oxidation of all organic matter in the sample. The weight loss by calcination gave the total organic matter content of the sample. The residue is the mineral part or ash (Stadlmayr *et al.*, 2020).

Minerals content evaluation

Microwave digestion was performed for the determination of other elements in the solid sample. 500 mg of sample was transferred to a TFM®PTFE vessel with 6 mL of 65% ultrapure concentrated HNO₃ (14.33 mol/L) and 1 mL of 30% H₂O₂. The sample was subjected to digestion using a microwave (MW-AD, Ethos EZ Microwave Digester, Milestone, Shelton, CT, United States). The heating program for digestion consisted of 4 phases: phase 1 (90°C for 7

min), phase 2 (170°C for 5 min), phase 3 (210°C for 5 min) and phase 4 (210°C for 20 min). In all phases, the power was set to 1000 W. The sample was resuspended with 25 mL of bi-distilled water to undergo analysis by graphite furnace atomic absorption spectrometry (GFAAS). Analyses for arsenic, boron, calcium, chromium, cobalt, iron, manganese, selenium, copper, and zinc were performed by atomic absorption spectroscopy (AAS) according to the AOAC International, (1995). An AA-6300 atomic absorption spectrophotometer (Shimadzu, Columbia, MD, United States), equipped with an ASC-6100 autosampler (Shimadzu, Columbia, MD, United States) and a GFA-EX7i graphite furnace atomizer (Shimadzu, Columbia, MD, United States) was used. Instrument control and data analysis were performed using Multi-Element Program software (WizAArd software, Shimadzu, Columbia, MD, United States). Argon was used as the carrier gas. The AAS instrument is equipped with a hollow cathode lamp for linear sources and a deuterium lamp as a background corrector. Graphite pyrolytically coated tubes were used for the atomization step. To optimize the analytical signal, various tests were performed with different lamp intensities, sample injection volumes, and temperature ranges (1600°C–1800°C for atomization).

Prior to analysis, a calibration line was performed for each analyte to be searched for using the multi-element standard prepared at concentrations of 0 µg/L (Cal Blk), 4 µg/L (Cal Std 1), 12 µg/L (Cal Std 2), and 20µg/L (Cal Std 3). For the determination of other elements in the liquid sample from the *T. vulgare* extraction process, the microwave extraction step was not carried out,

but dilutions and subsequent analysis by graphite furnace atomic absorption spectrometry (GFAAS) was performed. The results are reported in Table 6.

Cell cultures

To evaluate the biological effects and biocompatibility profile of samples under investigation, specific bioscreens were performed on preclinical skin models by using primary and continuous cultures of both epidermal and dermal origin, representing ideal models to study cellular responses to *in vitro* treatments as well as toxicological responses.

HaCaT cells, an immortalized, nontumorigenic human keratinocyte cell line, were received from CNR (courtesy of Dr Valeria Cicatiello) to accomplish *in vitro* bioscreens. HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, United Kingdom) containing high glucose (4.5 g/L), supplemented with 10% fetal bovine serum (FBS; Cambrex, Verviers, Belgium), L-glutamine (2 mM; Sigma, Milan, Italy), penicillin (100 U/mL; Sigma), and streptomycin (100 lg/mL; Sigma) at 37°C in a humidified 5% CO₂ atmosphere, according to ATCC recommendations (Miniaci *et al.*, 2016). Human adult dermal fibroblasts (HDFa) were acquired by ATCC after isolation from the skin of a white male donor (PCS-201-012™). They were cultured in Fibroblast Basal Medium (ATCC) supplemented with recombinant human fibroblast growth factor (rh FGF, 5 ng/mL), L-glutamine (7.5 mM), ascorbic acid (50 µg/mL), hydrocortisone hemisuccinate (1 µg/mL), rh Insulin (5 µg/mL) and fetal bovine serum (FBS, 2%). Penicillin-

Streptomycin-Amphotericin B Solution (Penicillin: 10 Units/mL, Streptomycin: 10 µg/mL, Amphotericin B: 25 ng/mL) was also added. HDFa cells were seeded at a density between 2, five to five $\times 10^3$ cells/cm² and have been passed when approximately 80%–100% confluence was reached in actively proliferating cultures. Cells were cultured in a humidified 5% carbon dioxide atmosphere at 37°C, according to supplier's recommendations (Gazzillo *et al.*, 2023).

Preparation of biological samples (LW and SW) for in vitro experiments

The different waste samples were subjected to distinct treatment methods based on their respective characteristics. Liquid waste (LW) samples were freeze-dried, while solid waste (SW) samples were extracted using the procedure for sprouted wheat (Hung, Hatcher and Barker, 2011), with some modifications. The resultant extract obtained from solid waste was dried using a rotavapor, reconstituted with 10 mL of water (UHPLC-MS grade), and subsequently freeze-dried.

Bioscreens in vitro

Cellular responses to samples application *in vitro* were investigated through the estimation of a “cell survival index”, arising from the combination of cell viability evaluation with automated cell count (Irace *et al.*, 2017). The cell survival index is calculated as the arithmetic mean between the percentage values derived from the MTT assay and the automated cell count. HaCaT and

HDFa were inoculated in 96-well culture plates at a density of 10^4 cells/well and allowed to grow for 24 h. The medium was then replaced with fresh medium, and cells were continuously treated for 24, 48 and 72 h with a range of concentrations (5→100 $\mu\text{g/mL}$) of biological samples, i.e., liquid waste (LW) and solid waste (SW). Using the same experimental procedure, cells were also incubated with cisplatin (cDDP) as positive control for cytotoxic effects (data not shown). After *in vitro* incubations, cell viability was evaluated using the MTT assay procedure, which measures the level of mitochondrial dehydrogenase activity using the yellow 3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate. The assay is based on the redox ability of living mitochondria to convert dissolved MTT into insoluble purple formazan. Briefly, after the treatments, the medium was removed, and the cells were incubated with 20 $\mu\text{L/well}$ of a MTT solution (5 mg/mL) for 1 h in a humidified 5% CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and by adding 100 $\mu\text{L/well}$ of DMSO to solubilize the obtained formazan. Finally, the absorbance was monitored at 550 nm using a microplate reader (iMark microplate reader, Bio Rad, Milan, Italy).

Cell number was determined by TC20 automated cell counter (Bio-Rad, Milan, Italy), providing an accurate and reproducible total count of cells and a live/dead ratio in one step by a specific dye (trypan blue) exclusion assay. Bio-Rad's TC20 automated cell counter uses disposable slides, TC20 trypan blue dye (0.4% trypan blue dye w/v in 0.81% sodium chloride and 0.06%

potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. Once the loaded slide is inserted into the slide port, the TC20 automatically focuses on the cells, detects the presence of trypan blue dye and provides the count. When cells are damaged or dead, trypan blue can enter the cell allowing living cells to be counted. Operationally, after treatments in 96-well culture plates, the medium was removed, and the cells were collected. Ten microliters of cell suspension, mixed with 0.4% trypan blue solution at 1:1 ratio, were loaded into the chambers of disposable slides. The results are expressed in terms of total cell count (number of cells per ml). If trypan blue is detected, the instrument also accounts for the dilution and shows live cell count and percent viability. Total counts and live/dead ratio from random samples for each cell line were subjected to comparisons with manual hemocytometers in control experiments. The calculation of the concentration required to inhibit the net increase in the cell number and viability by 50% (IC_{50}) is based on plots of data ($n = 4$ for each experiment) and repeated three times (total $n = 12$). IC_{50} values were calculated from a dose response curve by nonlinear regression using a curve fitting program, GraphPad Prism 8.0, and are expressed as mean values $\pm$ SEM ($n = 24$) of four independent experiments (Irace *et al.*, 2017; Ferraro *et al.*, 2023).

Cytomorphological analysis

HaCaT cells and HDF were grown on 60 mm culture dishes by plating 5×10^5 cells. After reaching the sub-confluence, the medium was replaced with fresh medium, and cells were continuously treated for 24, 48, and 72 h with a range of concentrations (5→100 µg/mL) of biological samples (LW and SW). After treatments, cells were examined by a phase-contrast microscope (Labovert microscope, Leitz) to monitor any morphological change. Microphotographs at a $\times 200$ total magnification ($\times 20$ objective and $\times 10$ eyepiece) were taken with a standard VCR camera (Nikon) (Ferraro *et al.*, 2023).

Statistical analysis

Statistical analysis of data was performed by two-way ANOVA analysis (SPSS 13.0) followed by the Tukey-Kramer multiple comparison test to evaluate significant differences; p-values ≤ 0.05 were considered as significant. All the determinations were undertaken in triplicate, and results were expressed as mean $\pm$ standard deviation (SD).

Results*Quantification of flavonoids and phenolic compounds*

The identified polyphenols ($n = 16$) in both solid and liquid waste were characterized using a UHPLC coupled to a high-resolution Orbitrap mass spectrometry. For the quantitative analysis calibration curves were built in appropriate solvents and regression coefficients higher than 0.990 were

obtained. Quantitative analysis showed that the dominant phenolic acid identified was ferulic acid, followed by dihydroferulic acid, apigenin-6-arab-8 hexoside, feruloyl quinic acid, apigenin-C-hexoside-hexoside, chlorogenic acid, caffeic acid and others (Table 4). In the solid waste polyphenols extract, ferulic acid represents up to 55.5% of the total polyphenol content identified. In the liquid waste, dihydroferulic acid showed a reduction by 75% compared to the solid waste. A clear reduction in the content of active compounds of up to 95.4% was observed between the solid and liquid waste.

Table 4. Polyphenol content in solid and liquid waste. The results are displayed as average value ($\mu\text{g/g}$) for solid waste and as average ($\mu\text{g/mL}$) for liquid waste and standard deviation (SD).

Bioactive Compounds	Solid Waste $\mu\text{g/g dw}$	Liquid Waste $\mu\text{g/mL}$
Ferulic acid	89.782 ± 1.626	<i>nf</i>
Dihydroferulic acid	24.934 ± 1.323	6.24 ± 0.212
Apigenin-C-hexoside-pentoside	12.398 ± 0.372	0.076 ± 0.052
Apigenin-6-arab-8-hexoside	12.167 ± 1.203	0.08 ± 0.055
Feruloyl quinic acid	7.917 ± 0.388	0.039 ± 0.02
Caffeic acid	2.715 ± 0.067	0.716 ± 0.010
Chlorogenic acid	2.299 ± 0.134	<i>nf</i>
Kaempferol-3-O-rutinoside	1.722 ± 0.095	0.022 ± 0.008
Coumaric acid-O-hexoside	1.264 ± 0.044	0.03 ± 0.002
Apigenin-C-hexoside-hexoside	1.259 ± 0.081	0.016 ± 0.008
Isoorientin-2-O-rhamnoside	1.259 ± 0.050	0.016 ± 0.007
Kaempferol-3-O-glucoside	0.768 ± 0.001	0.04 ± 0.000
Caffeic acid-O-hexoside	0.663 ± 0.082	<i>nf</i>
Naringenin	0.563 ± 0.020	0.037 ± 0.002
Sinapoyl-hexoside	0.528 ± 0.008	0.08 ± 0.002
Apigenin-7-O-glucoside	0.351 ± 0.017	0.002 ± 0.000
Total	160.697	7.394

*dw: dry weight

Antioxidant capacity and total phenolic content (TPC) of liquid and solid waste

The antioxidant activity of both liquid and solid wastes was assessed using two different assays (FRAP and ABTS assay). The total phenolic content (TPC) was evaluated through the Folin-Ciocalteu test. A calibration curve of inhibition, built with Trolox®, was employed as a positive control of the antioxidant assays, while gallic acid was employed to perform the calibration curve for TPC evaluation. Table 5 displays the data as millimoles of Trolox equivalent per kilogram or liter depending on the typologies of the sample is considered solid or liquid (average value and SD). In both antioxidant activity tests a higher antioxidant activity was observed for the solid extract. The total phenolic content in the liquid extract undergoes a significant percentage reduction compared to the solid waste.

Table 5. Evaluation of the antioxidant activity through ABTS and FRAP assay and the total phenolic content (TPC) (Folin-Ciocalteu Test). The analyses were performed in triplicate and data expressed as mean $\pm$ standard deviation.

Samples	ABTS		FRAP		TPC	
	mmol	mmol	mmol	mmol	mg	mg
	trolox/kg	trolox/L	trolox/kg	trolox/L	GAE/g	GAE/mL
Solid Waste	8.598 $\pm$ 0.053		7.262 $\pm$ 0.240		1.771 $\pm$ 0.005	
Liquid Waste	0.223 $\pm$ 0.011		0.285 $\pm$ 0.009		0.105 $\pm$ 0.000	

Nutritional composition of solid waste

Nutritional components of the solid waste were evaluated using different techniques. The Folch method was used to evaluate the content of fat within

the sample. Protein was ascertained through Kjeldahl method. The moisture of the sample was determined to express the data as content of dry material. The solid waste is composed of proteins 3.6 ± 0.3 g per 100 g, fat 2.3 ± 0.6 g per 100 g, and ash 4.1 ± 0.7 g per 100 g. The moisture in the sample was $39\% \pm 2.4\%$. The results shown were the average of three repeated measurements and were reported as average $\pm$ standard deviation.

Minerals content evaluation

Table 6 reports the results obtained from analysis by graphite furnace atomic absorption spectrometry (GFAAS). The solid waste sample was previously treated by microwave digestion, while the liquid waste was directly diluted and subjected to GFAAS analysis. All analyses were performed in triplicate, and results are expressed as averages of mg/kg and mg/L for solid and liquid waste, respectively, and are reported with standard deviations.

Table 6. Quantitative determination of minerals in the solid and liquid waste samples.

Minerals	Solid Waste mg/Kg	SD	Liquid Waste mg/L	SD
Ammonia (NH ₃)	9.25	±0.29	6.005	±0.3
Phosphate (PO ₄ ³⁻)	30.8	±1.1	40.85	±1.05
Calcium (Ca ⁺⁺)	29.33	±2.1	18.18	±1
Magnesium (Mg ⁺⁺)	18.89	±1.8	14.5	±1.05
Sulphate (SO ₄ ²⁻)	20.5	±0.8	41.9	±1.25
Arsenic (As ⁺⁺⁺)	0.0078	±0.02	0.01	±0.025
Boron (B)	<0.2	-	<0.2	-
Manganese (Mn ⁺⁺)	0.162	-	<0.0001	-
Zinc (Zn ⁺⁺)	0.159	±0.016	0.2555	±0.014
Copper (Cu ⁺⁺)	0.026	±0.005	0.0095	±0.003
Cobalt (Co ⁺⁺)	<0.0001	-	<0.0001	-
Iron (Fe ⁺⁺⁺)	0.0945	±0.004	0.015	±0.002
Selenium (Se)	<0.0001	-	<0.0001	-
Chromium (Cr ⁺⁺⁺)	0.0411	±0.008	0.0762	±0.005
Chloride (Cl ⁻)	3.3	±0.13	0.9	±0.065

All the determinations were performed in triplicate and results are expressed as mg/kg and mg/L means for solid and liquid wastes, respectively, and standard deviation.

Bioactivity evaluation in preclinical models of human skin

Both liquid (LW) and solid (SW) wastes were investigated for their potential toxicity by well-established *in vitro* preclinical models of human skin. Epidermal and dermal cultures were selected for biocompatibility analysis of the biological samples under investigation (Piccolo, Ferraro, *et al.*, 2019). Quantitative (i.e., automated cell counts) and functional (i.e., mitochondrial redox activity) assays allowed for the assessment of cellular responses to *in vitro* treatments (Ferraro *et al.*, 2021). Overall, by targeted bioscreens a “Cell survival index” to characterize cellular viability and responses to experimental conditions was established, as fully described in the experimental section (Irace *et al.*, 2017). Representative concentration-effect curves in the examined cellular models (HaCaT cells and HDFa) after 24, 48, and 72 h of exposure to the indicated concentrations ($\mu\text{g/mL}$) of the biological samples (LW and SW) are reported in Figure 7A. As can be clearly observed, bioscreens allow the examined samples to be defined as fully biocompatible. Indeed, no significant interference with cell viability, growth, and proliferation was observed. In line, no cytotoxic effect was noted even at the highest tested concentration (100 $\mu\text{g/mL}$) of both LW and SW following prolonged incubation times (72 h). Consequently, IC_{50} values calculated from concentration-effect curves are all higher than 100 $\mu\text{g/mL}$, indicative of a good safety profile in human cells. In addition, cytomorphological analyses do not show noteworthy alterations in cellular morphology (Figure 7B).

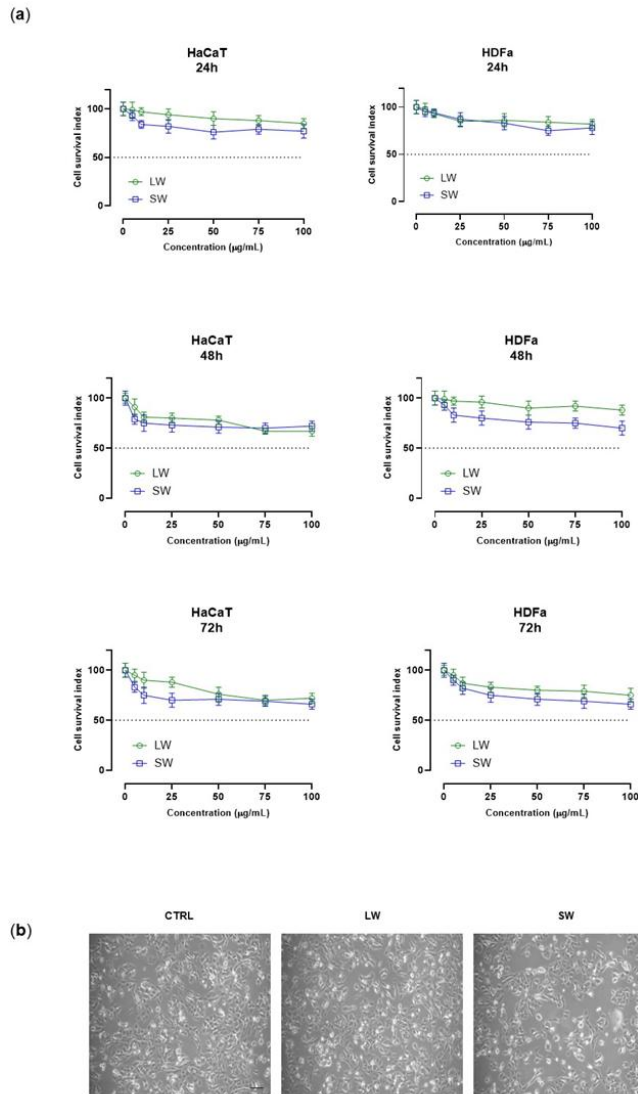


Figure 7. (a) Cell survival index, evaluated by the MTT assay and live/dead automated cell count, for HaCaT cells and HDFa following 24, 48, and 72 h of incubation with the indicated concentrations (0-100 $\mu\text{g/mL}$) of liquid waste (LW) and solid waste (SW), as reported in the legend. Data are expressed as a percentage of untreated control cells and are reported as mean of four independent experiments $\pm$ SEM ($n = 24$). The Cell Survival Index was calculated as described in the experimental section and plotted in line-graphs against the different concentrations of the tested samples. (b) Representative photomicrographs of HaCaT cells monolayers at 200 $\times$ total magnification (20 $\times$ objective and 10 $\times$ eyepiece) by phase-contrast microscopy after 48 h of treatment with 100 $\mu\text{g/mL}$ of LW and SW, as indicated in the legend.

Discussion

Recently, have been reported several studies aimed at valorizing the waste obtained from the natural matrix in the context of the upcycling economy and reduce the environmental pollution. The analysis and characterization of the phenolic compounds contained in aqueous extracts of fennel waste, pea pods and from spent coffee grounds have been already performed (Castaldo, Izzo, *et al.*, 2021; Castaldo, Lombardi, *et al.*, 2021; Castaldo, Izzo, Gaspari, *et al.*, 2022). Valid examples of optimization of biofuel production with reduction of final waste have been already reported in literature, demonstrating that a green production process brings not only environmental but also economic benefits for companies (Mridha *et al.*, 2024; Sarkar *et al.*, 2024). In this context, we were motivated to analyze waste generated from the production of the aqueous extract of the *Triticum Vulgare* (TVE) with the aim of promoting an eco-friendly circular economy operation.

The specific extract of *Triticum vulgare* (TVE) is manufactured by a recently implemented extraction process (U.S. Patent No. 9,895,392) (Riccio, 2018) of Farmaceutici Damor, and it is used as the active principle in pharmaceutical formulations for the treatment of decubitus ulcers, skin lesions, and burns. The company Farmaceutici Damor produces about 2-3 tons of waste each year, which compared to the production data is a relatively small amount. However, the cost of disposing of this waste could be onerous. As reported above, the extraction process adopted for the preparation of one of the most iconic pharmaceutical products based on phytostimulins, is green as it is

based on the use of ecological solvents characterized by a high extraction capacity. The aqueous extract of *Triticum vulgare* (TVE) was obtained through a simple water-based extraction process with non-potentially toxic or harmful solvents. The use of a green extraction process in production represents an essential point for the valorization of a by-product or waste and for its potential use in a subsequent production process. The solid and liquid waste analyzed in this study represent approximately 7.5% and 5% of the entire production process. Therefore, for the entire production process, a total loss between solid and liquid of 12.5% is estimated in terms of yield of the finished product. The ratio between the solid fraction and the liquid fraction in terms of mass is of the order of 1.5 to 1.

The approach of exploiting a by-product or waste obtained as a result of an industrial process to obtain a phytochemical preparation would not only significantly reduce waste, but could also create new sources of income, thus supporting the circular economy. Ensuring environmental and economic sustainability is crucial, especially if a technical-economic assessment of the benefits obtained is carried out. From an economic and environmental point of view, to enhance the by-products of the processing of natural products, the characterization of the chemical composition and the analysis of the chemical-physical characteristics are fundamental (Espro *et al.*, 2021).

Phenolic compounds are widely distributed in plants and have recently gained much attention due to their antioxidant activity and ability to scavenge free radicals with potential beneficial implications for human health (Muscolo

et al., 2024). Phenolic compounds can be classified as secondary plant metabolites, formed by plants during their secondary metabolism (Susanti *et al.*, 2024). Many of them are bioactive and can have antioxidant, antimicrobial, anticarcinogenic and many other beneficial health effects.

Wheat is an important component of the human diet and, therefore, may be an important source of phenolic antioxidants. In particular, ferulic acid is the predominant phenolic acid found in wheat bran, constituting approximately 90% of the total phenolic compounds (W. Li *et al.*, 2024). This observation pushed us to evaluate the by-products of wheat processing from a chemical-physical point of view to understand the potential of its reuse from a circular economy perspective. Literature shows that phenolic acids in wheat grains are mainly present in a form bound to other cereal components such as starch, cellulose, β -glucan, and pentosan (Yu, Vasanthan and Temelli, 2001; Vaher *et al.*, 2010). Insoluble bound phenols can be released by basic, acidic, or enzymatic treatment of samples prior to extraction (Liyana-Pathirana and Shahidi, 2006). The identification of the main chemical constituents and the quantification of the phenolic component, almost always present in nature and endowed with antioxidant activity, represents the starting point that can favor its potential industrial exploitation.

The good antioxidant activity of the analyzed wastes, mainly due to the presence of phenols, demonstrates the possibility of recovering or reusing the phytochemical components contained in these by-products for other production processes.

The antioxidant activity of the extract obtained by solid waste is associated, as already well known for wheat, mainly with ferulic acid (Zhou, Su and Yu, 2004; Klepacka and Fornal, 2006). The quali-quantitative analysis via UHPLC Q-Orbitrap HRMS of the two fractions of processing waste, i.e. solid and liquid, highlighted a different composition of the phenolic fraction. In fact, while in solid waste the phenolic fraction is mainly represented by ferulic acid, in the liquid component of waste the main phenolic component is made up of dihydroferulic acid.

Although solid and liquid waste are quite comparable in quantitative terms, the analysis carried out indicates that their chemical content is very different. This significantly affects their actual reuse value for other production processes. In fact, the in-depth chemical-physical analysis and characterization reported in this study highlighted that, regarding the nature of the phenolic substances, the solid component is mainly rich in ferulic acid (on average 90 $\mu\text{g/g}$ in dry weight) and dihydroferulic acid (on average 24 $\mu\text{g/g}$ in dry weight) while the liquid component is mainly represented by dihydroferulic and caffeic acid, among other things in quite limited quantities (on average 6.24 $\mu\text{g/mL}$ and 0.7 $\mu\text{g/mL}$, respectively).

Comparing the results of our study with recent literature concerning the reuse and characterization of industrial waste derived from various matrices, several notable differences arise, primarily attributable to the differing biological matrices and extraction methodologies employed. Nevertheless,

the existing literature presents numerous examples of waste reuse that is rich in bioactive compounds.

The investigation conducted by Sibhatu *et al.*, (2021) explored the utilization of brewery spent grain (BSG) residues for the extraction of ferulic acid, a naturally occurring antioxidant. This extraction was optimized through an acid treatment followed by alkaline hydrolysis using sodium hydroxide (NaOH), achieving a maximum yield of 46.17 mg/100 g of ferulic acid from BSG, as determined by the Box-Behnken design.

Concentrations of ferulic acid and other active ingredients are lower than those reported in the cited studies; in fact, the data indicate that the solid component is mainly rich in ferulic acid (90 mg/kg dry weight) and dihydroferulic acid (24 mg/kg dry weight), while the liquid component contains mainly dihydroferulic acid and caffeic acid, although in relatively limited concentrations (6.24 mg/L and 0.7 mg/L, respectively). However, it is worth highlighting that the polyphenol extraction method used in our study (Hung, Hatcher and Barker, 2011) does not use acids or bases and is a more environmentally friendly process.

In relation to the study on fennel waste conducted by Castaldo, Izzo, *et al.*, (2021), the extract demonstrated a ferulic acid concentration of 258 mg/kg but constituting only 4.43% of the total. In contrast, analysis of coffee waste (Castaldo, Lombardi, *et al.*, 2021) revealed a significantly lower ferulic acid content than our waste, 0.8 mg/kg compared to 89.78 mg/kg identified in our solid waste, indicating a ferulic acid concentration that is 100 times higher in

our samples. Despite this disparity, coffee waste showed a potential application in the preparation of fortified biscuits.

It is imperative to underscore that, in terms of bioactive compounds, particularly within polyphenolic extracts, it is essential to consider the full spectrum of compounds present, as they may exert synergistic effects. Consequently, we contend that the extraction of polyphenols without the isolation of a single bioactive compound is more advantageous, especially in light of our objective to adhere to principles of the circular economy, which advocate for the minimization of the number of steps and processes involved. The analysis carried out gives solid waste a higher recovery value than liquid waste due to the higher phenol content. In fact, ferulic acid contained mainly in solid waste could find use as a raw material in other production processes. For example, in the natural production of vanillin with biotechnological means and not with synthetic means, thus obtaining a product that can be labeled as "natural" and which could be an alternative both to the product obtained by extraction and to the additive obtained by synthesis, with costs of decidedly more contained production (Xu *et al.*, 2024).

In this context, evaluations conducted in preclinical models of human skin allow both solid and liquid waste from the processing of wheat (*Triticum Vulgare*) to be defined as highly biocompatible. Indeed, cellular responses observed after *in vitro* treatments indicate an excellent safety profile of the wastes under investigation, at least in skin-derived cell. Of note are outcomes

obtained in human primary cultures (HDFa) deriving directly from human skin, which represent preclinical cellular models very sensitive to xenobiotic effects and thereby particularly suitable for toxicology studies. Overall preclinical tests confirm the possibility of reusing and valorizing wastes from the industrial process to produce the phytostimulin-based therapeutics, paving the way for their potential upcoming applications in the biomedical and cosmetic field. Considering the positive feedback, we have attained in human skin cells further endorses a possible use of these materials in cosmetics, as well as raw material for the preparation of formulations for topical applications.

Recently, the use of waste was proposed for the development of cosmetic formulations. In particular, extracts obtained from clementine peels and olive leaves show to endowed 25% of antioxidant activity and no cytotoxic effects (*in vitro* studies on NCTC2544 Cells). Creams with these ingredients are stable at various temperatures and safe for human use, demonstrating good spreadability properties and pseudoplastic behaviour, promoting a circular economy and reducing environmental impact (d'Avanzo *et al.*, 2024). In a study by Wójciak *et al.*, (2024), they investigated the potential of defatted strawberry seeds, obtained after oil extraction, as a source of phenolic compounds with cosmetic applications. The extracts, rich in tiliroside, kaempferol 3-glucoside and ellagic acid, were obtained with a mixture of water and ethanol. Chemical analyses showed antioxidant properties and cytoprotective activity against oxidative stress in human fibroblasts. The

defatted strawberry seeds could therefore be used as beneficial additives for skin care products. Additionally, in a study reported by Draghici-Popa *et al.*, (2024), grape marc, a by-product of the wine industry, they explored the use of grape-seed polyphenolic extracts to produce sunscreens. Extracts, obtained from different grape varieties (Merlot, Fetească Neagră, Blaufränkisch, Isabella) using ethanol and acetone solutions, were analyzed for polyphenol content, antioxidant activity and sun protection factor (SPF). The best extract, from Merlot grape seeds with 70% ethanol, showed an SPF of 7.83, with stable and safe creams.

Based on this evidence reported in literature, the cosmeceutical applications represent one of a possible and potential use of the active ingredients contained in this waste. Moving in this direction, the herein-described outcome can be propaedeutic to further in-depth cellular studies aimed at a precise characterization of biocompatible waste recycling in the biomedical field. Certainly, one of the most economical valorization strategies for the reuse of this processing waste is to evaluate its potential use in the production of food supplements, and antioxidants, or used as a phytoprotective and anti-aging ingredient for cosmetic products. Therefore, the analysis and characterization of waste resulting from the production of a pharmaceutical preparation starting from wheat seeds can be seen as a strategic approach to a circular bioeconomy, where economic and practical advantages deriving from the valorization of waste can guarantee efficient recycling with a positive environmental impact.

Chapter 4

Specific Objective 2

4.1 Valorization of Wheat-Derived Pharmaceutical Waste via Sustainable NADES-Based Extraction and Untargeted Bioactive Profiling

Materials and Methods

Chemicals and reagents

All reagents used through this study were analytical reagent grade, and solvents were of UHPLC grade, unless otherwise specified. Methanol (MeOH), ethanol (EtOH), and water were purchased from Merck (Darmstadt, Germany). Formic acid ($\geq 99\%$, HiPerSolv CHROMANORM® for LC-MS) was obtained from VWR Chemicals (Radnor, PA, USA). Choline chloride was supplied by Fisher Scientific (Pittsburgh, PA, USA). L-(+)-Lactic acid was sourced from PANREAC (Barcelona, Spain). Glycerol (99%), thymol, decanoic acid, betaine, (+)-1,3-butanediol, D-(+)-glucose, and urea were supplied by Merck (Darmstadt, Germany). L-(-)-menthol was purchased from Fluorochem (Derbyshire, UK), while 2,3-butanediol was obtained from Cymit Química (Barcelona, Spain).

Analytical standard of trans-ferulic acid (99.0 %) was purchased from Merck (St. Louis, MO, USA). A standard stock solution of ferulic acid (at a concentration of 1000 mg L^{-1}) was prepared by weighing the appropriate amount of the powdered compound, which was dissolved in MeOH and

stored at $-20\text{ }^{\circ}\text{C}$ in an amber glass vial. An intermediate stock solution containing 10 mg L^{-1} was prepared by diluting the stock solution with MeOH and stored at $-20\text{ }^{\circ}\text{C}$ in an amber glass vial. The working standard solution (0.5 mg L^{-1}) was obtained by further dilution of the intermediate solution with MeOH to the required concentration.

Instrumentation

For NADES preparation, a magnetic stirrer MC-8 series (Bunsen, Madrid, Spain) and a glass thermometer (Scharlab, Barcelona, Spain) were employed. For sample preparation, an analytical BAS 31plus balance (BOECO, Germany), a vortex mixer Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), a DMT-2500 Multi-tube Vortex mixer (Hangzhou MIU Instruments Co., Ltd., Hangzhou, China), a VWR ultrasonic bath (VWR International, Radnor, PA, USA) and a Universal 320R centrifuge (Hettich, Germany) were used. Nylon syringe filters ($0.22\text{ }\mu\text{m} \times 13\text{ mm}$) from Phenomenex (Castel Maggiore, Italy) were used to filter sample extracts before injection into the UHPLC system.

The UHPLC-MS instrument consisted of an Agilent 1290 Infinity II Ultra-High Pressure Liquid Chromatography system (Santa Clara, CA, USA) coupled to an Agilent 6550 iFunnel Q-ToF LC/MS model (Santa Clara, CA, USA), equipped with a JetStream electrospray ion source (Dual AJS ESI). The Agilent MassHunter Data Acquisition and Mass Hunter Qualitative Analysis

(version 10.0) software were employed for data acquisition and processing, respectively.

Sampling

Triticum vulgare is subjected to standardized germination under controlled conditions of temperature, light, and humidity. The aqueous extract of *Triticum vulgare* (TVE) is obtained using a recently developed and patented extraction method by Farmaceutici Damor (Riccio, 2018). More specifically, liquid extraction is carried out on macerated germinated wheat, followed by a separation process of the liquid from the solid component by pressing, resulting in a clear crude extract. The residues from this production process are properly collected: in particular, a solid waste (SW) is generated as a by-product of the pressing phase. This solid waste was provided by the company Farmaceutici Damor (Italy). SW was dried (to permit homogenization), ground, and stored at $-80\text{ }^{\circ}\text{C}$ until the analysis.

NADES preparation

In this study, eight NADES, —both hydrophobic and hydrophilic— were prepared using different natural primary components at various molar ratios (Table 7). These NADES were selected to cover a wide range of physicochemical properties, including pH, polarity, and viscosity, which have been previously characterized. The composition of the NADES was as follows (molar ratio): lactic acid:glycerol:water (1:1:3), choline chloride:lactic

acid:water (1:3:6), lactic acid:glucose:water (5:1:9), thymol:menthol (1:1), decanoic acid:thymol (1:2), betaine:1,3butanediol:water (1:3:10.6), choline chloride:2,3 butanediol (1:4), choline chloride:urea:water (1:2:3).

The designated names correspond to the abbreviation of the two principal components of the mixture, while the addition of water was represented by adding "H" at the end of the nomenclature (hydrophilic nature). This nomenclature is consistent with that used in previous studies reporting the use of NADES (Carbonell-Rozas *et al.*, 2021). According to Dai *et al.*, (2013), NADES were prepared using the heating and stirring method while adding a predetermined amount of water (when included in the eutectic system). To create a uniform, transparent liquid, the natural ingredients of each NADES were placed in a dark bottle and heated to 40 °C for one hour while being stirred by a magnetic force. To ensure that recrystallization did not occur, the eutectic mixture was left at room temperature for twenty-four hours. The molecular structure and physicochemical properties of the NADES used in this study have already been characterized in previous studies (Turiel Trujillo, Díaz-Álvarez and Martín Esteban, 2024; Capilla-Flores *et al.*, 2025; Carbonell-Rozas *et al.*, 2025; Spaggiari, Carbonell-Rozas, *et al.*, 2025).

Table 7. NADES composition and physiochemical properties.

NADES	NAME	Component 1	Component 2	Water	molar ratio	Polarity (ENR) ^a (Kcal/mol)	pH	Viscosity [mPa·s] T=20 °C	Reference
NADES 1	LGLH	Lactic acid	Glycerol	yes	1:1:3	1.086	1.4	26	Carbonell-Rozas et al., 2025
NADES 2	ChCILH	Choline chloride	Lactic acid	yes	1:3:6	1.043	4.92	27	Carbonell-Rozas et al., 2025
NADES 3	TM	Thymol	Menthol	no	1:1	0.55-0.7	6.1	47.93	Spaggiari, Carbonell-Rozas, <i>et al.</i> , 2025
NADES 4	DAT	Decanoic acid	Thymol	no	1:2	0.41	5.6	16.02	Spaggiari, Carbonell-Rozas, <i>et al.</i> , 2025
NADES 5	Bet-1,3BDH	Betaine	1,3-Butanediol	yes	1:3:10.6	1.15	5.9	5.56	Spaggiari, Carbonell-Rozas, <i>et al.</i> , 2025
NADES 6	LGH	Lactic acid	Glucose	yes	5:1:9	-	1.07	44	Carbonell-Rozas et al., 2025
NADES 7	ChCl-2,3BD	Choline chloride	2,3-Butanediol	no	1:4	0.7	4.6	9.48	Capilla-Flores et al., 2025
NADES 8	ChClUH	Choline chloride	Urea	yes	1:2:3	0.955	7.7	9	Carbonell-Rozas et al., 2025

^a Expressed as emission energy of Nile Red.

NADES-based UAE extraction procedure

After NADES screening, ultrasound-assisted extraction (UAE) of phenolic compounds was performed by mixing 0.5 g of the dried sample with 7 mL of the optimum selected NADES (Betaine–1,3BDH). The mixture was vortexed for 20 s, followed by an ultrasound bath for 18 min at 28 °C. Finally, the tubes were centrifuged at 9000 rpm for 7 min at 5 °C. The supernatant (143 µL) was diluted with 857 µL of MeOH containing 0.1% (*v/v*) formic acid, resulting in a

final volume of 1 mL. The resulting solution was filtered through a nylon filter ($0.22\ \mu\text{m} \times 13\ \text{mm}$) prior to injection into the UHPLC-Q-ToF-HRMS system. The schematic procedure of the sample treatment is shown in Figure 8.

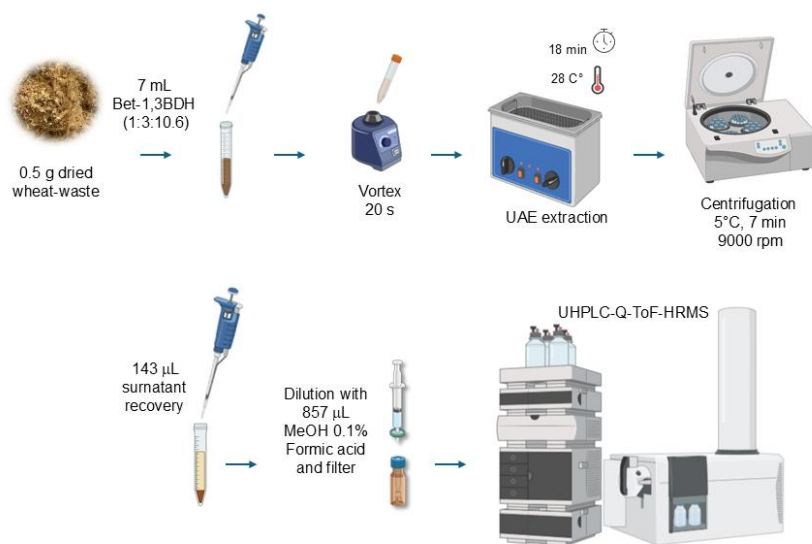


Figure 8. Schematic procedure of the proposed NADES-based UAE sample treatment.

Design of experiments (DoE) and statistical analysis

The multivariate optimization of the UAE extraction procedure was carried out using a reduced face-centered central composite design (reduced CCF) within the framework of response surface methodology (RSM), to evaluate the influence of the key parameters affecting the extraction. The multivariate study was conducted using MODDE™ Pro software version 13.1 (Sartorius AG, Goettingen, Germany). The four independent variables investigated in

the design were: sample mass/solvent volume ratio (from 0.05 to 0.250 g/mL), temperature of the ultrasonic bath (from 25 to 60 °C), extraction time (from 3 to 20 min) and centrifugation time (from 5 to 25 min). These ranges were defined based on the commonly used values in the UAE. The experimental data were statistically evaluated using analysis of variance (ANOVA) at the 95% confidence level. The adequacy of the fitted model was assessed using the coefficient of determination (R^2) and the lack-of-fit test. Response surface plots were constructed using the same software by selecting two variables at a time while keeping the others constant.

UHPLC-Q-ToF-HRMS conditions

Chromatographic conditions were based on a previously reported method for phenolic compounds detection (Carbonell-Rozas, Romero-González and Garrido Frenich, 2024). Chromatographic separation was performed on an Agilent 1290 Infinity II Ultra-High Pressure Liquid Chromatography system (Santa Clara, CA, USA) using a Hypersil GOLD™ aQ (100 × 2.1 mm, 1.9 μm) (Thermo Scientific, CA, USA). The mobile phases were water with 0.1 % formic acid (solvent A) and MeOH with 0.1 % formic acid (solvent B). The chromatographic gradient profile was as follows: 0–2 min, 5–30 % B; 2–8 min, 30–100 % B; 8–10 min, 100 % B. Subsequently, eluent A was re-established to 95 % in 1 min and maintained for 2 min. The total run time was 13 min, and the injection volume was 5 μL. The flow rate and the column temperature were set at 0.3 mL/min and 40 °C, respectively.

Mass spectrometric analysis was carried out on an Agilent 6550 iFunnel Q-ToF LC/MS model (Santa Clara, CA, USA), equipped with a JetStream electrospray ion source (ESI). The source operated in both positive (ESI⁺) and negative (ESI⁻) modes. Source parameters were set as follows: nebulizer pressure 35 psi, drying gas flow rate 15 L/min, drying gas temperature 160 °C (ESI⁺) and 150 °C (ESI⁻), sheath gas flow rate 10 L/min, sheath gas temperature 260 °C (ESI⁺) and 250 °C (ESI⁻), capillary voltage 3500V (ESI⁺) and 3000V (ESI⁻). Data were acquired in centroid mode using LC-MS and Auto MS/MS workflows. For both ionization modes, the acquisition range was set at m/z 50–1000 with a scan rate of 10 spectra/s. Absorbance thresholds were set at an absolute value of 200 and a relative value of 0.01 % for MS, and 5 and 0.01 %, respectively, for auto MS/MS mode. The collision energy was set to 0 V at 0 min and ramped to 20–30 V at 0.5 min during auto MS/MS acquisition for both polarities. Reference mass correction was applied using the following ions: m/z 121.050873, 322.048121, and 922.009798 for positive mode, and m/z 119.036, and 966.0007 for negative mode, with a detection window of 100 ppm and a minimum peak height of 1000 counts.

Data Preprocessing, Statistical Analysis and Data Mining

To perform the non-targeted analysis, raw data (*.d) were converted to abf format using Analysis Base File Converter version 1.3.8802.34458. to permit the automatic deconvolution and alignment using MS-DIAL version 5.5.250221 (Tsugawa *et al.*, 2015). Data from MS-DIAL were normalized using

the NOREVA pipeline for imputation of missing values and normalization (Fu *et al.*, 2022). Data were uploaded to the NOREVA platform, selecting a multi-class analysis approach without QC and internal standards. Missing values were imputed using the K-nearest neighbours (KNN) algorithm, with the number of neighbours set to 10 and a maximum of 1500 metabolites imputed. No data transformation was applied before normalization. Normalization was performed using EigenMS algorithm, followed by autoscaling as a metabolite-based preprocessing method. The normalized dataset was subsequently analyzed using SIMCA software (version 18, Sartorius), applying Pareto scaling and Principal Component Analysis (PCA-X) for initial unsupervised exploration. Supervised Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) was then performed to explore class discrimination between control (hydroalcoholic extract) and treated samples (NADES extract). The OPLS-DA models were also subjected to a permutation test, with 200 permutations, in order to prove their validity. Variables with a Variable Importance in Projection (VIP) score above 1 were selected for further annotation of significative features. Compound annotation was carried out using SIRIUS software (version 6.1.0). The tentative identification was conducted using multiple spectral libraries and databases integrated within SIRIUS, including COCONUT, ChEBI, LOTUS, PubChem, PlantCyc, among others (Dührkop *et al.*, 2019). Each identification was assigned a confidence level based on the available information-such as

molecular weight, fragmentation pattern, isotopic distribution and spectral similarity-according to the model proposed by Schymanski *et al.*, (2014).

Results and discussion

Selection of NADES as extraction solvent

To select the best extractant and evaluate how the NADES composition affects the extraction of phenolic compounds from wheat-based waste, eight different NADES were used as extraction solvents in an SLE procedure. Taking into consideration the relevance and the high presence of ferulic acid in wheat matrices (Leváková and Lacko-Bartošová, 2017; Ciriaco *et al.*, 2024), and also its scientific interest (Kobayashi *et al.*, 2025), we selected the mass signal of this compound (expressed as peak area) as representative compound to assess extraction yield. Two spectral identification points for ferulic acid were established: the adduct ion $[M-H]^-$ at a m/z of 193.0506 and its most representative fragment at a m/z of 178.0274. Both diagnostic ions showed a signal-to-noise ratio above 10, and the ion ratio was preserved throughout the analysis.

Initially, 0.5 g of dried and ground wheat waste and 5 mL of each NADES were mixed and vortexed for 20 s. The mixture was then placed under mechanical agitation for 15 min at maximum speed (2500 rpm). After the centrifugation step, 100 μ L of the supernatant was mixed with 900 μ L MeOH containing 0.1% (*v/v*) formic acid and filtered before its injection into the UHPLC-Q-ToF-HRMS system. In parallel, extraction was also carried out

using a traditional extraction solvent composed of 5 mL of EtOH:water (80:20, *v/v*). In all cases, two samples were submitted to the same sample procedure (experimental replicates), obtaining a relative standard deviation (RSDs) below 20 %. The results are expressed as the normalized peak area (decimal logarithm of the peak area) of ferulic acid (target compound of reference) in wheat-based waste with the corresponding relative standard errors (Figure 9).

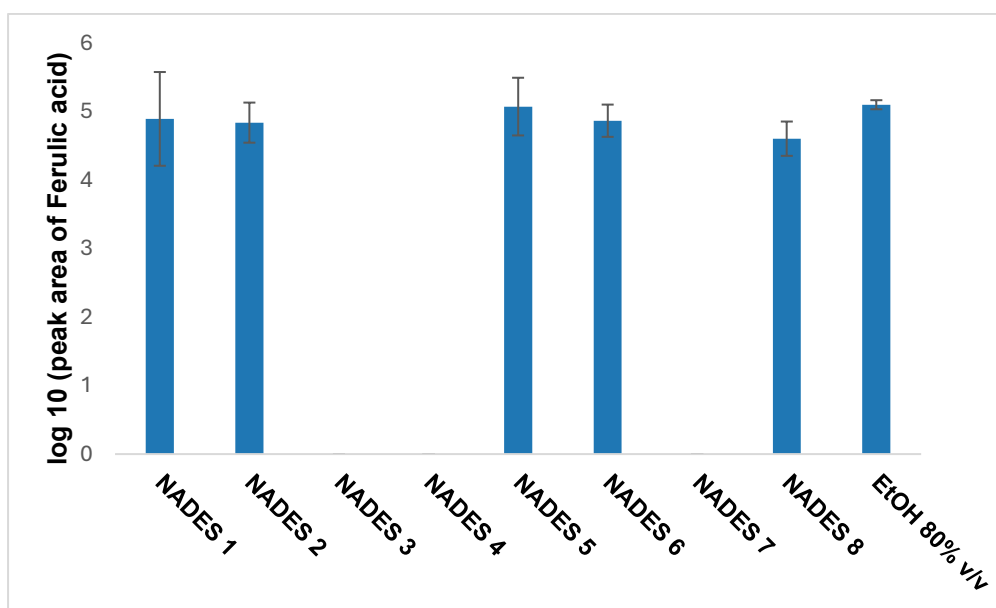


Figure 9. Extraction efficiency of ferulic acid in different NADES systems and a hydroalcoholic mixture. The analysis was performed in duplicate, and the results are represented as log10 of peak area of ferulic acid +/- relative standard errors.

It can be observed that most of the NADES tested (except for NADES 3, 4 and 7) were able to extract ferulic acid with a comparable yield as the

hydroalcoholic mixture of EtOH:water (80:20, *v/v*). The obtained data indicated that NADES, and especially NADES 5, composed by betaine:1,3-butanediol:water (Bet-1,3BDH) at a molar ratio of 1:3:10.6, can be used as an effective alternative to organic solvents to extract phenolic compounds from wheat-based pharmaceutical waste.

Therefore, Bet-1,3BDH was chosen as the extraction solvent for subsequent experiments, as it showed the highest efficiency and yielded the greatest ferulic acid concentration (peak area compared with standard dissolved in MeOH). This suggests superior solvation and mass transfer capabilities, likely attributed to its favourable combination of low viscosity and the presence of water in its composition, which is known to enhance hydrogen bonding interactions and reduce internal friction within the solvent matrix (Wang *et al.*, 2024). Additionally, it is formulated with betaine, a compound that is particularly advantageous compared to traditional NADES based on choline chloride (ChCl) because of its high biodegradability and low toxicity (Mohd Fuad and Mohd Nadzir, 2022).

In contrast, NADES 3, 4, and 7, which lack water in their composition, failed to extract detectable amounts of ferulic acid. In particular, NADES 3 and 4 were characterized as hydrophobic, raising the possibility of phase separation during the extraction process. To investigate this aspect, we also performed a salting-out test on NADES 3 by adding 1 g of NaCl. The results showed that there were no improvements in extraction with the addition of salts to NADES 3. The same test was also carried out with NADES 5, but to evaluate if

centrifugation improved with the addition of salt, allowing better recovery of the supernatant. In contrast, salt did not improve phase separation and even reduced the peak area, indicating lower ferulic acid recovery (Figure S1).

Our results confirmed the key role of water in NADES performance. In particular, NADES 5 (our optimum selected) contains a high-water content of 10.6 (molar ratio), in line with previous studies reporting that water reduces viscosity and improves analyte solubilization through hydration shells and increasing polarity (Vilková, Płotka-Wasyłka and Andruch, 2020). However, pure water extracted less ferulic acid; thus, this increase was not solely due to water but to the eutectic system as a whole, with its unique composition and properties (Figure S1).

In our procedure, pH is not the decisive factor for extraction failure. Although acidic environments may favour extraction kinetics, pH alone does not preclude extraction. Indeed, NADES 8, composed of urea and possessing a neutral to slightly basic pH (7.7), was still able to extract ferulic acid, although in significantly lower quantities compared to NADES 5.

The most critical parameter influencing extraction efficiency in this comparative study was viscosity. High viscosity hinders solvent penetration, reduces diffusion rates, and limits analyte mobility, ultimately impairing extraction performance (Benvenuti *et al.*, 2020). NADES 5, with the lowest viscosity among the solvents tested (5.56 mPa·s), consistently outperformed the others.

Generally, hydrophilic NADES exhibit strong matrix effects, which can interfere with the quantification of the analyte or the reproducibility of the extraction. Nevertheless, NADES 5 showed a relatively low matrix effect, especially when compared to the other hydrophilic NADES 1, 2, and 6 (Figure S2). To evaluate the intrinsic matrix effect of each NADES, the comparison was made by measuring the signal obtained when ferulic acid was dissolved in a traditional solvent (MeOH) versus when it was dissolved in the corresponding NADES.

In conclusion, the data support the hypothesis that hydrophilic, low-viscosity NADES containing water are optimal for the extraction of ferulic acid. Therefore, it is fundamental to balance solvent polarity, viscosity and water content for effective extraction using green solvents.

Optimization of the UAE conditions

After identifying NADES 5 as the optimal system, we decided to further evaluate its performance by comparing the traditional SLE technique with an UAE procedure, where the mechanical agitation step was replaced by sonication. UAE is generally recognized as a greener extraction method due to its lower energy consumption and potential to reduce extraction times (Panda and Manickam, 2019). The results showed that the extraction yields of ferulic acid using NADES 5 were comparable between the two techniques. Therefore, for all subsequent experiments, we focused on the use of NADES 5 in combination with UAE (Figure S1).

The chemometric experimental design was applied to optimise the conditions of UAE for the extraction of ferulic acid from wheat-based waste. This approach permits fewer experiments, lower solvent and waste levels, and energy and time consumption that is consistent with GSP principles (López-Lorente *et al.*, 2022). The four independent variables investigated in the design were: sample/solvent ratio, temperature of the ultrasonic bath, extraction time and centrifugation time. Regarding the sample/solvent ratio, the volume of extracting solvent was limited to a maximum of 10 mL to avoid excessive dilution and to be in line with GSP principle number 5. Moreover, higher volumes would require the injection of large amounts of NADES into the instrument, which could pose some concern, such as peak distortion and column backpressure, and also source fouling and blockages. The extraction time was set to a maximum of 20 min to ensure the method remains sufficiently short and aligned with GSP principle 8. In addition, a maximum temperature of 60 °C was set to avoid degradation of phenolic compounds. A reduced CCF design was suggested by MODDE™, considering the number of variables, a minimum of 15 degrees of freedom, and a total of 23 runs including 3 center points, ensuring model robustness and accurate error estimation. Thus, the matrix design was composed of twenty-three experiments, which were carried out randomly using wheat waste as a representative matrix. The response variable was the chromatographic peak areas of ferulic acid in the sample. The values of the independent variables in

each experiment and the responses obtained are shown in Table S1. The behaviour of the responses obtained was explained by a quadratic model.

ANOVA results showed that the model was statistically significant ($F=21.6325$ and $p\text{-value}=0.000$). The determination coefficient (R^2 of 0.910) and the cross-validated predictive coefficient (Q^2 of 0.837) indicate good model fit and predictive ability. Additionally, the lack of fit was not significant ($F=4.07908$ and $p\text{-value}=0.214$), confirming that the model adequately describes the experimental data. The quality and adequacy of the model were assessed using the coefficient of determination, R^2 , and the lack of fit $p\text{-value}$.

The criteria used to select the optimal values for the studied variables were the maximization of the peak area of ferulic acid, which was chosen as the response variable in the model. The three-dimensional (3D) response surface plot generated using MODDE™ (Figure 10) illustrates the effects of the sample/solvent ratio and extraction time on ferulic acid recovery. The curvature of the response surface reflects the interaction between these variables. The area of maximum curvature, coloured in red, corresponds to the highest peak area observed, indicating the conditions that lead to the most efficient extraction of ferulic acid. In the 3D plot, the other two factors, i.e. extraction temperature and centrifugation time, were held constant at their optimal values, 28.4 °C and 7 min, respectively, to better visualize the interactions of the two most impactful variables, i.e. sample/solvent ratio and extraction time. As shown from the model, ferulic acid extraction increased with longer extraction times, reaching its maximum at 18.3 min. Regarding

the sample mass/solvent ratio, the best performance was achieved at 0.07 g/mL, equivalent to 0.5 g of sample in 7.14 mL of solvent, which is close to the lowest value in the model range (0.05 g/mL). Higher ratios, which correspond to lower solvent volumes, were insufficient to ensure efficient extraction. For practical purposes, the optimal extraction values have been rounded to the nearest unit. Thus, the optimal values were: 28 °C as extraction temperature, 0.5 g of wheat waste sample, 7 mL of NADES as extraction solvent and 18 min as extraction time.

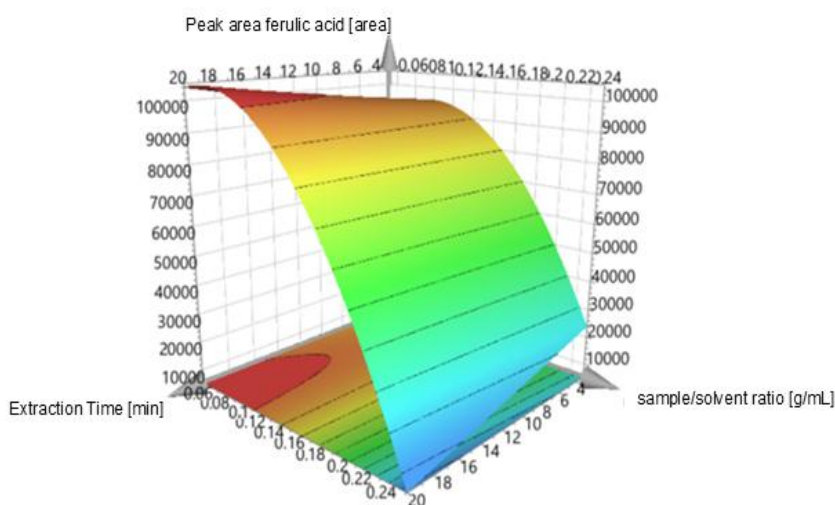


Figure 10. 3D response surface plot for the optimization of the proposed NADES-based UAE procedure.

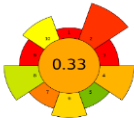
Greenness and practicability evaluation and comparison

The greenness and the applicability of the proposed sample extraction method were evaluated using the AGREEprep and BAGI metrics, respectively (Wojnowski *et al.*, 2022; Manousi *et al.*, 2023). For the evaluations conducted with these two tools, the NADES-based UAE optimised method was compared with two hydroalcoholic extraction approaches. Table 8 summarises the main operational characteristics of each sample treatment, together with the respective pictograms and scores. AGREEprep tool relies on the ten principles of GSP, which are quantitatively expressed on a standardized scale from 0 to 1 and visually through a pictogram (Wojnowski *et al.*, 2022). The reference method obtained the lowest final score (0.09) due to numerous critical issues, including the abundant use of hazardous solvents, a high number of extraction steps and high energy consumption due to rotavapor use and long centrifugation time. The energy consumption of each device is evaluated by Criterion 8. The hydroalcoholic mixture method, proposed as a control in this study, obtained an intermediate score (0.33), thanks to the reduction in the number of steps and the volume of sample and solvent used. Finally, the optimised UAE method obtained the highest score (0.57), thanks to the drastic reduction in energy consumption, especially reducing centrifugation time (the most energy-intensive step) and to the multiple advantages of using NADES. In fact, their safety use allows for achieving the maximum score (1) for criteria 2 and 10, unlike the hydroalcoholic mixture, which is less safe (ethanol can cause skin irritation)

and is neither sustainable nor renewable (criterion 3) (Spaggiari, Hiemstra, *et al.*, 2025). All three methods involve ex situ extraction in the laboratory, after transporting the sample from the company; consequently, criterion 1 scored 0 in all cases.

The BAGI score is a complementary tool based on the principles of white analytical chemistry (WAC) (Nowak, Wietecha-Posłuszny and Pawliszyn, 2021). This tool evaluates how practical and applicable a method is, and it is based on ten attributes. The result is expressed as a score in a pictogram with values in a range between 25 and 100, and if the value is >60, the method can be considered as practical. In our case, all three examined methods have a score higher than 60 and overall, the scores obtained are similar. One of the limitations of NADES that slightly reduces the blue score is the need to prepare reagents and materials in the laboratory (attribute 7), although their preparation is extremely simple (*Section NADES preparation*).

Table 8. Comparison of selected phenolic extraction methods based on their main features, greenness and practicability scores.

Reference	Extraction solvent	Sample (g)	Volume (mL)	Technique	Time (min)	°C	AGREEprep Score	BAGI Score
Ciriaco <i>et al.</i> , 2024	EtOH:H ₂ O (80/20, v/v)	1	30	SLE	40	25		
This study	EtOH:H ₂ O (80/20, v/v)	0.5	5	SLE	15	25		
This study	NADES Bet:1,3BDH : water (1:3:10.6)	0.5	7	UAE	18	29		

Abbreviations: SLE (solid liquid extraction); UAE (ultrasound assisted extraction); BAGI (Blue applicability grade index); AGREEprep (Analytical GREEnness metric for sample preparation).

Multivariate analysis

Model quality and robustness were evaluated using cross-validation ANOVA (CV-ANOVA), with R²_Y and Q² values above 0.9, indicating high model performance (Table 9). The model showed 100% classification accuracy in the misclassification test; however, this result should be interpreted with caution due to the limited number of sample groups included. The test provided values of R² intercept and Q² intercept of 0.299 and -0.623, respectively, in ESI(+) mode, and 0.313 and -0.702 for R² and Q² intercepts when assessing

the model built for ESI(-) dataset. These values indicate a worse performance when randomized, therefore proving the good prediction power of the model without being overfitted.

Table 9. Quality parameters built for a dataset of both polarities.

Dataset	Number of components	CV-ANOVA (p-value)	R2X (cum)	R2Y (cum)	Q2 (cum)	Classification accuracy
ESI(-)	2	3.67554×10^{-8}	0.996	0.998	0.995	100%
ESI(+)	2	1.65937×10^{-10}	0.599	1	0.993	100%

The OPLS-DA plots were generated using SIMCA software (version 18, Sartorius) and are reported in Figure 11 (ESI -) and Figure 12 (ESI +). In these figures, the control is represented by hydroalcoholic extraction (EtOH, 80%), while the treated is the extraction performed with our optimum NADES 5 (betaine-1,3butanediol-water 1:3:10.6, molar ratio). NADES and hydroalcoholic extracts showed clear separation, with OPLS-DA effectively discriminating the groups, supported by acceptable quality parameters and no overfitting (Permutation Test). VIP scores above 1 were selected and exported for further annotation in both polarity modes.

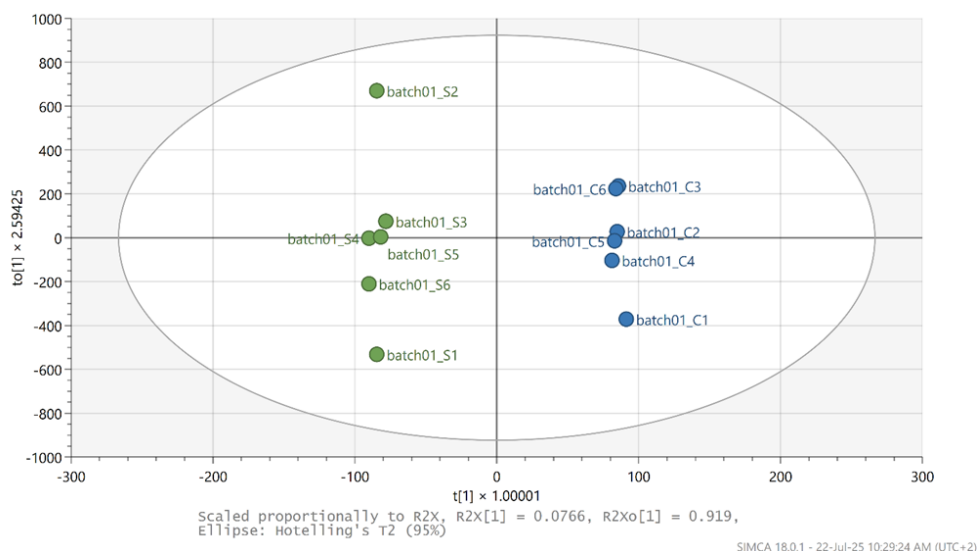


Figure 11. Orthogonal partial least squares – discriminant analysis (OPLS-DA) of the 2 datasets of samples extracted with NADES 5 (green dots) and extracted with EtOH 80% (v/v) (blue dots) in negative ionization mode (ESI-).

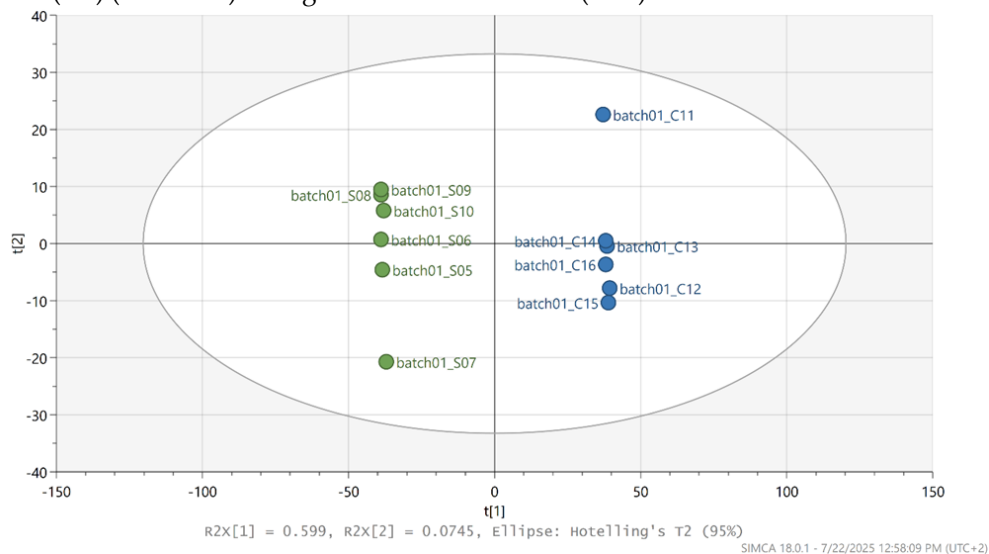


Figure 12. Orthogonal partial least squares – discriminant analysis (OPLS-DA) of the 2 datasets of samples extracted with NADES 5 (green dots) and extracted with EtOH 80% (v/v) (blue dots) in positive ionization mode (ESI+).

Tentative compounds identification and annotation using SIRIUS

The compounds were annotated using SIRIUS and its integrated multiple spectral libraries and databases. Results are represented in Table 10 (negative polarity) and Table 11 (positive polarity).

In negative polarity, 23 compounds were tentatively identified, distributed across different chemical classes, including phenolic acids, alkaloids, carboxylic acids and fatty acids. Only 4 metabolites were found to be more abundant in samples extracted with the innovative solvent (NADES) than in the hydroalcoholic mixture. These include the alkaloid 4-hydroxyquinoline, known for its antimicrobial properties and as a precursor of antibiotics and fungicides, and the phenolic compound ferulic acid, identified with a confidence level of 1 thanks to confirmation with the standard, which has antioxidant activity and is of great interest in nutraceuticals and cosmetics. The group of compounds extracted with NADES also includes two very long-chain fatty acids, tetracosanedioic acid and hexacosanedioic acid, which are among the lipophilic compounds of industrial interest, are also particularly noteworthy. In contrast, most of the bioactive compounds detected in negative polarity were more abundant with hydroalcoholic extraction. These include phenolic acids such as protocatechuic acid and p-coumaric acid, both of which have antioxidant and anti-inflammatory properties, and a wide variety of long-chain fatty acids and hydroxy acids, including ricinoleic acid, nervonic acid and oleic acid.

Overall, for positive polarity, 39 compounds belonging to different chemical classes were tentatively annotated. Comparative analysis indicated that the innovative solvent (NADES) enhanced the extraction of 18 out of 39 annotated compounds, particularly within specific chemical classes of bioactive compounds. In addition to ferulic acid, among the phenolic compounds and their derivatives, piperonyl acrolein, trans-cinnamyl alcohol, 6-methylcoumarin, angoletin, tyrosol 4-sulfate, cis-p-coumaroylagmatine and m-cresol, all known for their potential antioxidant and antimicrobial properties, were extracted in higher concentrations using NADES. Moreover, an increase in metabolites with specific activity was observed, such as compounds of cosmetic interest like allantoin and the flavouring agent heptenone. Furthermore, lipophilic molecules such as 13-docosenamide and N-hexadecanoyl-sphinganine were also recovered more efficiently using NADES. However, some relevant metabolites were more abundant with traditional extraction. These included choline (involved in lipid metabolism and membrane structure), glycerophosphocholine (membrane stabiliser), and ($\pm$)-cystine. Moreover, octadecanamide and other lipid derivatives were detected in the conventional extracts. Overall, the presence of lipophilic compounds in the analysed waste showed that this material could be used as a source of high-value phytochemicals, with potential applications in various industrial sectors, particularly in lignocellulosic biorefineries (Benito Jareño *et al.*, 2025). Furthermore, thanks to the use of the non-targeted approach, it was possible to identify and annotate bioactive compounds that were already

known but never reported in wheat. This result further emphasises and confirms the crucial role of untargeted strategy, together with NADES-based extraction, in plant metabolomics studies (Spaggiari, Hiemstra, *et al.*, 2025).

Table 10. ESI (-) tentative compound annotation.

m/z [M-H] ⁻	RT	Annotated compound	Confidence level	Class	Function or biological role	Concentration in extract with NADES
137.02 46	6.139	3-Hydroxybenzoic acid	2A	Salicylic acids	In corn, citrus, bilberry, beer; gut metabolite of catechins; used in adhesives, resins, oil drilling, rubber.	Lower
137.03 62	0.986	Urocanic acid	3	Imidazole alkaloids derived from histidine	Histidine metabolite; UV protection, immune modulator.	Lower
144.04 47	4.533	4-Hydroxyquinoline	3	Alkaloids	Microbial product; Present in cereals, antimicrobial; antibiotic/fungicide precursor.	Higher
153.01 88	3.49	Protocatechuic acid	2A	Benzene and substituted derivatives	Antioxidant, anti-inflammatory; nutraceutical use.	Lower
163.04 02	5.075	Coumaric acid	2A	Phenolic compound (Hydroxycinnamic acid derivative)	Plant phenolic; antioxidant, hepatoprotective, antimicrobial; food additive.	Lower
193.05 07	5.721	Ferulic acid	1	Phenolic compound (Hydroxycinnamic acid derivative)	Multi-functional antioxidant; used in cosmetics, nutraceuticals.	Higher
195.02 92	5.043	4-Methoxyisophthalic acid	3	m-Phtalate esters	Lignin breakdown product; minor role.	Lower
243.19 63	9.753	2-Hydroxytetradecanoic acid	3	Fatty acyls	Stabilizes membranes; in energy, nutrition, and LPS synthesis.	Lower
267.19 61	10.056	Monooxyhexadecanoic acid	4	Fatty acids	-	Lower

271.22 82	10.404	2-Hydroxypalmitic acid	3	Long-chain fatty acids	In sphingolipids; membrane, energy, signaling roles.	Lower
277.21 79	10.316	Octadeca-9,13,16-trienoic acid	4	Unsaturated fatty acids	-	Lower
279.23 46	10.518	Mangiferic acid	3	Linoleic acid derivatives	Antioxidant/anti-inflammatory; inhibits angiogenesis.	Lower
281.24 95	10.757	(E)-Oleic acid	3	Long-chain fatty acids	Monounsaturated FA; nutrient, stabilizer; in oils, soaps, pharma.	Lower
297.24 41	9.767	Ricinoleic acid	3	Long-chain fatty acids	Castor oil component; in lubricants and pharma.	Lower
299.25 89	9.973	12-Hydroxyoctadecanoic acid	3	hydroxy fatty acid	Plant metabolite	Lower
299.25 92	10.773	10-hydroxystearic acid	3	Long-chain fatty acids	Hydroxy FA; bioplastic/lubricant intermediate.	Lower
307.26 35	10.843	6-Icosynoic Acid	3	Fatty acyls	Acetylenic FA; antifungal (from plant <i>S. sabiceoides</i>).	Lower
309.27 95	11.092	11-Eicosenoic acid	4	Fatty acyls	Monounsaturated FA; nutrient, stabilizer; in einkorn wheat; in jojoba oil, skincare.	Lower
353.34 18	12.204	Tricosanoic acid	4	Lipids and lipid-like molecules	Saturated FA; nutrient, stabilizer; used in cosmetics, lubes.	Lower
365.34 26	11.874	Nervonic acid	4	Steroids and steroid derivatives	Key for myelin and nerves in animals; membrane, lipid, stress in plants.	Lower
369.33 79	11.705	2-Hydroxytricosanoic acid	3	Long-chain fatty acids	Hydroxy FA in waxes; supports cuticle.	Lower
397.33 3	11.328	Tetracosanedioic acid	4	very long-chain fatty acids	Found in potatoes, plants, wheat (post-depolymerization).	Higher
425.36 37	11.719	Hexacosanedioic acid	4	Lipids and lipid-like molecules	Dicarboxylic acid; nylon-like polymer precursor.	Higher

Table 11. ESI (+) tentative compound annotation.

m/z [M-H] ⁺	RT	Annotated compound	Confidence level	Class	Function or biological role	Concentration in extract with NADES
104.10 726	9.603	Choline	2A	Alkaloids	Cell membrane, lipid metabolism, present in wheat	Lower
109.06 445	8.26	M-cresol	3	Anisoles	Plant phenolics, antimicrobial	Higher
113.09 589	4.792	Heptenone	3	Enones	Flavoring agent	Higher
118.08 664	8.773	Betaine	2A	Quaternary amines	Osmolyte, present in wheat	Higher
135.08 04	8.529	Zimtalcohol (trans-Cinnamyl alcohol)	3	Benzene and substituted derivatives	natural aromatic, antiox, antimicrobial in blueberries, other plants (incl. Poaceae)	Higher
146.05 962	4.529	4-Hydroxyquinoline	3	Alkaloids	Microbial product; Present in cereals, antimicrobial; antibiotic/fungicide precursor.	Lower
159.04 967	4.73	Allantoin	3	Carboxylic acids	Saccharomyces and E.coli metabolite. Found in rice. Metabolite of uric acid. Used in cosmetic for epithelium regeneration	Higher
161.05 719	6.009	6-Methylcoumarin	3	Coumarins and lignans	Allergen & fragrance; potential antioxidant	Higher
177.05 467	5.723	Piperonylacetolein	3	Hydroxycinnamic acids and derivatives	Found in teas and various plants	Higher
181.12 213	7.196	Limonenecarboxylic Acid	3	Monoterpenoids	Present in Viridiplantae	Higher
195.06 662	5.723	Ferulic Acid	1	Phenolic compound	Multi-functional antioxidant; used in cosmetics, nutraceuticals.	Higher
207.09 952	5.052	Senkyunolide F	4	Oxacyclic compounds	Natural phthalide anti-inflammatory/antibacterial (<i>Ligusticum</i> , <i>Angelica</i>)	Lower
211.14 415	5.543	Cyclo(D-Leu-L-Pro)	3	alpha amino acids and derivatives	-	Lower
219.03 14	4.091	Tyrosol 4-sulfate	4	Aryl sulfate	Tyrosol metabolite (olive oil), antioxidant, cardioprotective	Higher
230.24 84	9.51	Lauramine oxide	2B	Long-chain alkyl oxides	rice metabolite, surfactant in detergents/shampoos	Lower

241.03 22	7.052	(±)-Cystine	4	cysteine and derivatives	Most abundant in red peppers, Italian sweet peppers, and zucchinis; less in green peppers and beer. Studied for skin-lightening and anti-dark spot effects.	Lower
258.11 102	0.979	Glycerophos phocholine	2B	Phosphocol ines	Membrane stabilizer, energy source/storage	Lower
258.28 644	9.511	N-[2- hydroxyethyl} tetradecan- 1-amine or 2- (tetradecyla mino)ethano l	2A	amino alcohol	Found in root of <i>Populus trichocarpa</i> (poplar)	Lower
261.07 346	5.442	3- Hydroxyuridi ne	4	Carboxylic acids	Nucleoside plant compound, allelopathic (<i>Baillonella toxisperma</i>)	Lower
268.22 498	7.742	4- Morpholinyl(4- pentylcycloh exyl)methan one	5	Carboxylic acid amides	Metabolite in <i>Arabidopsis thaliana</i>	Lower
277.16 565	4.701	Cis-P- Coumaroyla gmatine	2A	Coumaric acids and derivatives	Nutrient in cereals	Higher
284.29 483	9.867	Octadecana mide	2B	Fatty amides	Surfactant/emulsifier; nutrient, membrane stabilizer, energy source/storage	Lower
293.16 071	4.2	Caffeoylagm antine	4	Styrenes	Phenolic derivatives, antioxidant/antimicrobial (rice and barley)	Lower
294.23 987	9.275	Lanyuamide I	4	isobutylami des	N-alkylamides in plant defense	Lower
295.22 757	8.912	12-Oxo-9- octadecynoi c acid (stearolic acid)	3	long fatty acids	W. trilobata extracts, natural herbicides	Lower
300.29 025	9.596	(-)- Sphingosine	2A	Sphingolipi ds	Found in wheat (<i>Triticum</i>)	Lower
301.14 252	9.325	Angoletin	3	Aryl ketones	Extract from <i>C. quadrangularis</i> and other plants, antioxidant	Higher
302.30 634	9.843	L-threo- sphinganine	3	Organonitro gen compounds	L-threo-sphinganine in mustard, tea; PKC/PI3K inhibitor, oncology studies	Lower
308.07 895	1.522	Narciclasine (Lycorcinol)	5	benzodioxol es	Metabolite of <i>Narcissus pseudonarcissus</i> , induces apoptosis (oncology)	Higher

318.30 13	9.66	Phytosphingosine	2A	Organonitrogen compounds	4-hydroxysphinganine are membrane lipid in fenugreek, citrus, chestnut; cosmetic use	Lower
319.17 432	4.89	Kankanoside D	3	Terpenoids	Present in <i>Cistanche tubulosa</i>	Higher
338.34 283	10.688	13-docosenamide	3	Fatty amides	antioxidant, anticancer; from <i>Penicillium</i> or plants (<i>Neolitsea</i> , <i>Wagatea</i>)	Higher
340.28 366	8.667	3-O-acetylcassine	4	Alkaloids	Alkaloid in <i>Senna spectabilis</i>	Lower
413.37 827	11.827	Sitostenone	2A	oxosteroids	Present in Poaceae (<i>Triticum</i>)	Lower
520.34 076	11.622	Lysopc(18:2(9z,12z))	3	glycerophosphocholines (lipids)	Wheat lipid	Lower
540.53 577	10.877	N-hexadecanoyl-sphinganine	4	ceramides	Ceramide-like lipids restore skin barrier, promote corneocyte maturation	Higher
543.13 116	0.979	Tetrahydroa mentoflavone	4	Flavonoids	Biflavonoid, antioxidant, antiviral, neuroprotective	Lower
575.28 418	9.785	Asclepin	5	Steroidal glycosides	Metabolite of <i>Asclepias curassavica</i>	Higher
825.42 969	12.051	Licoricesaponin J2	5	terpenoid saponins	Found in <i>Glycyrrhiza uralensis</i>	Higher

Chapter 5

Reflections and Perspectives

The agri-food and pharmaceutical sectors generate substantial amounts of by-products and waste that, if not properly managed, can contribute to pollution, resource depletion, and greenhouse gas emissions. To address these challenges, production processes must be revised and increasingly aligned with the principles of sustainable development, promoting innovations that minimise waste generation and encourage the recovery and reuse of by-products. In this context, research plays a crucial role in connecting scientific knowledge with industrial practice, thereby facilitating the transition to more environmentally responsible production models.

This doctoral thesis fits within this framework, focusing on the study of solid and liquid residues produced during the production of the *Triticum vulgare* aqueous extract by Damor Farmaceutici. Through an in-depth and integrated chemical, physical, and biological analysis, the project provided the essential foundation for assessing the feasibility of reusing these materials. Chemical characterization revealed the presence of bioactive compounds, including phenolic acids such as ferulic acid, known for their antioxidant and health-promoting properties. Furthermore, cytotoxicity tests confirmed their safety and absence of harmful effects, which is a key prerequisite for any valorisation strategy.

At the same time, the project emphasised the importance of adopting green extraction strategies. A significant contribution of this work was the exploration of Natural Deep Eutectic Solvents (NADES) as alternative and sustainable extraction media. These solvents, characterised by low toxicity and biodegradability, have shown strong potential for the efficient and environmentally friendly recovery of bioactive molecules. Moreover, the possibility of obtaining ready-to-use extracts without additional purification steps further enhances their applicability and eco-efficiency.

The results of this research highlight that the valorisation of agro-industrial residues necessarily requires a multidisciplinary approach. However, large-scale applications still demand further optimization, particularly concerning the intrinsic viscosity of NADES. Future studies should therefore focus on improving process scalability to ensure that environmental performance aligns with industrial practicality. Although the preparation of NADES is straightforward and economically accessible, as described in Section 4.1, their preparation nonetheless requires specific operational expertise. Making such solvents commercially available could therefore represent a significant advantage, facilitating their dissemination and accelerating their adoption as green alternatives in industrial contexts.

In conclusion, the findings of this research demonstrate that the waste generated during TVE production remains rich in bioactive compounds and that their exploitation through sustainable extraction strategies represents a promising approach for the development of new bio-based products. The

valorization of agro-industrial by-products thus emerges not only as a scientific and technological opportunity, but also as an ethical and environmental choice aimed at promoting a more responsible use of resources.

Chapter 6

Conclusions

Wheat is a crop of primary importance, not only for its nutritional value, but also as a source of bioactive compounds with potential therapeutic applications. Among these, *Triticum vulgare* aqueous extract (TVE) is well known and used for its regenerative properties in wound healing. However, the industrial production of TVE by Damor Farmaceutici generates solid and liquid wastes that have remained poorly explored for a long time. This doctoral thesis contributed to filling this gap by providing an in-depth characterization of these residues and highlighting their potential value within a circular economy framework.

Our results evidenced that, although the amount of these by-products is not large enough to pose a critical disposal issue, they nonetheless represent a precious resource. The solid fraction proved particularly rich in phenolic compounds, with ferulic acid identified as the main constituent (89.782 mg/kg). This bioactive compound, recognized for its antioxidant properties and established use in protecting skin from oxidative damage, confers a significant added value to the solid fraction. Antioxidant activity, measured by ABTS and FRAP assays (8.598 and 7.262 mmol Trolox/kg), further confirmed the relevance of the solid over the liquid fraction, which, despite

its lower phenolic content, still contained bioactive compounds such as dihydroferulic acid (6.24 mg/L).

Mineral content analysis provided additional insights. Notably, potentially harmful elements such as arsenic and chromium were detected only at trace levels. As regards toxicity, both liquid and solid wastes were investigated *in vitro* preclinical models of human skin (HaCaT cells and HDFa) after 24, 48, and 72 h of exposure. Results confirmed no cytotoxic effects even at the highest concentration tested (100 µg/mL) at 72h, supporting the biocompatibility of both waste types.

A further key outcome of this research was the evaluation of NADES as an alternative extraction system. Among the eight tested formulations, which cover a wide range of physiochemical characteristics (pH, viscosity and polarity), the betaine:1,3-butanediol:water system (1:3:10.6, molar ratio) achieved the highest extraction yield of ferulic acid. The optimized UAE-NADES-based extraction method allowed for a reduction in extraction time and toxic solvent consumption. Results obtained with two tools, AGREEprep and BAGI scores (>60), confirmed the sustainability advantages of this approach compared to conventional methods. Moreover, non-targeted metabolomic analyses demonstrated that the choice of solvent strongly influences the recovery profile, enabling the identification of different classes of bioactive compounds with potential applications in cosmetics, nutraceuticals, and pharmaceuticals. Although NADES present some limitations, including high viscosity and limited commercial availability, in

this study, they have emerged as a safer, sustainable, and versatile alternative to conventional solvents, opening new perspectives for low-impact industrial extraction processes.

In conclusion, this thesis has demonstrated both the intrinsic value of waste derived from *Triticum vulgare* extract production and the feasibility of innovative strategies for its valorization. These results represent a significant starting point for future studies aimed at transforming the evidence gathered into concrete applications, promoting the integration of by-products into circular economy models and a more sustainable use of agro-industrial resources.

Chapter 7

Appendix

7.1 Supplementary Materials related to Chapter 4 (Specific Objective 2)

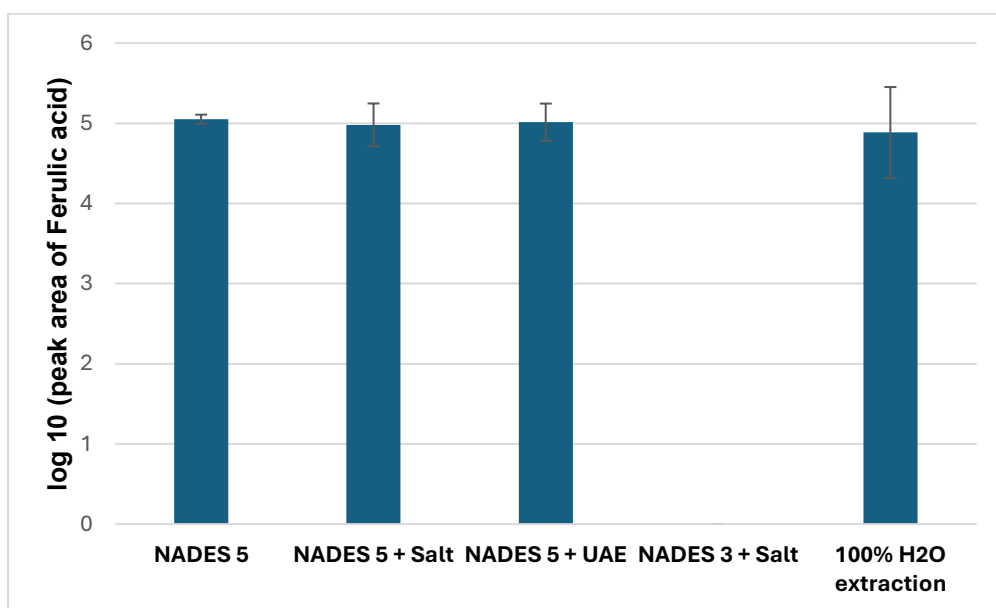


Figure S1: Results of extraction with NADES 5, NADES 5 + salt, NADES 5 with UAE, NADES 3 + Salt and 100% water as extraction solvent. The analysis was performed in duplicates, and the results are represented as log₁₀ of the peak area of ferulic acid +/- relative error standard.

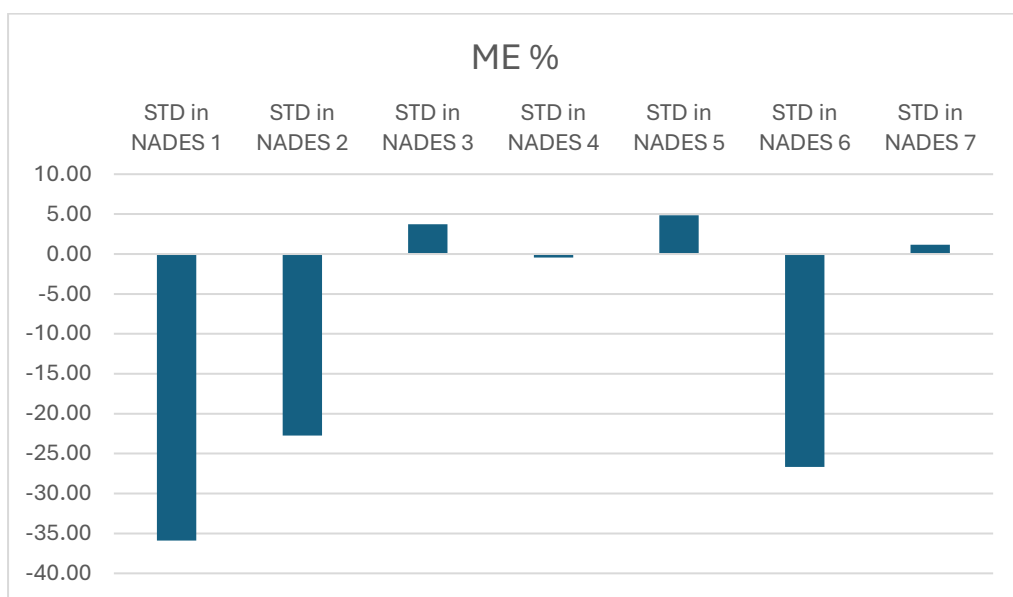


Figure S2: Matrix effect (ME, %) of NADES 1–7, determined by comparing the signal of a 500 $\mu\text{g/L}$ ferulic acid standard solution prepared in each NADES to that of an equivalent solution prepared in methanol. The percentage of the matrix effect was calculated as the difference between the peak area of the standard in NADES and the peak area of the standard in methanol at the same concentration, divided by the peak area of the standard in methanol, and multiplied by 100.

Table S1: Reduced CCF, indicating experiments with the level of the studied variables and experimental responses expressed as peak area of ferulic acid.

Exp No	Exp Name	Run Order	Incl/Excl	T (°C)	Centrifugation Time (min)	Extraction Time (min)	sample/solvent ratio (g/mL)	Peak are ferulic acid
1	N1	20	Incl	25	5	3	0.05	8809
2	N2	17	Incl	60	5	3	0.05	80409
3	N3	4	Incl	25	25	3	0.05	82973
4	N4	6	Incl	60	5	20	0.05	8426
5	N5	19	Incl	25	25	20	0.05	96142
6	N6	1	Incl	60	25	20	0.05	74893
7	N7	10	Incl	25	5	3	0.25	25653
8	N8	15	Incl	25	25	3	0.25	23348
9	N9	9	Incl	60	25	3	0.25	2440
10	N10	22	Incl	25	5	20	0.25	6160.5
11	N11	13	Incl	60	5	20	0.25	13568
12	N12	12	Incl	60	25	20	0.25	
13	N13	3	Incl	25	15	11.5	0.15	61994
14	N14	23	Incl	60	15	11.5	0.15	10134
15	N15	21	Incl	42.5	5	11.5	0.15	95241
16	N16	2	Incl	42.5	25	11.5	0.15	70037
17	N17	5	Incl	42.5	15	3	0.15	62223
18	N18	18	Incl	42.5	15	20	0.15	99654
19	N19	16	Incl	42.5	15	11.5	0.05	10395
20	N20	11	Incl	42.5	15	11.5	0.25	7480.6
21	N21	8	Incl	42.5	15	11.5	0.15	76173
22	N22	7	Incl	42.5	15	11.5	0.15	78810
23	N23	14	Incl	42.5	15	11.5	0.15	88754

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LIST OF ABBREVIATIONS

(±)-6-Hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid (Trolox)
 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)
 2,2-diphenyl-1-picrylhydrazyl (DPPH)
 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ)
 α-linolenic acid (ALA)
 accelerated solvent extraction (ASE)
 Active Pharmaceutical Ingredients (APIs)
 AGREE preparation (AGREEprep)
 agro-industrial waste (AIW)
 alkylresorcinols (ARs)
 American Type Culture Collection (ATCC)
 anaerobic digestion (AD)
 analysis of variance (ANOVA)
 analytical greenness metric (AGREE)
 Analytical Method Greenness Score (AMGS)
 Aqueous extract of Triticum vulgare (TVE)
 arbitrary units (arb unit)
 assessment of green profile (AGP)
 atomic absorption spectroscopy (AAS)
 Bioactive Compounds (BACs)
 blue applicability grade index (BAGI)
 brewery spent grain (BSG)
 carbon dioxide (CO₂)
 Chloroform-Oriented Toxicity Estimation Scale (ChlorTox Scale)
 chlorogenic acids (CGAs)
 choline chloride (ChCl)
 Circular Analytical Chemistry (CAC)
 circular economy (CE)
 cisplatin (cDDP)
 coefficient of determination (R²)
 cross-validation ANOVA (CV-ANOVA)
 deep eutectic solvents (DES)

LIST OF ABBREVIATIONS

Dietary fiber (DF)
Dimethyl sulfoxide (DMSO)
docosahexaenoic acid (DHA)
dried avocado peels (DAP)
dry matter (DM)
Dulbecco's modified Eagle's medium (DMEM)
eicosapentaenoic acid (EPA)
electrospray ionization (ESI)
Enzyme-Assisted Extraction (EAE)
Ethanol (EtOH)
European Food Safety Authority (EFSA)
eutectic molecular liquids (EMLs)
Ferric reducing antioxidant power (FRAP)
ferrous chloride (FeCl₂)
fetal bovine serum (FBS)
Food and Agriculture Organisation of the United Nations (FAO)
Food and Drug Administration (FDA)
food loss (FL)
Food waste (FW)
formic acid (FA)
fruit and vegetable waste (FVW)
gallic acid equivalents (GAE)
Gas chromatography (GC)
Generally Recognised as Safe (GRAS)
graphite furnace atomic absorption spectrometry (GFAAS)
Green Analytical Chemistry (GAC)
green analytical procedure index (GAPI)
Green chemistry principles (GCP)
Green Sample Preparation (GSP)
greenhouse gases (GHG)
Gross Domestic Product (GDP)
Half-maximal inhibitory concentration (IC₅₀)
High sensitivity of human epidermal keratinocytes (HaCaT)
High-Density Lipoprotein (HDL)

high-performance liquid chromatography (HPLC)
High-voltage electrical discharge (HVED)
Human adult dermal fibroblasts (HDFa)
hydrogen bond acceptors (HBAs)
hydrogen bond donors (HBDs)
insoluble dietary fiber (IDF)
ionic liquid (ILs)
K-nearest neighbors (KNN)
Lignocellulosic residues (LRs)
Liquid waste (LW)
Low-Density Lipoprotein (LDL)
magnetic DES (MDES)
Metabolomics Standards Initiative (MSI)
metal halides (MCl_x)
metalloproteinases (MMP)
Methane (CH_4)
Methanol (MeOH)
Microwave-Assisted Extraction (MAE)
millions of tonnes (Mt)
minimum inhibitory concentration (MIC)
3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)
municipal solid waste (MSW)
National Environmental Methods Index (NEMI)
National Fire Protection Association (NFPA)
Natural Deep Eutectic Solvents (NADES)
nuclear magnetic resonance (NMR)
Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA)
Pareto scaling and Principal Component Analysis (PCA-X)
persistent, bioaccumulative and toxic chemicals (PBT)
polyhydroxybutyrate (PHB)
polymeric DES (polyDES)
polymerizable DES (PDES)
polyunsaturated fatty acids (PUFAs)
Pressurized Liquid Extraction (PLE)

Principal Component Analysis (PCA)
Public Private Partnerships (PPP)
Pulsed Electric Field (PEF)
reactive nitrogen species (RNS)
reactive oxygen species (ROS)
red-green-blue (RGB)
reduced face-centered central composite design (reduced CCF)
relative standard deviation (RSD)
response surface methodology (RSM)
rutin equivalents (RE)
safety, health and environmental (SHE)
Sample Preparation Metric of Sustainability (SPMS)
sodium hydroxide (NaOH)
Solid waste (SW)
Solid-liquid extraction (SLE)
solid-phase extraction (SPE)
soluble dietary fiber (SDF)
Soxhlet extraction (SE)
spent coffee ground (SCG)
standard deviation (SD)
standard error of the mean (SEM)
standing for gallic acid equivalents (GAE)
Steam Explosion (STE)
Subcritical Water Extraction (SWE)
sun protection factor (SPF)
Supercritical Fluid Extraction (SFE)
Sustainable Development Goals (SDGs)
ternary deep eutectic solvents (TDES)
therapeutic deep eutectic solvents (THEDES)
thin-layer chromatography (TLC)
three-dimensional (3D)
total dietary fibers (TDFs)
total phenolic content (TPC)
Trolox Equivalents (TE)

LIST OF ABBREVIATIONS

Ultra-High performance liquid chromatography (UHPLC)
Ultra-high performance liquid chromatography coupled with
ORBITRAP high-resolution mass spectrometry (UHPLC-Q-Orbitrap
HRMS)
Ultrasound-Assisted Extraction (UAE)
Variable Importance in Projection (VIP)
Wheat bran extract (WBE)
White Analytical Chemistry (WAC)