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THESIS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY:

SUSTAINABLE TECHNOLOGIES FOR THE ANALYSIS OF BIOACTIVE COMPOUNDS IN AGRI-FOOD PRODUCTS: AN INTEGRATED APPROACH FOR SUSTAINABILITY ASSESSMENT

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"The hardest thing to find in life is balance - especially the more success you have, the more you look to the other side of the gate. What do I need to stay grounded, in touch, in love, connected, emotionally balanced? Look within yourself".
– C.D.

To all those who are able to find beauty in the little things. –

ABSTRACT

Agri-food systems (FSs) address 'one of the basic human needs' to ensure a steady food supply (intended as food security), food safety, and quality, thus guaranteeing dietary-balanced variety, while reducing related – environmental impacts. It is worth mentioning that FSs generate about 16 Gt CO_2 eq., thus representing an average contribution of 31% to global anthropogenic GHGs. In this framework, the Ph.D. research project aims to valorize the main representative agri-food products and related by-products of the Italian Market (i.e., durum wheat, pasta, wine, olive oil, etc.), through an integrated approach for sustainability assessment. In particular, the Life Cycle Assessment (LCA) methodology, using SimaPro 9.5. software has proven to be a useful tool for assessing the environmental performances of major agri-food products and processes, identifying critical hotspots in up- and downstream activities, thus foreseeing possible waste-recovery strategies to mitigate impacts along the food chain.

Moreover, within the field of sustainability, quality assessment of different food samples was carried out using emerging and sustainable methods, such as Ultrasound-Assisted Extraction (UAE), Deep Eutectic Solvents (DESs), for the sustainable extraction of target analytes from agri-food products and by-products, focusing on both analytical parameters as well as sustainability performances. In particular, the project investigated the analysis and analytical determination of different food bioactive compounds (BCs), namely polyphenols, antioxidants, biogenic amines (BAs), as well as essential fatty acids (EFAs), that are naturally occurring compounds, exerting both health-promoting properties on human health (i.e., prevention of cancer and cardiovascular diseases, etc.), as well as being valuable product and/or process markers for food quality. However, to mitigate intrinsic drawbacks for the environment and operator safety, (i.e., flammability, high toxicity, and non-biodegradability), related to conventional procedures, the use of alternative methods (i.e., UAE), and *green* solvents (i.e., DES) has been proposed. To this end, the implementation of a comparative LCA has enabled the quantification of environmental impacts, thus representing real-time analysis for pollution prevention in the product or process design.

Keywords

Agri-Food Systems; Bioactive Compounds; Sustainable Chemistry; Deep Eutectic Solvents; Life Cycle Assessment; Agri-Food Sustainability; Waste Recovery Strategies.

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INTRODUCTION

Agri-food systems (FSs) address 'one of the basic human needs' to ensure a steady supply, food security, and quality, thus guaranteeing dietary-balanced variety and improved nutritional composition. Nowadays, food production fulfills more than just a human requirement; it also addresses a plethora of social, economic as well as environmental concerns. Nevertheless, according to the United Nations (2019), the global population is expected to reach 9.1 billion by 2050, an 83% increase over the current level. This rapid growth will lead to an estimated +47% increase in food demand by 2050 (Costa et al., 2022), resulting in increased anthropogenic pressure on natural resources. In this framework, FSs have been expanding to fulfill this growing market demand, thus representing a social, economic, and environmental issue.

According to the reviewed literature, a third of global greenhouse gas emissions (GHGs) are mainly attributable to food systems, generated within the entire supply chain from production to final consumption (Crippa et al., 2021), (Rosenzweig et al., 2020). Considering the FAO global estimates, in 2020, GHGs from agri-food systems were 16 Gt CO_2 eq, (−3% from 2019), thus representing an average contribution of 31% to global anthropogenic GHGs, amounting to 52 Gt CO_2 eq, in 2020. Considering the food production chain complexity, nearly 71% of GHGs from the food system are associated with agricultural and land use activities, mostly attributable to agronomic practices and intensive use of fertilizers and pesticides, as well as energy-related activities (Crippa et al., 2021). Among the activities of the food supply chain, about 18–20% of total GHGs are attributable to waste and side-stream activities, mainly related to industries, storage, and logistics, thus having a key role in addressing sustainability challenges and promoting circular economy principles. Concerning the environmental dimension, food waste leads to several impacts along the food supply chain, including inefficient use of water resources (89% of total GHGs), and land use (3.3 $GtCO_2$ equivalents per year), which in turn can lead to the degradation of natural ecosystems and a progressive reduction of the valuable goods and services they provide. As a practical matter, the ecological footprint of food waste affects the total bio-capacity deficit by 58% worldwide, 30% in the Mediterranean, and 18% in Italy (Chen, et al., 2020). In this context, it is alarming to note that FSs are among the main responsible factors for the depletion of bio-physical processes that regulate climate resilience. In these regards, identifying and assessing the most impactful activities in the agri-food sector is the first step toward bringing food

systems within the limits of environmental and socio-economic bio-capacities.

Several resilience strategies have been proposed by institutions and governments, at the global level, to proactively contribute to this goal. From a perspective of integrated collaboration between environmental resources, government institutions, and economic resources availability, among the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda, proposed by UN member states, No. 2 *"End hunger, achieve food security, improve nutrition and promote sustainable agriculture"*, and No. 12: *"Ensure sustainable patterns of production and consumption"*, represent the core of a possible change aimed at reducing FSs related–environmental burdens. In this framework, linear economy models re-configure to a circular economy perspective with increasingly innovative solutions in the valorization of agri-food products; besides, this would allow the valorization of processing by-products as *"second raw materials"* for new markets, thus limiting over-production while extending food shelf-life.

Indeed, several agri-food products are considered exploitable matrices to recover valuable bioactive compounds (BCs), such as polyphenols, antioxidants, essential fatty acids (EFAs), and phytochemicals (Panzella et al., 2020). To extract and recover value-added compounds from food matrices for possible reuse as food additives or natural ingredients for the development of functional foods, it is necessary to conduct an efficient and sustainable extraction process. Traditionally, the extraction process for food analyses was carried out by using organic solvents (i.e., methanol, ethyl acetate, n-hexane, etc.), nevertheless, their significant impacts on the environment, operator safety, and high toxicity. Therefore, in recent years, conventional methods have been joined or replaced by innovative green methods, focusing on both analytical parameters, in terms of extraction yield, purification, as well as sustainability concerns (Ruesgas-Ramo et al., 2017; Vauchel et al., 2018). One of the promising green solvents for efficient extraction of BCs from food matrices are Deep Eutectic Solvents (DESs). Due to their chemical properties and non-toxicity, DESs could be suitable for food products formulated with natural ingredients or several applications, from cosmetics to pharmaceuticals as bio-based alternatives (Panzella et al., 2020).

When considering a chemical procedure, it is also necessary to include environmental criteria in process design, control, and optimization, to develop sustainable and green processes. In these regards, Life Cycle Assessment (LCA) based approaches can be considered useful tools for multi-criteria optimization of the chemical process based on its environmental performances.

AIMS OF Ph.D. RESEARCH PROJECT

This research considers the possibility of valorizing agri-food products and related processing by-products through an integrated approach for a multi-dimensional assessment of sustainability. In particular, the Ph.D. project focused on the recovery of bioactive compounds from food matrices, through the development and application of emerging and sustainable technologies, such as Deep Eutectic Solvents (DESs), ultrasound-assisted extraction (UAE), etc., which enable the rapid extraction of target analytes (i.e., polyphenols, antioxidants, biogenic amines, essential fatty acids, etc.) from agri-food products, that are of particular interest for both human nutrition and in the Italian Market. The matrices analyzed included samples from the *"ancient" Senatore Cappelli* durum wheat chain (grains, flour, pasta, and processing by-products or husks), psychoactive foods and beverages (tea, coffee, and chocolate), and wine. The extraction procedures were then tested focusing on analytical parameters, which were then characterized by the application of chromatographic techniques (HPLC-UV/Vis and LC-ESI-MS), and UV-Vis spectroscopy; as well as in terms of sustainability performances. In particular, the environmental impacts of sustainable technologies compared with conventional analytical procedures for the determination of bioactive compounds in food matrices, were assessed through the application of the LCA methodology, using the SimaPro 9.5 software. In addition, the application of the Life Cycle Thinking (LCT) approach allowed the sustainability assessment of the major Italian agri-food production chain (durum wheat, olive oil, wine), thus identifying critical hotspots in the supply chain, while assessing environmental sustainability performances. Moreover, the research greatly emphasized upstream and downstream process management in the food chain, to promote resource efficiency and to valorize processing by-products that still have high potential, through their reuse in other production chains, thus foreseeing the possibility of developing *"circular"* strategies. In this framework, the main results of the proposed research project were the object of no. 11 publications in scientific International Journals, and the published editorial versions (PDF original copy, and DOI addresses), are presented in the Experimental Section (Chapter III).

CHAPTER I

THEORETICAL BACKGROUND OF THE RESEARCH PROJECT IN THE NATIONAL AND INTERNATIONAL CONTEXT

1.1 Global food systems and enhancements toward sustainable food production

The global food system (FS) is recognized nowadays as a complex system that influences diets, human health, and other outcomes, including economic growth, natural resources, environmental resilience, and sociocultural factors (Westhoek et al., 2016). Important drivers involved in food systems (FSs) are the environment, governance, institutions, economy, and societal aspects, whose activities are linked in a *'cascade'* to agricultural production. Taking into account the complexity of FS, it is worth noting that all elements (environment, people, inputs, infrastructures, institutions, etc.), and activities involved in the production, processing, distribution, and consumption of food, are strictly interconnected with the natural environment, and its related ecosystem conditions (i.e., climate, resource availability, genetics, etc.) (Vermeulen et al., 2012), in a dynamic model that constantly exchanges with other systems, (i.e., health-care, economic and energy sectors). In particular, the agri-food system is mainly influenced by the natural environment, both in terms of agricultural productivity for providing ecosystem services, as well as related environmental impacts arising within the food chain (Westhoek et al., 2016). Figure 1.1 shows the complexity model of the agri-food systems, according to a societal-integrated and dynamic model.

In recent decades, the agri-food sector has undergone a series of remarkable changes leading to the evolution of systems involved in food production. In particular, FS is rapidly transforming, driven by urbanization and changes in technology, policy, and industrialization processes, encompassing the interaction of agricultural and industrial processes to fulfil the requirements of a constantly growing and ever-changing global population and disposable income.

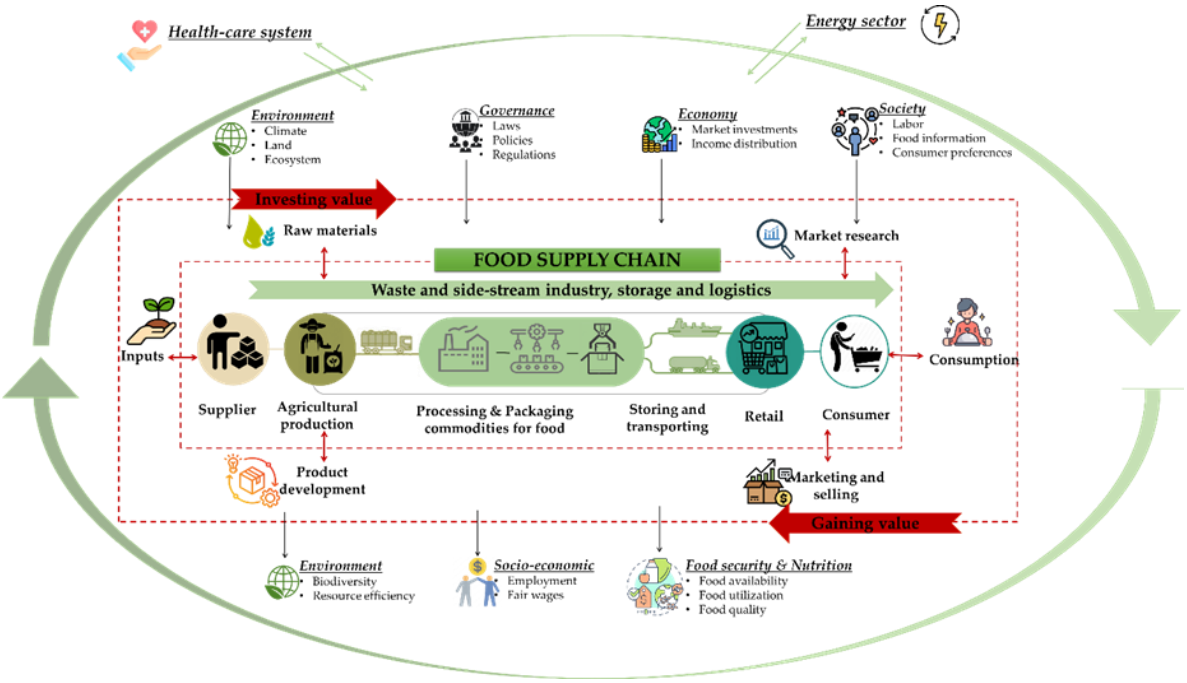


Figure 1.1. Components of the agri-food system: main actors and activities. Source: author's elaboration

These latter represent significant challenges for FSs, worldwide, to be addressed from several perspectives: *i)* agricultural intensification to meet the rising demand for food; *ii)* diversification of food sources, to ensure nutritional variety; *iii)* distribution networks, infrastructure, and transportation systems to ensure equitable access to food; *iv)* sustainable practices aimed at maximizing yields while minimizing environmental impacts; *v)* reducing food losses and waste across production chain; *vi)* adapting food systems to be more resilient to climate change; *vii)* investing in research and development of new agricultural technologies, crop varieties, and farming practices to boost food production, technology, and efficiency; *viii)* international cooperation to address global food security challenges; *ix)* responsible consumption for healthier food choices and more sustainable food systems (Teigiserova et al., 2019). These changes require, therefore, a multi-faceted approach involving technological innovation, policy reform, education, and international cooperation. In these regards, it is possible to distinguish three main stages leading to transitions in the development of the agri-food sector:

1. The first stage comprises the period 1990-1994 when ownership changes in state-owned farms and food industry enterprises occurred for the adaptation to the market economy. In 1990, the Agricultural Market Agency was established, being responsible for the implementation of the

policy of state intervention in the market for agricultural products. During this period, negotiations on the regulation of world foreign trade within the GATT Uruguay Round were concluded.

2. The second stage related to the period 1995-2003, which was marked by an intense process of adjustment to integration with the EU regulations and market strategies, concerning the implementation of the Common Agricultural Policy (CAP), and the required food safety standards for farms, food industry enterprises and institutions. In 1995, as a result of the GATT Uruguay Round negotiations, the World Trade Organisation (WTO) was established and world trade in agri-food products was liberalized. The intensification of globalization processes in the world economy was one of the consequences of global foreign trade liberalisations. In particular, the increase in foreign direct investment (FDI) by multinational food corporations has been expressed in the globalisation processes of the sector.

3. The third stage for the evolution of the agri-food sector includes the adoption by EU member countries of the CAP measures, to consolidate the role of European agriculture for the future. CAP actions mainly concern: *i)* income support measures through direct payments to ensure income stability and remunerate farmers for environmentally friendly farming; *ii)* market measures to cope with challenging market situations, such as a drop in prices due to a short-term oversupply in the market; and *iii)* rural development measures with both national and regional programs to cope needs and challenges of rural areas. During the period of EU membership, farms, and food industry enterprises benefited from support (i.e., European Recovery Investment or EURI, European Agricultural Fund for Rural Development or EAFRD, etc.), which all enabled large modernization investments. As a result, an improvement in management agricultural production efficiency was noted, thus representing an opportunity to reinforce both farms and food industry enterprises. The possibility of constantly assessing CAP performances and improving its efficiency has been achieved with the Common Monitoring and Evaluation Framework (CMEF) Monitoring and evaluation results

generate valuable information, thus representing an opportunity to help in setting policy and program long-term objectives.

In June 2021, following extensive negotiations between the European Parliament, the Council of the EU, and the Commission, a transitional regulation on CAP was introduced for the period 2021-2027 (European Commission (EU)., 2023). The transitional period aimed to provide EU countries with proper design and prepare for the implementation of their CAP strategic plans, also including new elements to enforce environmental and circular economy aspects, as a result of the *"European Green Deal"*, and *"Farm to Fork"* strategies.

According to the implementation of the CAP action plans with strategies involving environmental and circular economy aspects, farm and food industry enterprises will undergo deep changes in food production processes, in particular focusing on resource efficiency, renewable energy use, reduction in the use of chemicals for fertilization and pest management in agriculture, as well as reducing overall environmental impacts and climate-altering emissions associated with food production and processing (Ammirato et al., 2021). These evolving changes allowed the integration of the past agri-food system models (i.e., agro-industrial model) with sustainable development systems, as the *"integrated-territorial model"*.

The integrated-territorial model in agri-food systems refers to a holistic approach that integrates the whole food supply chain stages, within a defined territory, taking into account social, economic, and environmental factors associated with food production and consumption. In particular, the model aims to promote sustainable and resilient agri-food systems through a framework for implementing local development plans. It recognizes the diversity and specificities of different regions and encourages the adaptation of strategies to local contexts, to build a shared interpretation among the local community, both in terms of enterprises and citizens, on the relative positioning of the territory. In the theoretical integrated-territorial approach proposed by (Galeano-Barrera et al., 2022) (Figure 1.2.), the use of land and agriculture represents one of the most important forms of territorial or regional development (Lanfranchi et al., 2014). Moreover, the possibility of promoting territorial development was achieved by an integrated policy approach enhancing smallholder productivity, as well as a short supply chain, for social transformation, sustainability, and collaboration in agricultural extension systems (Galeano-Barrera,

et al., 2022). Finally, the integration of rural and territorial model would be also promoted by both geographical and technological proximity (Joltreau et al., 2020). The former simplifies product distribution, transport, and logistics, and reduces the number of intermediary actors that are involved in the production process; while, the latter promotes the use and adoption of technologies, to facilitate the food production process.

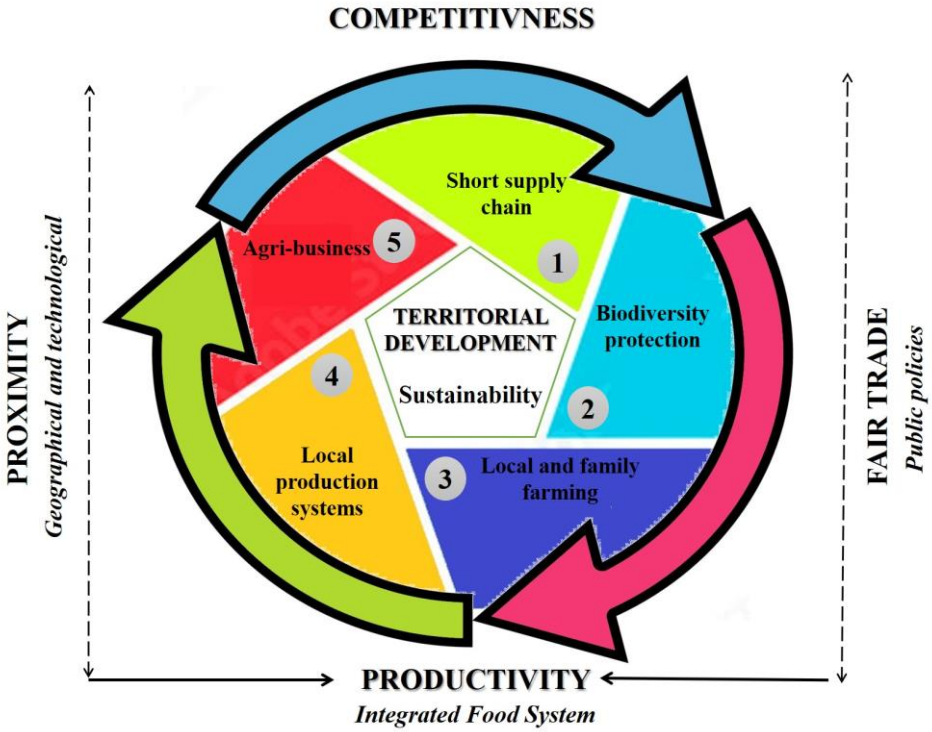


Figure 1.2. Theoretical model for the integrated-territorial development. Source: author's elaboration

In particular, the aforementioned model and its holistic vision of territorial development closely linked to the entire territory (in terms of resources, communities, and ecosystems) aims to create more resilient and equitable food systems that benefit both rural and urban areas, while promoting sustainability and cultural preservation.

1.2 Market of Agri-food Sector in International and National context

The agri-food sector is a highly integrated value chain (including both alcoholic and non-alcoholic beverages), for the entire economy abroad, involving all three sectors of the economic activity: the primary sector with the production of raw materials, the secondary through the processing industry, and the tertiary with distribution, marketing, and other services. Agri-food production is set to occupy an important position on the

upcoming decades' agenda, as the challenge remains to meet global demand, along with the powerful downstream (food) retail sector. Agri-food has almost not experienced rapid shifts of uncertainty due to consistent consumption over time, driven by increasing global population and disposable income.

Nowadays, global food and beverage sales registered a growth of 7.4% in 2023 (until June), amounting to USD 6.93 trillion, and showing an increase of +22.8% compared to the year 2020 (Allianz Trade, 2023) as shown in Figure 1.3. The war between Russia and Ukraine threatened the chances of global economic recovery from the COVID-19 pandemic, thus leading to economic sanctions on several countries, increased commodity prices, and supply chain breakdowns, at least in the short term. The food and beverage market is expected to grow to USD 9.37 trillion by 2027, at a compound annual growth rate (CAGR) of 6.3% (Reportlinker, 2023).

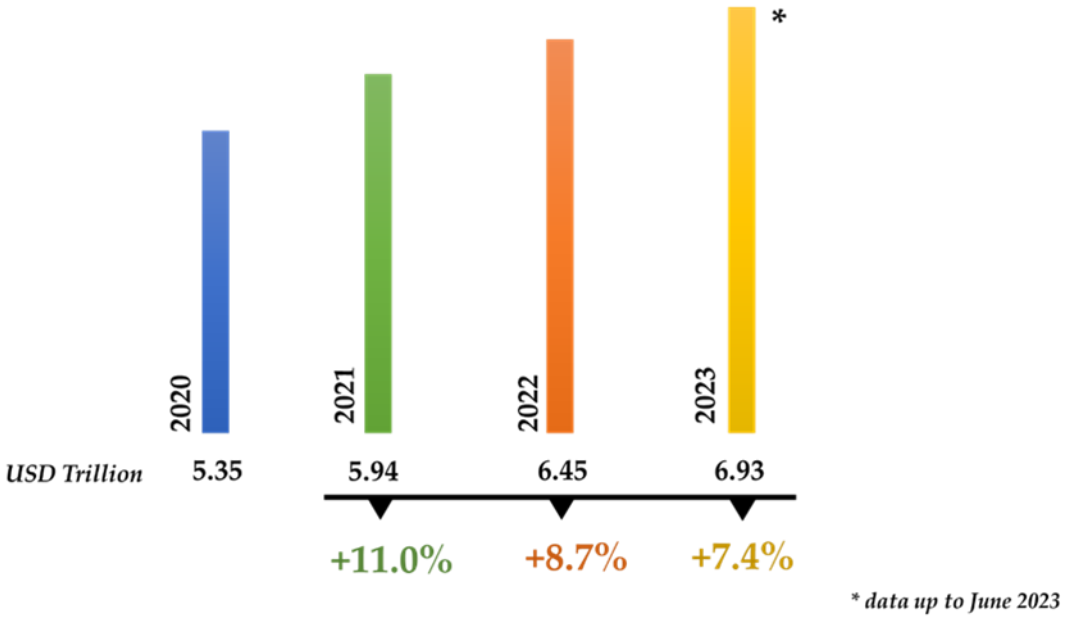


Figure 1.3. Global food sales (USD trillion) within the years 2020 to June 2023. Source: author's elaboration

China was by far the world's leading food producer in 2021, with annual output valued at $1.5 trillion, followed by India, with $422.5 billion attributable to food production, United States ($340.6 billion), and Brazil, with $149.2 billion attributable to food production (FAO, 2021). In terms of food amount production (million tons, MT) (Figure 1.4.), China has a history of being a world leader in agriculture and food production with 53.24 MT produced in 2020, thus representing about 57% of the total

production, worldwide. Agriculture as well as farm activities for food production are then mainly located in the United States with 7.98 MT (8.41%) of food produced in 2020, Turkey with 5.83 MT (6.22%), India with 5.74 MT (6.12%), and Brazil with 2.68 MT (2.85%).

The food and beverage industry represents also a prominent contributor to the European economy, generating a turnover of $1.1 trillion (corresponding to 15.68% of the global food production), with about 30.7 million employers in the sector (11.1% share of the food supply chain in EU employment), thus making it one of the largest manufacturing industries, worldwide (FoodDrinkEurope, 2022).

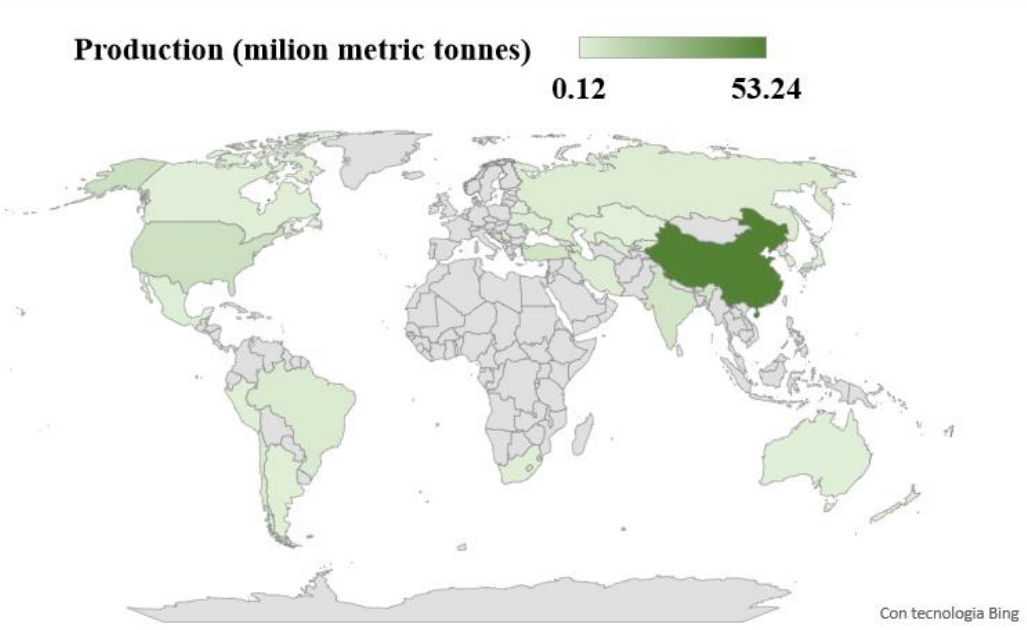


Figure 1.4. Major food-producing countries worldwide (2020). Data are expressed in million metric tonnes. Source: author's elaboration, based on (FAO, 2021).

Over one-half (56.9%) of the total production value of the EU agricultural industry in 2022 stems from the *'big four'*: France (23% of the value of agricultural production), with an economic revenue of EUR 96.6 billion, Germany (15% of the total production value), with EUR 74.5 billion, and Spain (EUR 63.2 billion) (Figure 1.5.). Italy is confirmed as the first European country for added value in agriculture, and the third for production value (15%), corresponding to a revenue of EUR 71.2 billion. The next group of EU countries were Poland (EUR 40.0 billion), the Netherlands (EUR 36.3 billion), and Romania (EUR 22.8 billion) (EUROSTAT, 2022a).

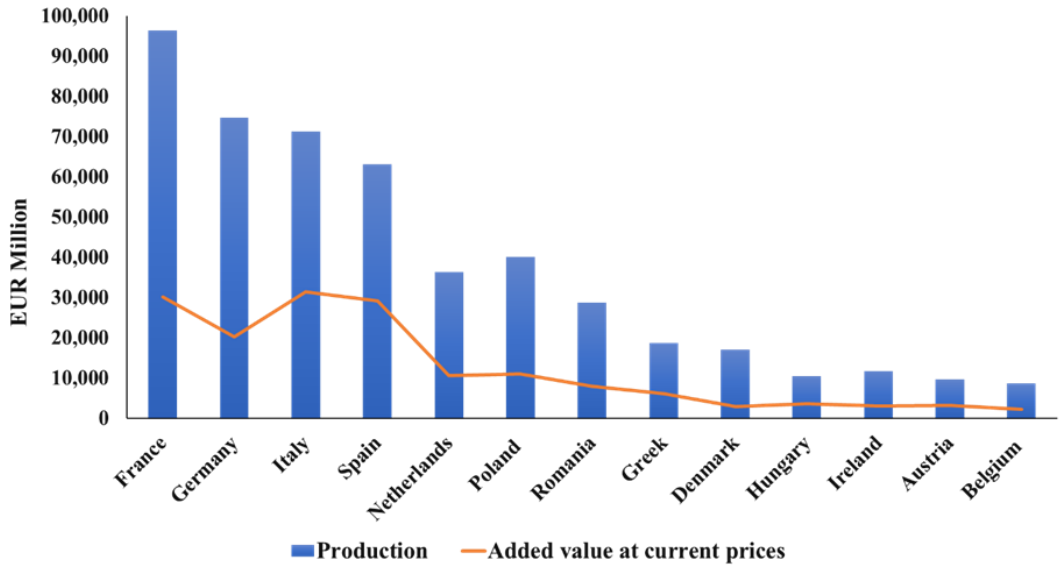


Figure 1.5. Main food-producing countries at the European level (2022). Source: author's elaboration based on (EUROSTAT, 2022a) estimates.

In 2022, the economic value of the food industry (in terms of crops, animals, and agricultural services) was EUR 536.7 billion, of which 54% came from crops (with cereals, and vegetables the main ones), with an economic value of EUR 287.3 billion; 38.5% was allocated to livestock and animal products (mainly pork meat, and milk), with an economic value of EUR 206.7 billion; and finally, the remaining 8% consisted of agricultural services (EUR 23.0 billion), and secondary activities (EUR 19.8 billion), as shown in Figure 1.6.

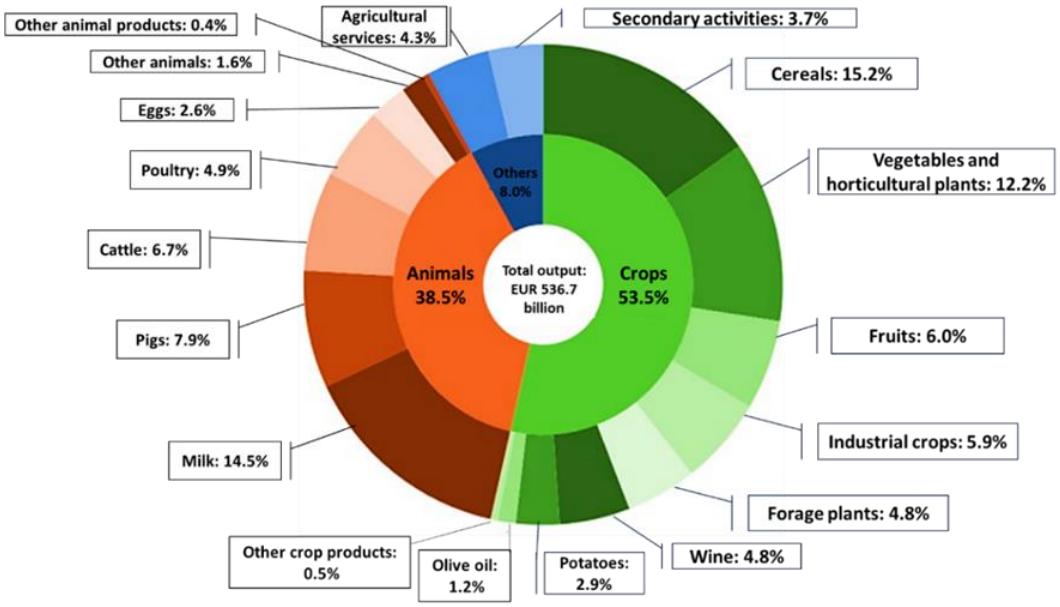


Figure 1.6. Main food products of EU agricultural industry, in 2022. Source: author's elaboration adapted from (EUROSTAT, 2022a).

Among all food products, it is also worth mentioning the evolution in the production of the major crops for food, scheduled as an action plan in the EU's Farm to Fork Strategy (EU, 2018). In 2021, the main crops harvested in the EU include soft wheat and spelt (129.9 million tonnes produced), sugar beet (113.3 million tonnes), grain maize (73.0 million tonnes), and fresh vegetables (67.2 million tonnes, of which tomatoes represent 25.6%), barley (52.1 million tonnes), and potatoes (50.4 million tonnes) (Figure 1.7.). Comparing the period 2001–2021, there was a considerable increase in the EU's harvested production for rape and turnip rape seeds with a total production of 16.9 million tons in 2021 (average growth +62% from 2001), for common wheat and spelt (+6%). By contrast, the EU's harvested production of potatoes and of rye, winter cereal mixture (maslin), and durum wheat were notably lower in 2021, thus denoting a –10.1%, –18.1%, and –16.9% decrease respectively, compared to 2001. These fluctuations in production can be attributed to weather conditions during the growing season and at harvest time, as well as to other factors such as soil quality, nutrient availability and pests, which affect both yield and food quality (Alhashim et al., 2021).

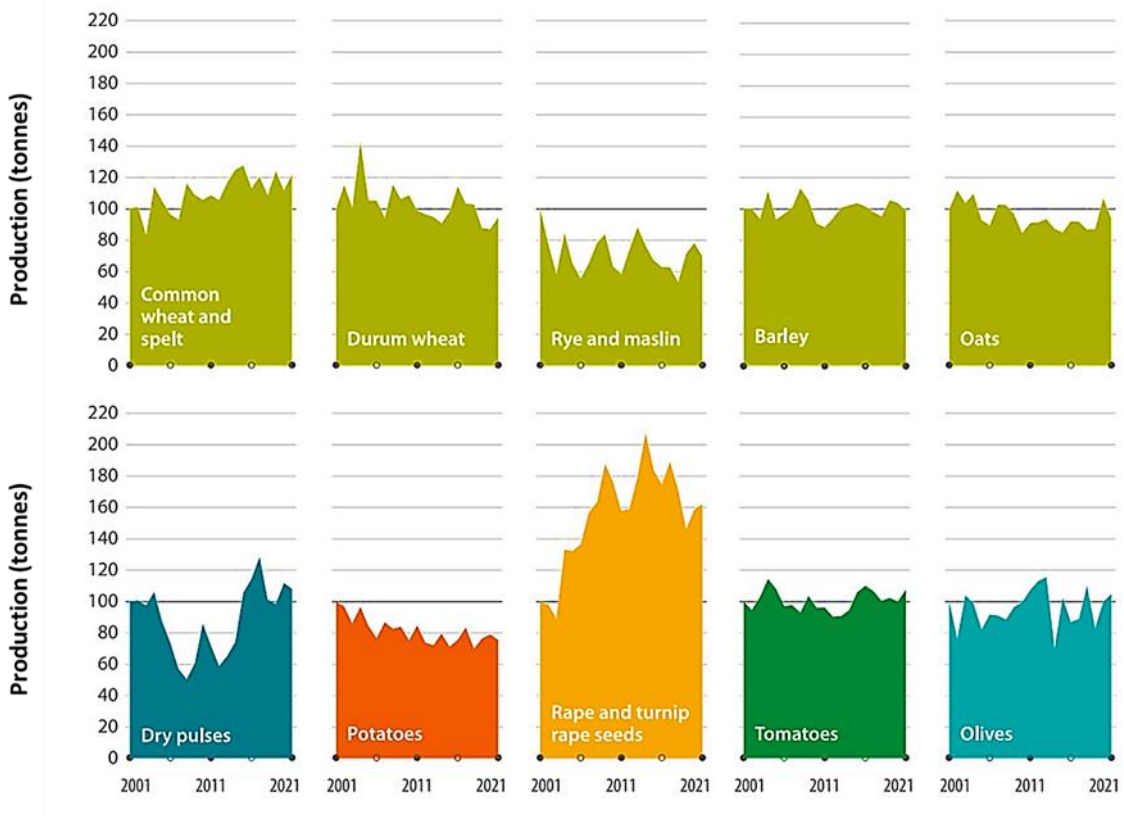


Figure 1.7. EU evolution of major crop production between 2001 and 2021. Data referred to the production (expressed in tonnes). Source: Eurostat, 2022 (online data code: apro_cpsh1).

As the EU covers a large area with a wide range of climates, farmers have to consider all the agronomic factors (i.e., crop rotation and soil conditions), input costs (i.e., seeds and fertilizers), and expected yields, as well as policy incentives or restrictions, which overall tends to have a knock-on impact on both agricultural productivity and prices over the years (EU, 2023). Nevertheless, this annual decision-making process is less relevant for farmers of permanent crops, such as olives, apples, and grapes.

In 2022, EU agri-food trade (exports + imports) totaled EUR 402 billion with the rest of the world, +23% compared to the year 2021. This is mainly due to an increase of + 34% in import value, as well as the value of EU agri-food export (+ 17%), compared to the year 2021 (EUROSTAT, 2022a). Considering the balance in EU agri-food trade, EU agri-food exports reached EUR 229.8 billion, an increase of 31% compared to 2021. Wine and beverages resulted as the most exported food commodity, with an economic value of EUR 64.07 million in 2022, + 12.3% compared to the year 2021. In addition, cereals, and milling products, recorded the second largest increase in total EU exports (+7%), compared to 2021 (Figure 1.8.), thus reaching 31.9 million tons (increase by 8% year-to-year) in 2022. As well as, for animal products which EU exported EUR 45 billion in 2022, representing an increase of 10% year-to-year. The primary exports are dairy products exports (mostly cheese, curd, and whey), with an economic revenue of EUR 20.4 million, and pig meat (EUR 13.8 million) (EUROSTAT, 2022a).

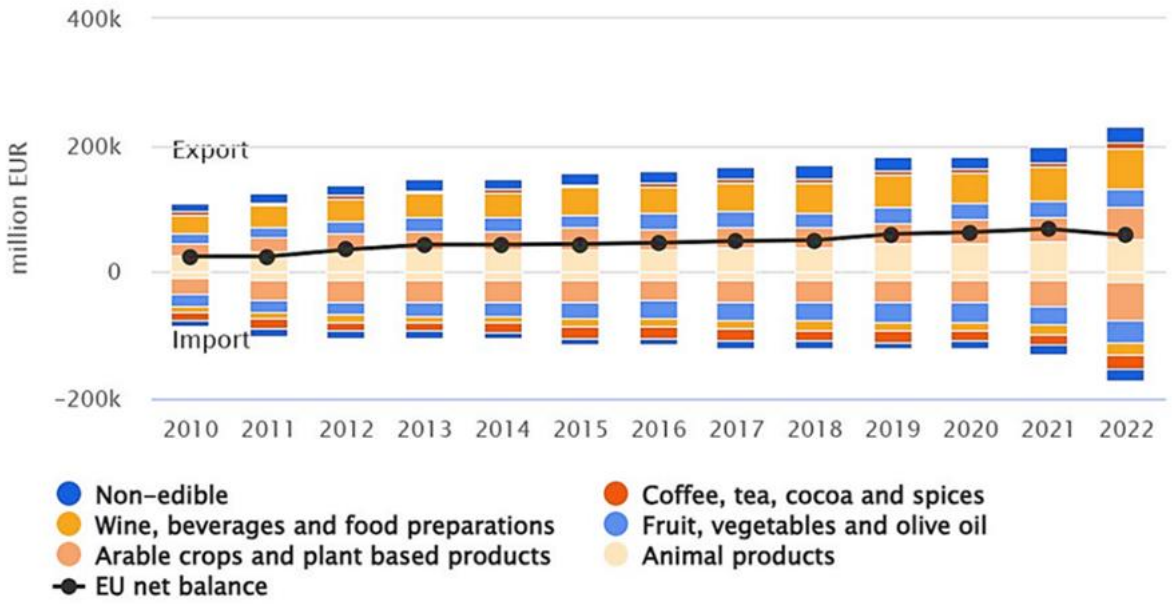


Figure 1.8. Structure of EU-27 agri-food trade within the years 2010-2022.

Source: (EU, 2023).

While, EU imports' economic value reached EUR 172 billion in 2022, +32% compared to 2021, as a result of an unexpected rise in global prices over the year for a large number of commodities, notably coffee and soya meals, to the drought in the summer of 2022 (Figure 1.8). Oilseeds and protein crops (such as grains and legumes) are the first imported food commodities, corresponding to an economic value of EUR 25.8 billion in 2022, followed by fruits and nuts, coffee, tea, cocoa, and spices.

Brazil is the leading source of imports, accounting for 12% of total EU imports. The United Kingdom ranks second with 9% of EU imports, and Ukraine represented the EU's third largest source of agri-food imports, increasing by 88% in 2022 compared to 2021 and accounting for 8% of the EU's total agri-food imports (EUROSTAT, 2022a).

1.2.1 The Italian agri-food market and trends toward sustainable agriculture

The agri-food industry in Italy is one of the leading sectors of both the national economy and cultural heritage. In the rankings of Italian manufacturing sectors, it is first in terms of turnover, second in terms of number of companies, number of employees, and also in terms of export in value. In 2022, the Italian agri-food sector reached an annual turnover of EUR 179 billion, with more than 60 thousand companies and 464 thousand employees involved in the sector and more than 50 billion in exports in value annually. Important values that in the last decade have recorded an increase of 24.7% in real terms, the number of employees by 12.2%, and the value of exports in real terms by 60.3% (Rapporto Federalimentare – Censis, 2023). Moreover, the Italian agri-food sector, including farms, food processing industries, wholesalers, large distribution areas, small retail shops, and catering operators, represents 31.8% of the Italian GDP (corresponding to EUR 607 billion), with a growing trend since 2013 (+12%). These numbers are more than adequate to consider the food industry a national asset and national heritage and support for its needs as a major component of Italy's national interest. Furthermore, Italy can boast a dynamic and diversified agri-food industry, deeply embedded in its cultural heritage and characterized by a wide range of quality products, with traditional agricultural practices and culinary competencies handed down through generations. In terms of agriculture, it should be noted that Italy has a great agricultural vocation owing, in particular, to the soil and climatic conditions favourable to crops, livestock, and human settlement. In Italy, the Utilised Agricultural Area (UAA) of 12,598.16 hectares (42% of the total national territory) is mainly allocated to the production of arable land (57.4%), cultivation of agricultural woody crops (17.4%),

permanent grassland (35%) and family gardens (0.1%) (ISTAT, 2021). 46% of the UAA is concentrated in five regions: Sicilia (1,387,521 ha), Puglia (1,285,290 ha), Sardegna (1,153,691 ha) Emilia-Romagna (1,064,214 ha), and Lombardia (1,010,780 ha). Figure 1.9. shows the UAA and Agricultural farms in terms of composition %, within Italian regions.

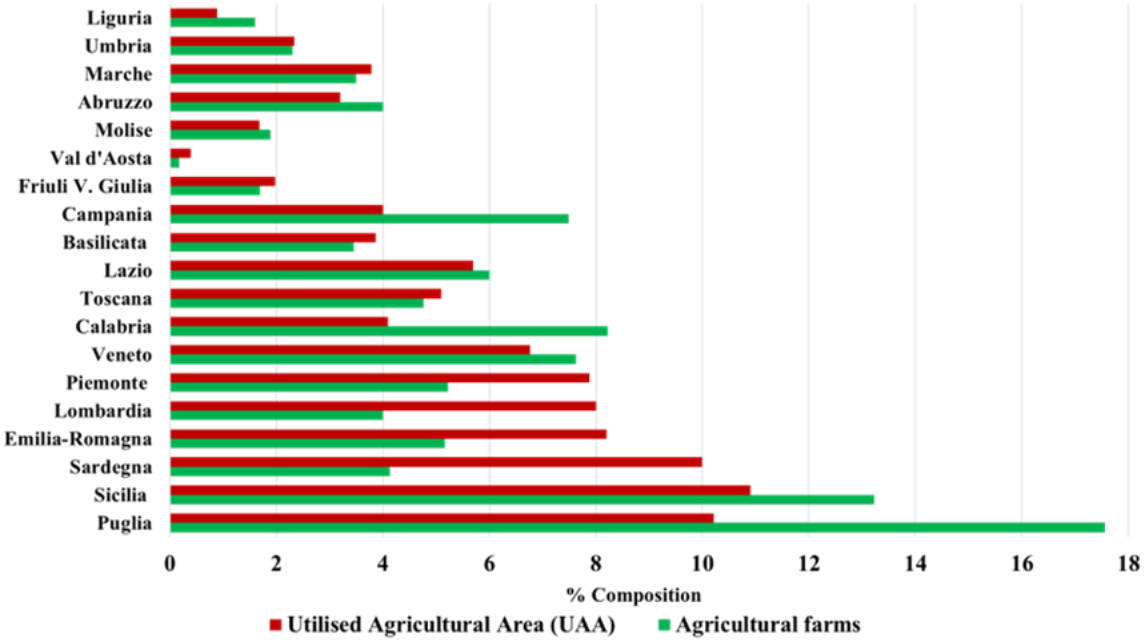


Figure 1.9. Agricultural farms and Utilised Agricultural Area (UAA) in Italy, 2020. Composition percentage (%) within Italian regions. Source: author's elaboration based on (ISTAT, 2021).

In 2021, Italian food production value ranked EUR 60.3 billion and it is expected to grow annually by 3.62% by 2028 (CAGR 2023-2028). The market's largest segment is represented by meat and meat products with a market volume of EUR 9.7 billion (16.2% of total production value), followed by vegetable crops with a market volume of EUR 8.9 billion (14.9%), and wine (9.6%), as shown in Figure 1.10.

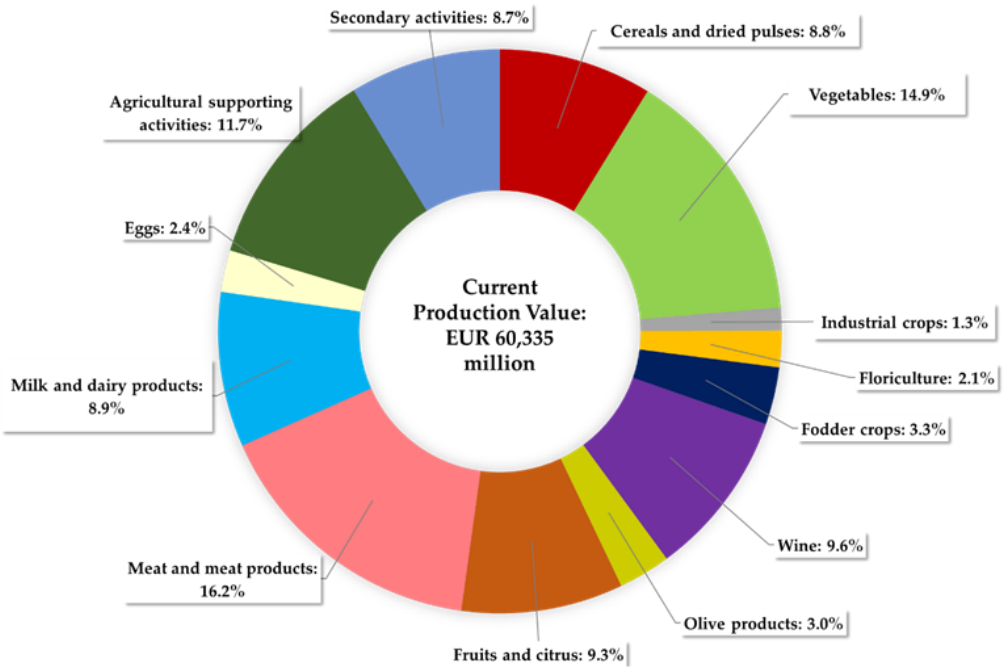


Figure 1.10. Production of food commodities in Italy, 2021. Values are expressed in EUR million, at current prices. Source: author's elaboration based on (ISTAT, 2021).

Crop production is confirmed as the major contributor to Italian agriculture, accounting for 53.1% of total agricultural production. Within food crops, it is worth highlighting wine (21,900 hectolitres), grapes delivered and sold (3,887 thousand tonnes), soft and durum wheat (3,568 and 3,000 thousand tonnes respectively), maize (6,086) and tomatoes (5,500), as shown in Table 1.1.

Table 1.1. Main crops production in Italy, 2022. Personal elaboration based on (CREA, 2022) and (ISTAT, 2021).

Crops	Quantity (1,000 tons)	Economic Value (1,000 €)	Var. % 2021-2022
Wine (1,000 hl)	21,900	3,797,802	–6.9
Oil	209	1,576,752	23.7
Grapes delivered and sold	3,887	1,354,448	5.3
Fodder (in straw)	185,674	2,008,346	17.6
Durum wheat	3,800	2,012,024	41.4
Flowers and ornamental plants	137,104	1,294,819	5.2
Hybrid maize (corn)	6,086	1,603,557	24.8
Tomatoes	5,500	1,266,460	–0.4
Potatoes	1,280	712,939	–3.7
Apples	2,211	963,037	4.1
Lettuce	497	680,325	4.1
Oranges	1,636	643,610	3.9
Courgettes	166	605,805	18.0
Soft wheat	3,568	687,936	36.4
Artichokes	378	265,430	6.4
Pears	470	448,544	–16.5

In 2021, crops showed a complex trend, as a synthesis of a slowdown in production volumes (–3.7%), a surge in prices (+9.8%), and a subsequent increase in value (+5.7%). Above all, woody crops suffered from unfavourable climatic conditions (i.e., spring frosts and summer droughts) that particularly affected crop-growing activities, thus recording a sharp drop about all fruit species (–18.9% in volume and –8.6% in value). Wine production also declined in both volume and value (–6.7% and –2.4% respectively), with even marked changes. The exceptions were citrus fruit (stable in volume and driven up by prices) and olives, which, thanks to the positive phase in the cyclical nature of production typical of the sector, recorded increases in volume of close to +10%, at the same time as a consistent increase in olive oil prices (+23.7%). Fodder crops show a very slight reduction in volume against a significant increase in value (+17.6%), which is entirely attributable to the price trend.

The EU's Farm to Fork strategy (F2F) aims to encourage a more sustainable and resilient agriculture, where all food production-related impacts (both in terms of environment, society, and economic resources) are minimized (EU, 2018). In these regards, the Italian agricultural sector is increasingly showing its *"green"* aspect, thus highlighting an ever-increasing leadership in the production of high-quality and excellent products that are overall valued in foreign markets. The cultivated area with organic farming has reached 2.2 million hectares in 2021, thus corresponding to 17.4% of the total UAA. This growing trend, compared to the last two years, has highlighted a 4.4% increase in the UAA, and a 5.4% increase in the number of operators involved in agricultural production. It is of particular interest that maintaining the upward trend of these two indicators becomes a priority, given the establishment at the EU level of reaching 25% of organic production of total UAA by 2030 (EU, 2018).

The distribution of organic UAA between the different macro-areas - arable crops grassland and permanent crops - shows a decreasing trend in the incidence of the area under arable land, fields, and pastures in favour of the remaining crop types, as shown in Table 1.2. In particular, the organic area under arable crops increased by 8.6%. Although to a lesser extent, the total organic area under permanent crops also increases, except for citrus fruits (–11%).

Furthermore, the reviewed literature highlighted some inconsistencies about the F2F target of allocating 25% of land to organic agriculture, which is expected to further reduce crop yields.

Table 1.2. Organic surface areas by agricultural production orientation (2021). Source: Personal elaboration from (SINAB, 2022).

Agricultural Production Orientation	SAU ha (2021)		Var. % SAU 2021/20	
	in conversion	**organic**	**in conversion**	**organic**
Total arable land	**183,641**	**851,510**	**21.1**	**6.2**
of which:				
Cereals	66,927	275,799	22.7	–1.2
Protein crops, legumes, grains	8,014	47,747	33.4	16.3
Root crops	467	3,394	2.6	11.7
Industrial crops	6,008	36,924	8.4	–3.6
Fresh vegetables, strawberries, cultivated mushrooms	10,140	49,652	11.4	–17.7
Forages	81,294	342,538	18.8	–4.4
Other arable crops	10,791	95,454	43.3	335.0
Permanent fields and pastures	**115,735**	**463,649**	**16.6**	**–4.3**
Total permanent	**109,526**	**429,956**	**36.6**	**4.5**
of which:				
Fruits	7,536	34,625	2.2	9.1
Nuts	10,101	44,737	21.0	0.0
Citrus Fruits	4,999	26,718	493.7	14.5
Olive	39,425	208,212	13.0	–1.6
Grape Vine	24,552	103,576	2.0	11.0
Other permanent	1,194	7,088	19.6	164.5
Resting land	**14,716**	**44,558**	**–2.7**	**–6.8**
Total	**401,898**	**1,792,325**	**22.3**	**2.5**

Different studies have pointed out that the impact of the F2F strategy, depending on the specific assumptions made, leads to a significant reduction in agricultural productivity, notably affecting cereals (from –15% to –48.5%), oilseeds (from –15% to –60.7%), and vegetable and fruit crops (from –5.2% to –13.0%) (Barreiro-Hurle et al., 2021), (Beckman et al., 2020), (Bremmer et al., 2021). This is mainly attributable to the reduced use of fertilisers (nearly about –20%), as well as the different compositions of fertilisers and pesticides in organic production, that affect crop yield. In these regards, the findings of Bremmer et al., (2021) calculated a decrease in the value of agricultural production by 2030 of approximately EUR 92 billion, a +20% increase in food prices, and an increase in land use of between two and three million hectares (Bremmer, et al., 2021), thus being in line with similar reporting literature studies (Wesseler, J., 2022) (Beckman et al., 2020). In addition to the challenges related to agricultural innovation, targets to reduce pesticide and/or fertiliser use and nutrient emissions could be perceived as a disincentive to switch to organic production. Moreover, to further reduce/overcome these negative impacts, in particular for permanent crops, there is a greater need for innovations (R&D, as well as new plant breeding techniques) that make crop production

more sustainable in the medium term for annual crops, and in the long term for permanent crops (Bremmer et al., 2021). In this context, it should be emphasized that the implications discussed are based on the assumption that no further radical changes in technology and institutions are expected. Therefore, over the long-term, the F2F strategy would imply a reallocation of inputs and allocative efficiency in EU agriculture. Nevertheless, achieving the targets under the F2F strategy is expected to increase soil cultivation, thus reducing climate-altering emissions associated with the use of fertilisers, as well as mitigating impacts on biodiversity losses (Wesseler, J., 2022). Ultimately, to ensure and maximize the environmental sustainability, climate, and diversity benefits of F2F strategies, it is important to consider the potential negative trade-offs concerning trade, indirect land use, and agricultural income.

1.3 Food systems and by-products: environmental dimensions and related impacts

Nowadays, new trends are changing the functioning of quality assurance process in the agri-food sector and related strategic priorities. To this purpose, the evolution of agri-food production is an ongoing process, driven by the quest for improved efficiency, sustainability, and meeting the global demand for safe and nutritious food. In these regards, according to the United Nations (2019), the world's population is expected to reach 9.1 billion by 2050, an 83% increase over the current population. This rapid growth will lead to an estimated +47% increase in food demand by 2050 (Gouel et al., 2018), resulting in increased anthropogenic pressure on natural resources. In this framework, food systems (FSs) have been expanding to fulfill market demand and waste production, thus representing a social, economic, and environmental issue.

According to the reviewed literature, a third of global greenhouse gas emissions (GHGs) is mainly attributable to food systems, generated within the entire supply chain from production to final consumption (Crippa et al., 2021; Rosenzweig et al., 2020). Considering the FAO global estimates, in 2020, GHGs from agri-food systems were 16 Gt CO_2 eq, (–3% from 2019), thus representing an average contribution of 31% (range 25% to 42%) to global anthropogenic GHGs, amounting to 52 Gt CO_2 eq, in 2020 (–4% from 2019). As well as FSs emissions per capita likewise decreased over the year 2020, from 2.4 t CO_2 eq/per capita to 2 t CO_2 eq/per capita, reflecting a well-documented reduction in economic activities due to the COVID-19 pandemic (FAOSTAT, 2021).

Agriculture, forestry, and land use currently represent the only sector showing major potential to become a net emission sink - pulling more GHGs from the atmosphere than

it emits - through the creation and protection of carbon sinks in forests, oceans, and soil.

Considering the agri-food chain components, in 2020, farm-gate emissions (related to agricultural production) with 7.4 Gt CO_2 eq were nearly 39% of the total GHGs, followed by emissions from pre- and post-production (5.6 Gt CO_2 eq), thus corresponding to 32% of total GHGs and finally land-use change (LUC) emissions (3.1 Gt CO_2 eq). It is worth-noting a decrease of −11% for LUC, and −4% for pre-and post-production compared to the year 2019, due to a reduction in deforestation and fossil fuel energy use, in line with the pandemic of COVID-19. In particular, climate-altering emissions from food systems are mostly attributable to agronomic practices and intensive use of fertilizers and pesticides, as well as energy-related activities (Crippa et al., 2021). In addition, CH_4 emissions accounted for 35% of the food system-related GHGs, mainly due to livestock production, animal breeding, and waste treatment.

Among the components of food supply chain, about 18-20% of total GHGs are attributable to waste and side-stream activities, mainly related to industries, storage, and logistics, thus having a key role in addressing sustainability challenges and promoting circular economy principles. When considering the existing plethora of studies on Food Loss and Waste (FLW), one of the most relevant aspects is represented by the considerable quantity of food, still of good nutritional quality, that is destined to become waste (food waste), and by the loss in quality (nutritional and/or organoleptic) of the food along all stages of the entire production chain, "*from field to fork*" (food loss), as included in the Circular Economy Package included legislative proposals on food waste (EU, 2018). Contextualizing the phenomenon at the national level, in Europe, wasted food is equal to 54% of the total food production (88 million tons (Mt) of food per year and about 173 kg/capita/year), for an economic value of EUR 8.5 billion (FAO, 2019). According to the national estimates on food waste (EUROSTAT, 2022b), the highest average amount of food waste collected in 2020 was in Cyprus (397 kg per person/year), and Denmark (221 kg per person/year). Only 7 of the 27 EU Member States collected less than 100 kg of food waste per person on average in 2020, with the lowest amounts in Croatia (71 kg per person), and Slovenia (68 kg per person), as shown in Figure 1.11. In almost all EU-27 Member States, total activities by households accounted for on average about 53% compared to all collected data, thus representing the largest source of food waste.

As regards the Italian context, data from Waste Watcher Observatory Report 2022 highlight an amount of 4.2 Mt/year and 65 Kg/capita/year of food wasted, for an economic value of EUR 9.3 billion (Waste Watcher International Observatory, 2022). In

particular, Italy records a high amount of waste mainly at the household level for about 54% - almost all of which is represented by completely unused food (43%), with a lesser extent by meal leftovers (11%). This is followed by waste in catering (21%), commercial distribution (15%), agriculture (8%), and processing (2%) (Waste Watcher International Observatory, 2022). According to the Report Waste Watcher, the most wasted food products in Italy were found to be those that are easily perishable and thus: fruit and vegetables (31%), followed by dairy products (21%), cooked pasta (19%), bread (16%) and meat (13%). While, globally, the greatest wastage is concentrated on: roots and tubers, fruits and vegetables (45% of global production); fishery products (35%); cereals (30%); dairy, meat, legumes, and oilseeds (20%) (FAO, 2019).

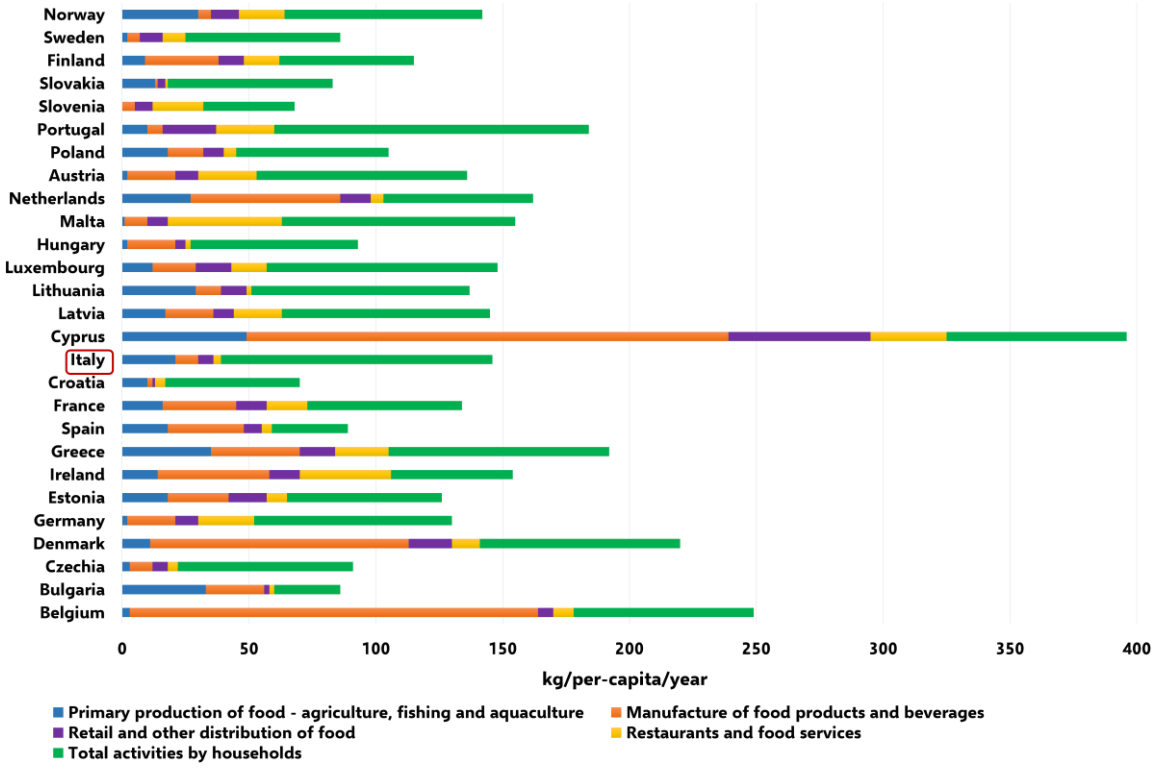


Figure 1.11. Food waste distribution among EU-27 Member States, in 2020. Data are expressed in kg/per-capita/year and are based on Eurostat estimates (online data code: env_wasfw). Source: author's elaboration.

In recent times, the COVID-19 emergency has greatly contributed to the food waste balance. The new 'residential' lifestyle has, in fact, substantially changed food purchasing and spending, highlighting, in the first phase of mobility restrictions, a significant change in food habits. An 8.34% average growth in purchases in Italy was recorded, driven by the North West, where the increase was +11.20%, and the North East with +9.66% compared to the same period in 2019. The largest purchases concerned long-life products,

such as canned and frozen food (+37% compared to fresh food), pasta, rice, and flour. In this context, the problem of food waste affects in a two-way manner*: i)* the food industries which, faced with an increase in demand and sales recorded with peaks of 60-70%, have been confronted with real difficulties in supply management; *ii)* consumers who, during the collective quarantine, have been attempted by erroneous planning of expenditure, marked by abundant and excessive purchases and the consequent increase in food surpluses and waste. To bring food systems within the limits of environmental and socio-economic capacity, waste would have to be structurally reduced to at least one-third of the current global estimates and one-quarter of those recorded in Italy (Galioto et al., 2022). Concerning the environmental dimension, food waste leads to several impacts along the food supply chain, including inefficient use of water resources (89% of human water consumption is attributable to food use only), land use (3.3 $GtCO_2$ equivalents per year) and other inputs required along the entire supply chain, which in turn can lead to degradation of natural ecosystems and a progressive reduction of the valuable goods and services they provide. As a practical matter, the ecological footprint of food waste contributed from 15 to 24% of total GHGs, thus affecting the total bio-capacity deficit by 58% worldwide, 30% in the Mediterranean, and 18% in Italy (Chen et al., 2020). In this context, it is alarming to note that food systems are among the main factors responsible for the depletion of the bio-physical processes that regulate the resilience of the planet, leading to an alteration to such an extent as to exceed the safety threshold within which human activities are guaranteed.

Global food production is estimated to increase up to 15% in the forthcoming decades to meet rapid population growth, and FSs emissions could increase by up to 80% from 2010 to 2050 (Costa et al., 2022). Table 1.3. shows the global food production and consumption divided for the main food groups in 2010, and projections in food production and consumption in the years 2030 and 2050, calculated by the IMPACT model, which is an integrated system of linked economic, climate, water, and crop models that allows for the exploration of such scenarios (IFPRI, 2022). Baseline projections indicate that global food production will grow on average by nearly 60% over the levels of 2010 by 2050 under environmental pressures. Production and demand are projected to grow especially for fruit and vegetables, with +91.2% for production and +53.3% for consumption; for meat (+67.4% for production, and +19.0% for consumption), and legumes with +83.3%, and +47.5% for production and consumption, respectively.

Table 1.3. Global food production in 2010 and projections within 2030 and 2050 differentiated by main food groups

Food groups	Total production (Metric tons)			Per capita food consumption (kg per capita per year)			Increase Percentage (%)			
							Production		Consumption	
	2010	2030	2050	2010	2030	2050	Δ 2010/ 2030	Δ 2010/ 2050	Δ 2010/ 2030	Δ 2010/ 2050
Meat	274	381	460	225	255	268	+39.2%	+67.4%	+13.1%	+19.0%
Cereals	2,155	2,746	3,235	1,267	1,290	1,299	+27.6%	+50.1%	+1.8%	+2.5%
Fruits & Vegetables	1,592	2,334	3,044	182	229	279	+46.6%	+91.2%	+25.8%	+53.3%
Oils	685	1,055	1,325	64	75	72	+54.0%	+93.4%	+17.2%	+12.5%
Legumes	66	94	121	61	75	90	+42.4%	+83.3%	+22.9%	+47.5%
Roots & Tubers	780	1,006	1,185	150	165	174	+28.9%	+51.9%	+10.0%	+16.0%

Data elaboration from projections from IFRIP's impact model: climate change and food systems, 2022 (https://www.ifpri.org/project/ifpri-impact-model). **Meat** includes pork, beef, poultry, sheep, and goats; **Cereals** include barley, maize, millet, rice, sorghum, wheat, and aggregated other cereals; Fruits & Vegetables: bananas, plantains, aggregated temperate fruits, aggregated tropical fruits, and aggregated vegetables; **Oils** include groundnuts, rapeseed, soybean, sunflower, and aggregated other oilseeds; **Legumes** include beans, chickpeas, cowpeas, lentils, pigeon peas, and aggregated other legumes; **Roots & Tubers** include cassava, potato, sweet potato, yams, and aggregated other roots and tubers.

Among all food groups, cereals represent the most produced food commodities, denoting a two-fold increase in production by 2050 (+50.4%).

In addition, the way that foods are produced, including the agroecological and farming system characteristics, contribute to their environmental impacts, and therefore the various food groups will result in different environmental pressures. Figure 1.12. shows climate-altering emissions attributed to food at various stages along the supply chain. Values are expressed in terms of CO_2 equivalents, considering not only CO_2 but all GHGs. Carbon dioxide equivalents are calculated for each greenhouse gas by multiplying the mass of emissions of that gas by its global warming potential (GWP) value. So, GWP measures the amount of warming a gas produces as compared to CO_2 (Forster et al., 2007).

It is evident from the graph, that the production of animal products generates the majority of feed-related GHG emissions (72–78% of total agricultural emissions), mainly attributable to low feed conversion efficiency, enteric fermentation in ruminants, and manure-related emissions; feed-related impacts of animal products also contribute to blue water use (about 10%) and pressure on cropland, as well as nitrogen and phosphorous fertilizers application (20–25% each). In comparison, staple crops (such as wheat, rye,

rice, olives, etc.), generally have lower environmental footprints (impacts per kg of product) than animal products, particularly in terms of GHGs.

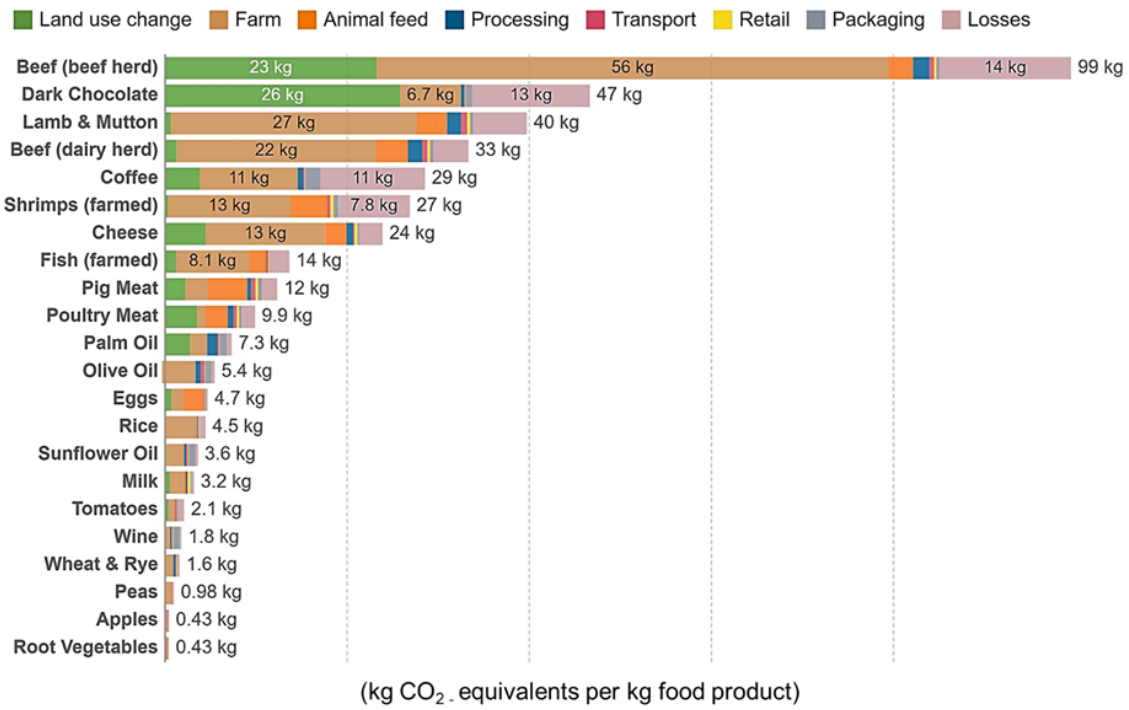


Figure 1.12. Greenhouse gas emissions per kg of food product across the supply chain (expressed in kg CO_2 eq per kg food product). Source: (Poore and Nemecek, 2018) OurWorldInData.org/environmental-impacts-of-food • CC BY

According to the findings of Springmann et al., (2018), staple crops grown for human consumption are responsible for one-third to one-half (30-50%) of cultivated land use, blue water use, and nitrogen emissions; therefore, regarding their major production volumes, they may result in higher total impacts than individual crops (Springmann et al., 2018). Furthermore, considering the projected population growth between 2010 and 2050 (Table 1.3.), it is worth noting that this contributes to an overall increased impact of each food, with a shift towards animal products (+7% to +16% in all environmental domains), fruit and vegetables (+2% to +28%), and a smaller proportion from staple crops (–7% to –19%).

1.4 Sustainability in the agri-food sector

In a world marked by rapid population growth, increasing demand for resources, and rising environmental challenges, the concept of sustainable development has emerged as a guiding principle for future collective living. The concept of *'sustainable development'* encompasses a visionary approach that seeks to meet the needs of the current generation

without compromising the ability of future generations to meet their own needs. Sustainable development harmonizes three essential pillars: economic progress, social equity, and environmental protection. It acknowledges the interplay between economic development, social welfare, and environmental protection, recognizing the strict interconnection of these dimensions.

Starting in the 1970s, the issue of environmental protection became increasingly prominent as a result of several environmental disasters (such as the first great oil spill caused by the Canyon oil tanker in 1967), which led to the establishment of ecological movements among the population. Thus in 1972, the global framework was laid to start a path of awareness towards sustainable development. June 1972 marked the first United Nations World Conference on the Human Environment, held in Stockholm. For the first time, states addressed environmental issues by adopting a sectoral policy to avoid ecological repercussions. An international body with a universal character was established to deal with environmental protection, namely the United Nations Environment Programme (UNEP). In 1972, the Report on the Limits to Development commissioned by the Club of Rome was published with the help of scientists, intellectuals, and governors, in which the importance of natural resources was emphasized. In particular, it highlighted the finite nature of the resources available in nature, since as T.R. Malthus stated, *"the population grows in geometric progression while natural resources grow in mathematical proportion; as a result, there will be a depletion of these resources".*

Sustainable development was first defined in 1987 with the publication of the Brundtland Report entitled *'Our Common Future'*. This report was prepared by the World Commission on Environment and Development established in 1983 by the United Nations General Assembly, as: *"the satisfaction of the needs of present generations without compromising the possibility of satisfying the needs of future generations"* (Brundtland, 1987). The environment acquires economic importance with this definition and is no longer considered a self-contained reality but rather linked and interconnected to economic development. Figure 1.13. shows the most important stages towards the achievement of sustainable development.

On 25 September 2015 in New York (USA), the UN General Assembly, represented by 193 member countries, signed the document entitled: *'Transforming our world. The 2030 Agenda for Sustainable Development'.* It represents an agenda of action for people, the planet, and prosperity, introducing the 17 Sustainable Development Goals (SDGs),

and 169 sub-goals, to be achieved over the next fifteen years, by 2030 (UN, 2015) ((Kretschmer et al., 2021).

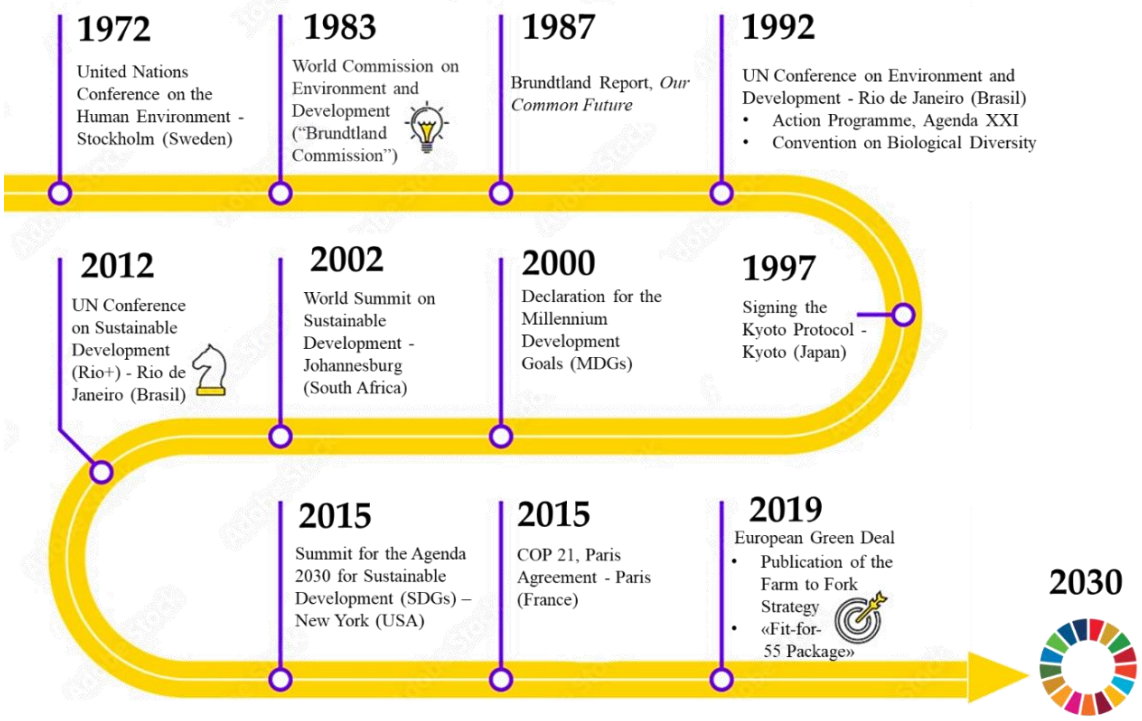


Figure 1.13. Steps towards sustainable development. Source: author's elaboration

The global SDGs provide an evidence-based framework for national, regional, and global sustainable development aimed to address a balance between economic, social, and environmental dimensions thus promoting a holistic change towards a sustainable future. In particular, focusing on food systems, sustainable development actions are achieved by multiple interconnections within SDGs, which are directly or indirectly focused on improving food security, nutrition, sustainability, and related issues. Figure 1.14 shows the contribution of the agri-food system and actions for promoting sustainable development. The food systems-related objectives, in further detail, mainly concern action plans for:

- Food security and nutrition: addressing major food systems (including fisheries and aquaculture), and their challenges, such as hunger and malnutrition eradication, while ensuring food security.
- Multifunctional agriculture and agro-ecology: move towards regenerative, ecological and multifunctional farming systems that protect soil fertility and biodiversity, including through more efficient use of water and fertilizers, reducing emissions and increasing greenhouse gas uptake, and adapting to the

impacts of climate change (Herrero et al., 2021).

- Water use: increase the efficiency of water use in agriculture through the recovery of wastewater as well as by reducing food losses and waste across the supply chain.

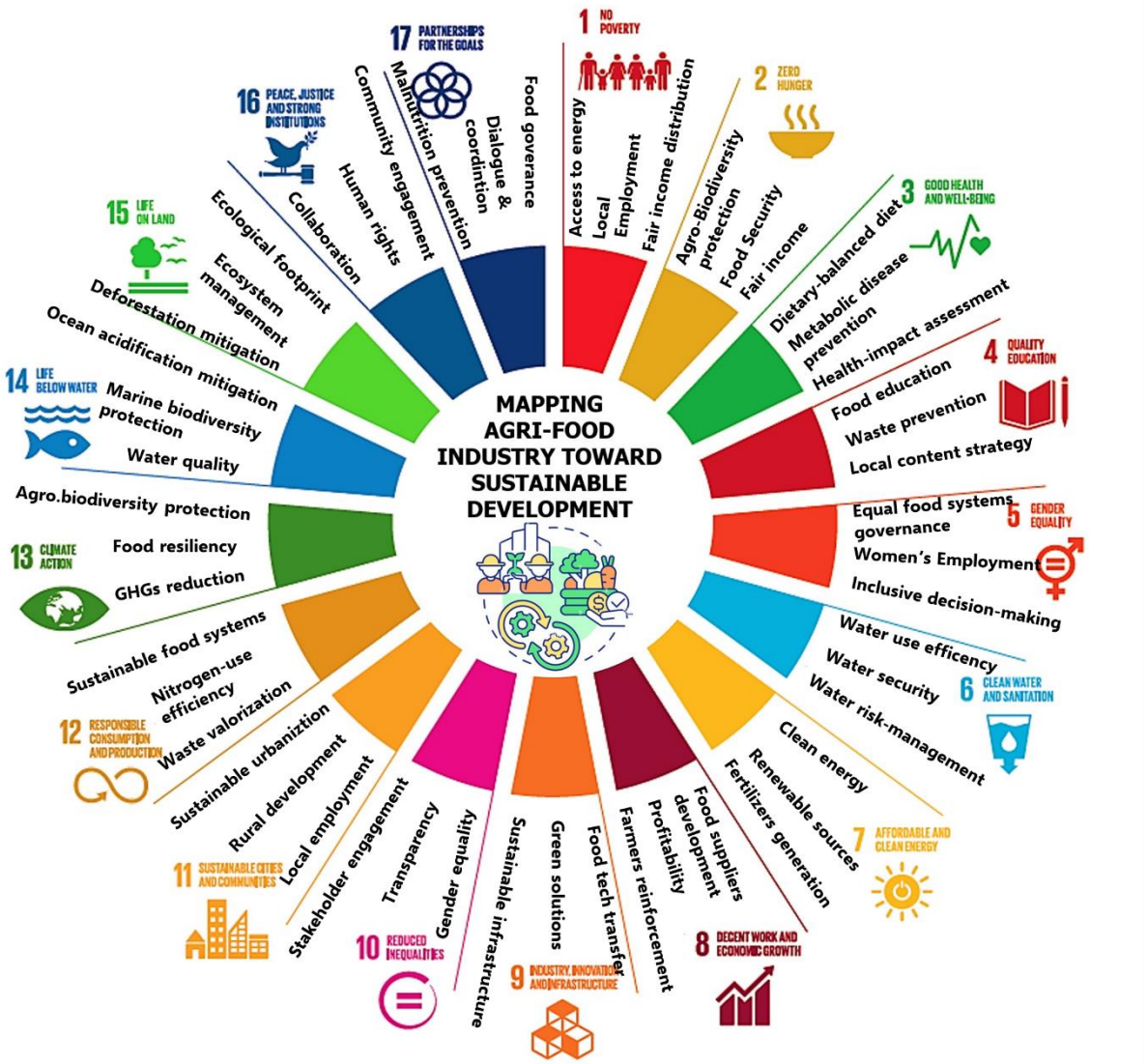


Figure 1.14. Agri-food systems and SDGs. Source: author's elaboration

- Sustainable agricultural practices for stakeholders: increase agro-ecological practices, and improve the quality of linkages between local entrepreneurs, governance, and consumers, thus strengthening, and building the capacity of food systems actors (Kretschmer et al., 2021).
- Reform both food production and supply and promote healthier diets: develop the infrastructure and systems to produce sufficient quantities of nutrient-rich foods, and affordable staple foods, reducing agri-food by-product losses in

processing, storage, and transport. As well as, reforming food supply along the agricultural production, processing, transport, and consumption chain, making supply chains more resilient, agriculture more productive and sustainable, and diets healthier.

- Sustainable food systems and nutrition: promote healthier diets and discourage excessive consumption of animal-origin foods as well as sugars, also by acting with nutritional education plans, since childhood aimed to prevent cardio-metabolic diseases.

In addition, in the current context, where severe environmental pressures have been worsened by the COVID-19 pandemic, the wars, and violent conflicts in Ukraine, pathways towards sustainable food production have been also marked by the main object for the European Green Deal (EGD), proposed by the European Commission on 11 December 2019, as "*a strategy to transform the EU into a fair and prosperous society, with a modern, resource-efficient and competitive economy where there are no net emissions of greenhouse gases in 2050"* (Fetting, C., 2020).

1.5 Life Cycle Assessment for the Sustainability evaluation in the Agri-food Sector

In recent years, different pathways have been proposed by researchers, technologists as well as governments and institutions to address sustainability in agri-food systems. Among the possibilities of addressing the complementary role of sustainable food production (i.e., agriculture practices, crop management systems, reduced use of fertilizers, etc.), and food consumption patterns (i.e., dietary shifts towards healthier foods, reduced meat consumption, etc.), different studies have been focused on combining both environmental and nutritional aspects to increase the sustainability of food systems (Chen et al., 2020),(Crippa, et al., 2021). Moreover, the interest in sustainability concerns has also highlighted the need to balance food industries, as well as chemical process engineering to include environmental criteria in processes design, validation, and optimization for the development of more sustainable and green processes (Vauchel et al., 2018). In this context, an adapted methodology is required to improve the sustainability of food systems and create a synergy that glues the sustainable production and consumption, while ensuring greener industrial processes.

Life Cycle Assessment (LCA) is a standardized and well-established methodology to quantify environmental impacts associated with a product, a process, and/or a service throughout its entire life cycle (Notarnicola et al., 2017). LCA is a tool providing

qualitative and quantitative data concerning environmental impacts based on a holistic approach that encompasses all phases of the life cycle, rather than only the production process itself. Accordingly, it is useful for product development and design with improved environmental performance by promoting the conservation of natural resources, as well as for reducing costs and improving business competitiveness. Following ISO standards 14040:2006 and 14044:2006, an LCA analysis consists of four phases (ISO, 2006a);(ISO, 2006b) as illustrated in Figure 1.15.

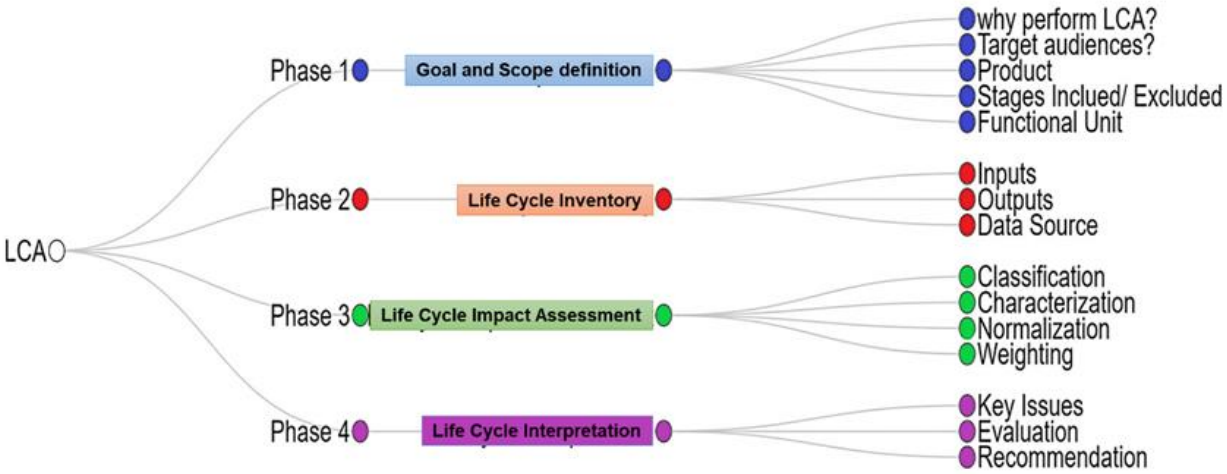


Figure 1.15. Life Cycle Assessment phases and main issues. Source: author's elaboration

1. **Goal and scope definition**: this first phase defines the main aims of the LCA study, thus considering different features, such as the target audience, the analyzed case study (product, process, and/or service), the life cycle stages to consider in the study (defined as system boundaries), as well as the functional unit of the scope, to which all elementary input flows from the inventory have to refer. According to the literature, LCA studies can be divided into two main categories based on the scope: *i.* descriptive-LCA, aimed at describing a chosen case study; and *ii.* comparative-LCA, aimed at comparing two or more products, systems, and/or services, while differentiating between these frameworks. It is worth mentioning that the reliability of the LCA results are strictly related to data quality requirements, in particular, temporal dimension (selected years), geographical area, spatial scale of the analysis (global, regional, or local), and technology used in the processes stages (Alhashim et al., 2021). Figure 1.16. shows the main characteristics of the goal and scope definition phase.

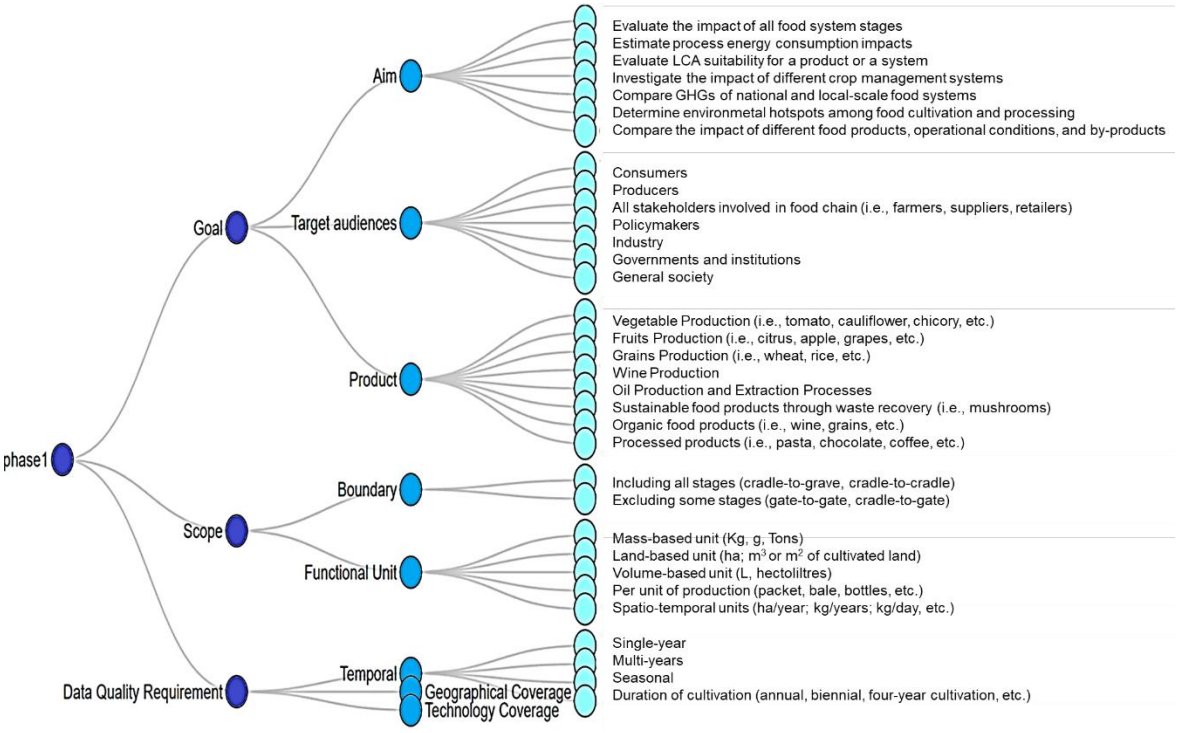


Figure 1.16. Phase 1 (Goal and Scope definition): main features. Source: author's elaboration

2. **Life Cycle Inventory (LCI):** the second phase is the product's life cycle inventory (LCI), which is obtained by summing up all the input and output from each unit process, and workflows involved in the production system. LCI provides quantitative environmental information about a product throughout its entire life cycle, on which the LCA analysis has to be performed. Considering the main LCI characteristics related to LCA analysis of food systems, the most cited input materials encompass water, raw materials (i.e., seeds, plastic materials, etc.), fertilizers, energy use (i.e., electricity, diesel, fossil fuels, etc.), packaging materials, transport facilities, as well as processes, outputs both in terms of the primary product, waste, and emissions to soil, air, and water. Furthermore, it is important to mention that the data sources of LCI could be: *i.* primary data, when obtained from specific processes within the life cycle of the investigated product or process activity (i.e., physical measures of a process, direct emissions data, and data averaged across all sites containing the specific process); or *ii.* secondary data sources, when collected from governmental and organizational records, national or international databases as well as from previous studies gathering information from primary sources. Figure 1.17. Shows the main characteristics of the LCI phase in the LCA analysis of food systems.

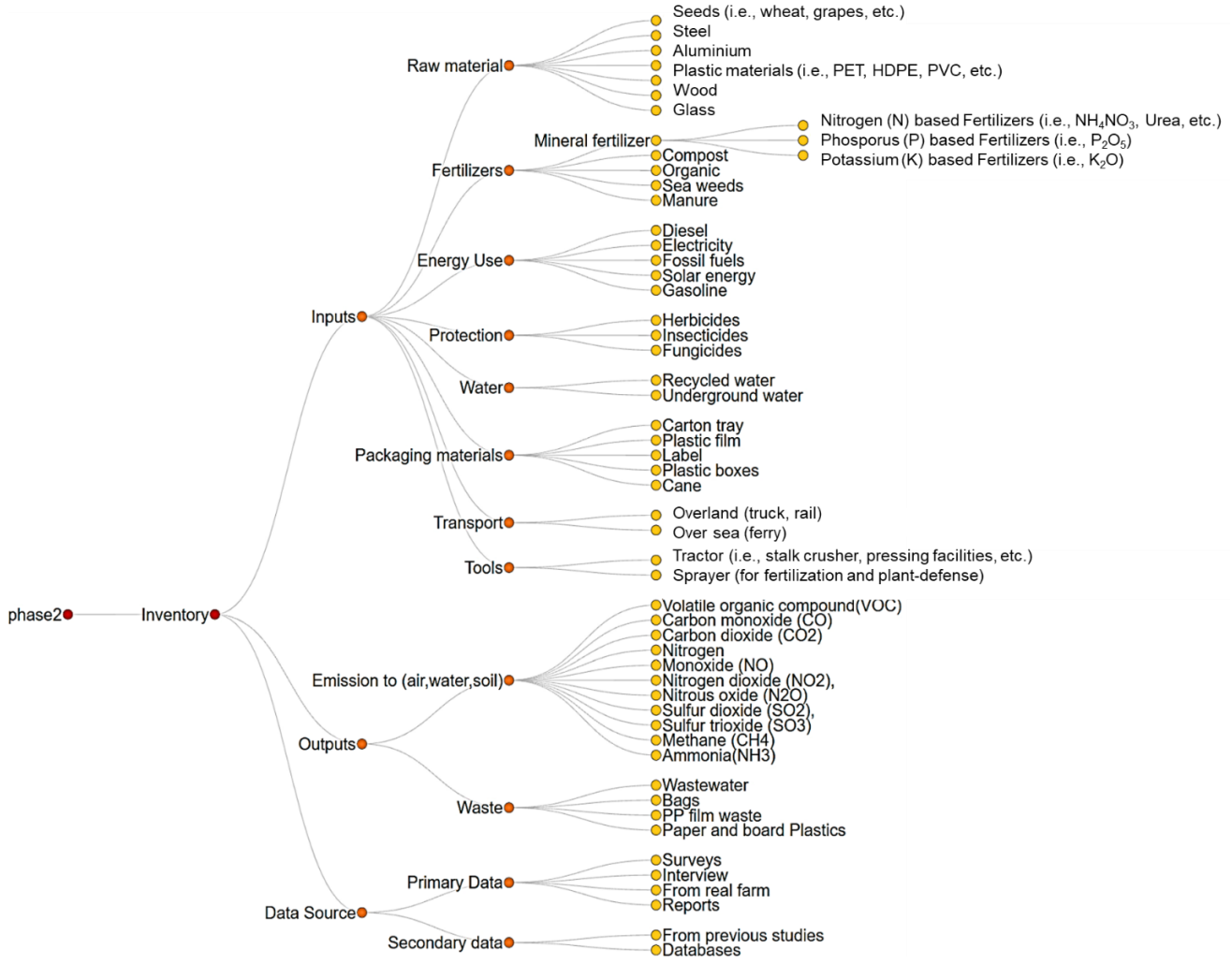


Figure 1.17. Phase 2 (Life Cycle Inventory): main features. Source: author's elaboration

3. **Life Cycle Impact Assessment (LCIA):** The third phase is the life cycle impact assessment (LCIA), which provides a quantitative analysis of the potential environmental impacts of a product or a system, based on life cycle inventory results. The LCIA consists of several elements: classification, characterization, normalization, and weighting. Of these four elements, normalization and weighting are considered optional, while the first two are mandatory elements in LCIA (Lee et al., 2004). Figure 1.18. Shows the main features concerning the LCIA phase in the LCA analysis. The most common standard method was the CML with different versions, such as CML 2 baseline 2000 V2/world, as well as the Recipe Midpoint method that expresses the relative severity of an environmental impact category, taking into account 18 midpoint indicators. In addition, the second most used methods in LCA studies on food systems are those

based on ISO standards 14044 (2006), ISO (2000), and ISO 14040 (2006), followed by many other methods, such as IPCC 2013 GWP 100, proposed by the Intergovernmental Panel on Climate Change, for the carbon footprint calculation (IPCC, 2023); (Forster et al., 2007). However, it is worth noting that the choice of an impact calculation method is strictly related to the impact category as well as the complexity of the case study under investigation (Alhashim et al., 2021).

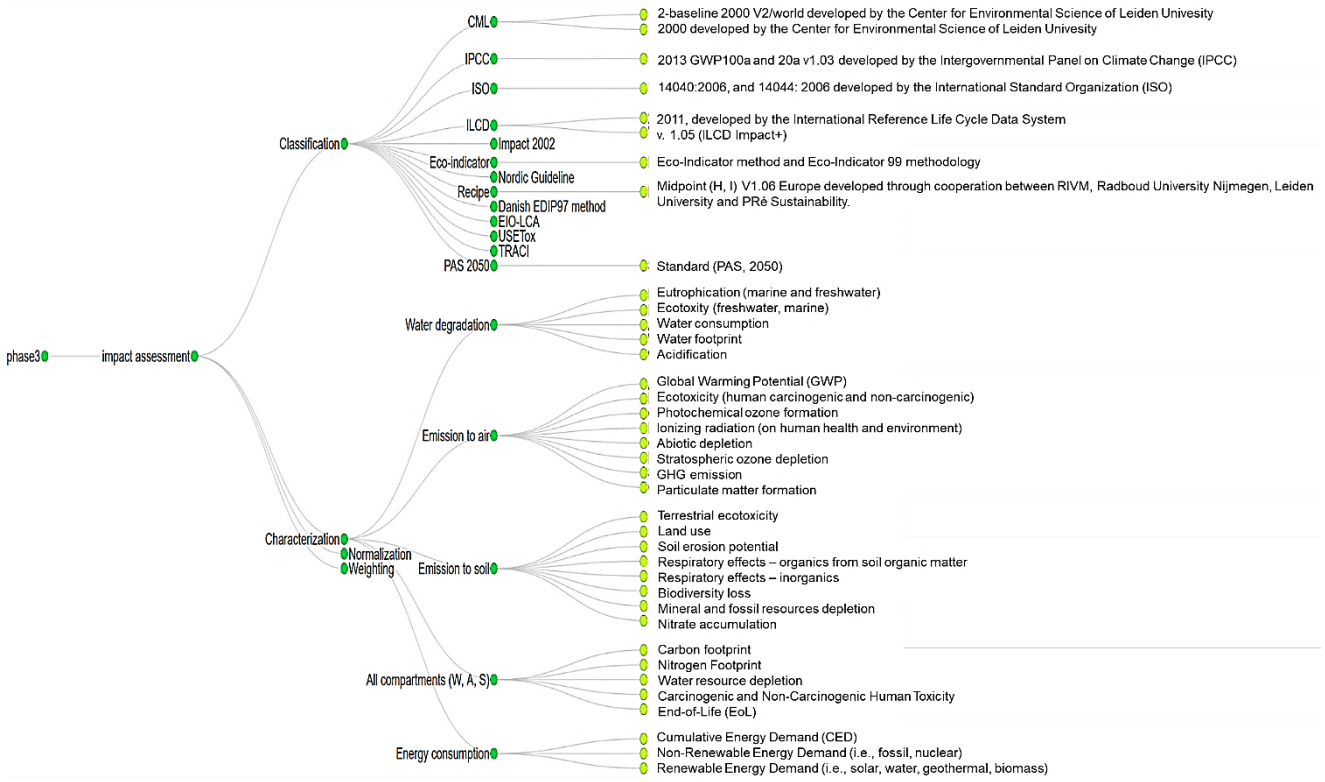


Figure 1.18. Phase 3 (Life Cycle Impact Assessment): main features. Source: author's elaboration

4. **Life Cycle Interpretation:** this last phase of LCA is aimed at explaining and contextualizing life cycle impact assessment results as a starting point for product improvement and potential options to mitigate environmental impacts. In these regards, it is suitable to identify key issues (i.e., input materials, activities, process stages, etc.) of the investigated product to underline the process tree and material flows. This represents the primary purpose of the interpretation phase, which is followed up by improvement recommendations to propose ecological alternatives and/or process modification. Figure 1.19. highlights the main characteristics of the interpretation phase in the LCA analysis.

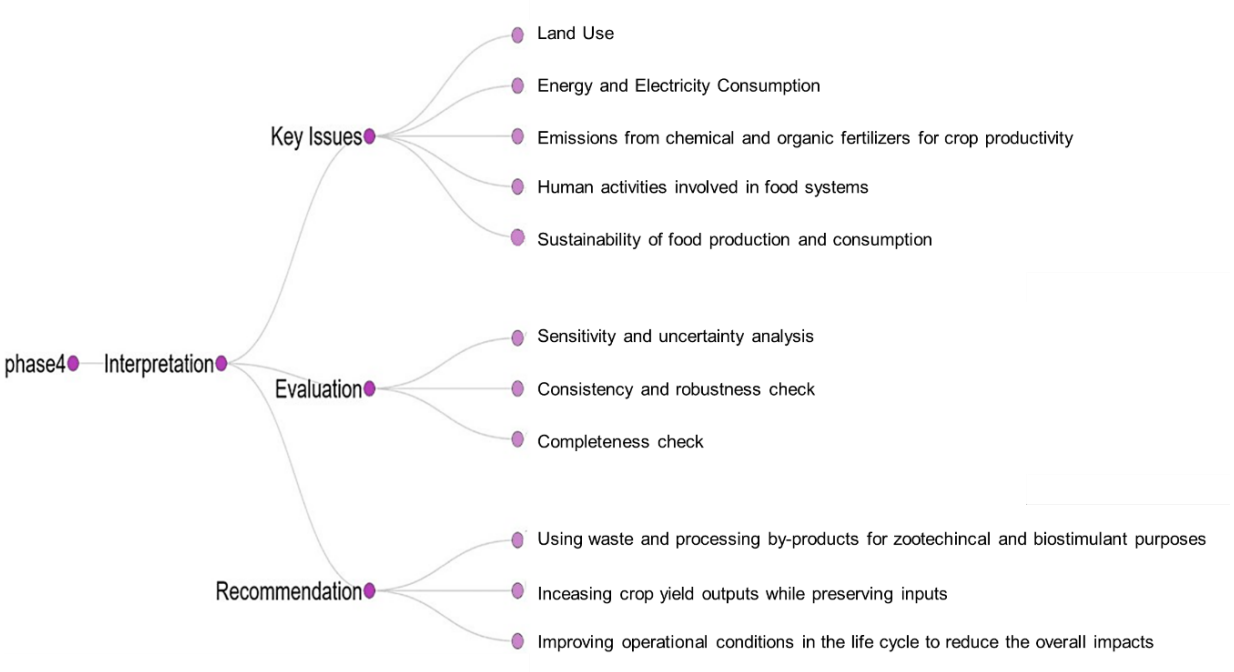


Figure 1.19. Phase 4 (Life Cycle-Interpretation): main features. Source: author's elaboration

1.6 Bioactive compounds in food products

Quality assessment in agri-food products involves the evaluation of specific attributes such as taste, aroma, texture, and appearance, which are closely tied to the content and composition of bioactive compounds (BCs). Natural bioactive components refer to naturally occurring compounds (normally from the secondary metabolism of plants) in different foodstuffs, that have the potential to affect human health beyond the basic nutritional value. Although these secondary metabolites are slightly less important compared to primary metabolites (i.e., carbohydrates, nucleic acids, proteins, amino acids, etc.) for living organisms, nonetheless, they exert beneficial physiological, behavioral, and immunological effects, as well as stress-response and natural defense mechanisms (Zainal-Abidin et al., 2017). Furthermore, it is worth noting that the biological activity of these compounds can be also associated with adverse effects on human health, beyond the recognized beneficial effects. This includes harmful effects such as toxicity, allergenicity, and mutagenicity, which usually depend on the amount and bio-availability of a certain substance (Biesalski et al., 2009).

To date, the range of naturally available BCs is numerous in terms of origin, structure, and bioactive effects, so it would be impossible to explore it all in detail. Therefore, in this overview, the most abundant BCs will be mentioned most briefly and attention will be focused in particular on biogenic amines, polyphenols, and essential fatty acids in

subsequent sections, thus representing the target analytes investigated in the research project.

The main BCs are those produced by plants and can be grouped as polyphenols, triterpenes and phytosterols, terpenoids, polysaccharides, carotenoids and tocopherols, phytosterols, as well as nitrogenous based-compounds (i.e., alkaloids, biogenic amines, etc.), and sulphur based-compounds (i.e., glucosinolates). Figure 1.20. highlights the main classes of bioactive compounds that can be found in food products.

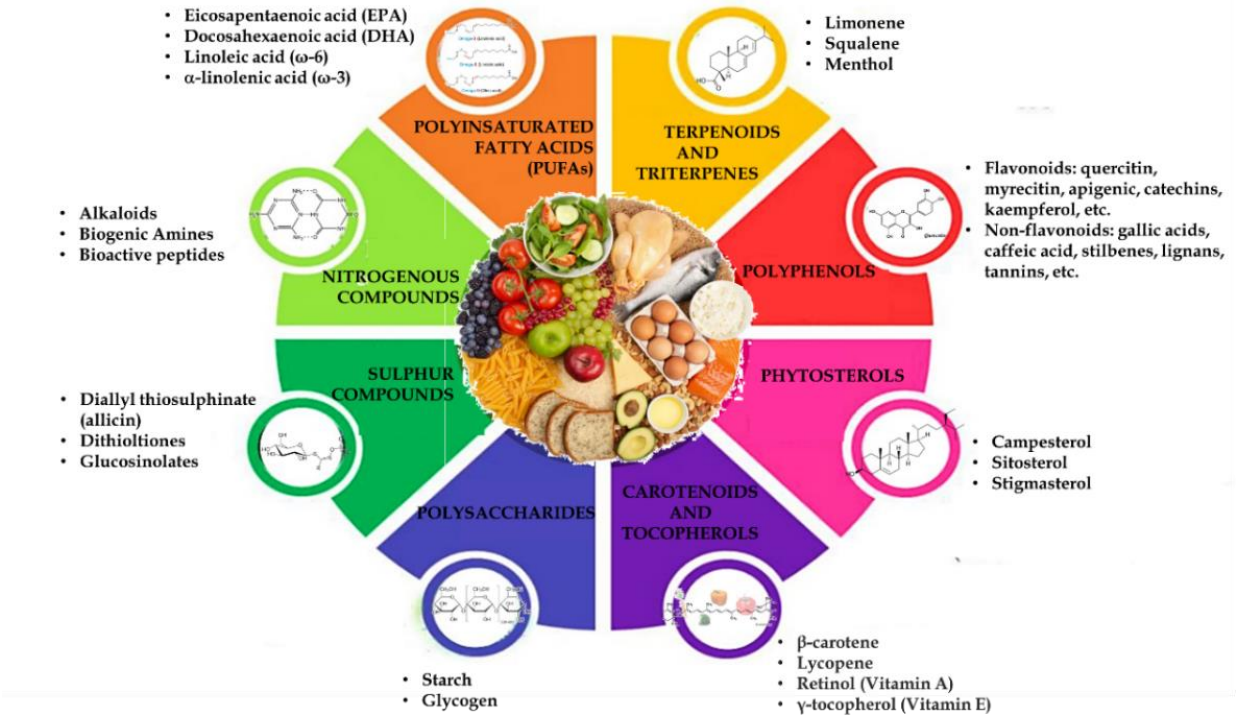


Figure 1.20. Overview of main bioactive compounds in food products. Source: author's elaboration.

Owing to the abundance of naturally occurring BCs, their characterization in food represents a major challenge that is addressed by various researchers, worldwide. In this regard, three main databases are currently aggregating data reported in the literature on bioactive components of different foods: *i.* the USDA flavonoid database (Bhagwat et al., 2016); *ii.* Phenol-Explorer (Neveu et al., 2010) for polyphenols; *iii.* and eBASIS (Bioactive Substances in Food Information Systems) (Plumb et al., 2017). Currently, the latter distributes information on over 300 foodstuffs, and 17 classes of compounds, including for the first time in its platform, the bioactive composition of meat and meat-based products. As a result of these databases' schematization, it is emphasized that the origin of BCs is not exclusively related to plants (such as glucosinolates, which are responsible for the taste and flavour of cruciferous plants), but also to animal foods. These include bioactive peptides such as carnosine, taurine, and creatine, which have been

identified in various types of meat (i.e., pork, beef, or chicken), and in various species of fish and marine organisms, whose effects can be attributed to the attenuation of muscular atrophy diseases, the reduction of metabolic syndrome risk, and blood pressure homeostasis. (Stadnik et al., 2015).

Fermented foods (i.e., chocolate, coffee, tea, wines, dairy products, etc.) are highly valued compounds in the human diet, due to several recognised bioactive effects, particularly antihypertensive and antioxidant properties. These effects are mostly attributable to probiotics produced by the microorganisms responsible for the fermentation process, usually yeasts and bacteria, such as *Lactobacillus spp.*, and *Bifidobacterium spp.* (Mamo, 2016).

1.6.1 Biogenic Amines

Biogenic Amines (BAs) are low molecular weight organic bases, which originate from the decarboxylation of amino acids by microorganisms or through amination and transamination reactions of organic compounds, such as aldehydes and ketones. Amines can be classified based on their carbon chain structure *i)* aliphatic: putrescine, cadaverine, spermine, and spermidine; *ii)* aromatic: phenylethylamine and tyramine; and *iii)* heterocyclic: tryptamine and histamine); as well as based on their functional groups *i)* monoamines: phenylethylamine, tyramine, and serotonin; *ii)* diamines: putrescine, cadaverine, and tryptamine; *iii)* polyamines: agmantine, spermine, and spermidine (Halász et al., 1994), (Silla Santos, 1996). Several amines have been identified in food, each characterized by specific origin pathways, and functions:

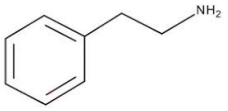
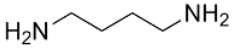
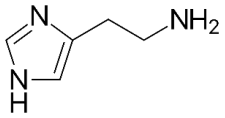
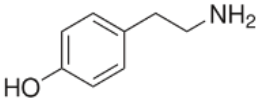
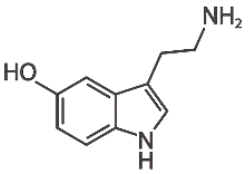
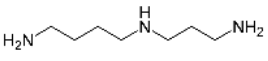
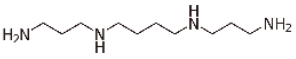
- Heterocyclic amines: that originate from the pyrolysis phenomena of proteins and amino acids, following extended cooking treatments. They exhibit potentially carcinogenic activity;
- Nitrosamines: carcinogenic, that are formed by binding the amine group with nitrites or nitrates present in food, as additives;
- Natural polyamines: which are naturally synthesized by cell metabolism;
- Biogenic amines: produced by decarboxylase-positive microorganisms.

Table 1.4. reported some of the major BAs occurring in foodstuffs, their chemical structure, and relative amino acid precursors. The occurrence of BAs in food can be variable depending on the protein composition of food, and it is mainly associated with:

- The presence of contaminating microorganisms and/or microbial clusters (decarboxylase-positive microorganisms) added during the preparation steps;

- The availability of free amino acids (FAA), i.e., availability of BAs precursors;
- Optimal conditions for both the growth (i.e., pH, temperature, NaCl concentration, etc.) of decarboxylase-positive micro-organisms and the activity of decarboxylase enzymes (Vinci et al., 2021); (Doeun et al., 2017).

Table 1.4. Biogenic Amines and their chemical structure. Source: author's elaboration

IUPAC nomenclature	***Amino acid Precursor***	***Molecular formula***	***Skeletal formula***	***Molar mass (g/mol)***
1-Phenyl-2-aminoethane **(B-PEA)**	Phenylalanine	$C_8H_{11}N$		121.18
Butane-1,4-diamine **(PUTRESCINE)**	Ornithine	$C_4H_{12}N_2$		88.15
Pentane-1,5-diamine **(CADAVERINE)**	Lysin	$C_5H_{14}N_2$		102.18
2-(1H-Imidazol-4-yl) ethanamine **(HISTAMINE)**	Histidine	$C_5H_9N_3$		111.15
4-(2-Aminoethyl) phenol **(TYRAMINE)**	Tyrosine	$C_8H_{11}NO$		137.18
3-(2-Aminoethyl)indol-5-ol **(SEROTONIN)**	Tryptophan	$C_{10}H_{12}N_2O$		176.22
N'-(3-aminopropyl)butane-1,4-diamine **(SPERMIDINE)**	Methionine	$C_7H_{19}N_3$		145.25
N,N'-bis(3-aminopropyl)butane-1,4-diamine **(SPERMINE)**	Methionine	$C_{10}H_{26}N_4$		202.35

Following these factors affecting BAs occurrence, food products that exhibit high levels of BAs are both fresh and perishable foodstuffs, being directly exposed to decarboxylase-positive micro-organisms (i.e., meat and meat-based products, seafood, fruit, vegetables, etc.), as well as fermented and/or processed foods (i.e., wine, beer, cheese, coffee, chocolate, etc.) as a direct consequence of the processing along the food

supply chain (i.e., alcoholic and lactic fermentation) (Önal, 2007). Therefore, hygienic standards are the first aspect that is required to be monitored to limit BAs formation in food commodities, as these procedures could limit microbial contamination (Vinci, et al., 2002). Nevertheless, their synthesis can also be monitored by means of environmental factors (such as temperature, humidity or a_w, pH, etc.) and technological factors, consisting in the addition of technological adjuvants, such as *i.* microbial starters (i.e., *Staphylococcus carnosus* in processed meats), *ii.* food additives (i.e., essential oils, spices), and *iii.* natural food additives (i.e., salt, sugar, and vinegar). Figure 1.21. summarizes the main BAs synthesis pathway, and its influencing factors in major food

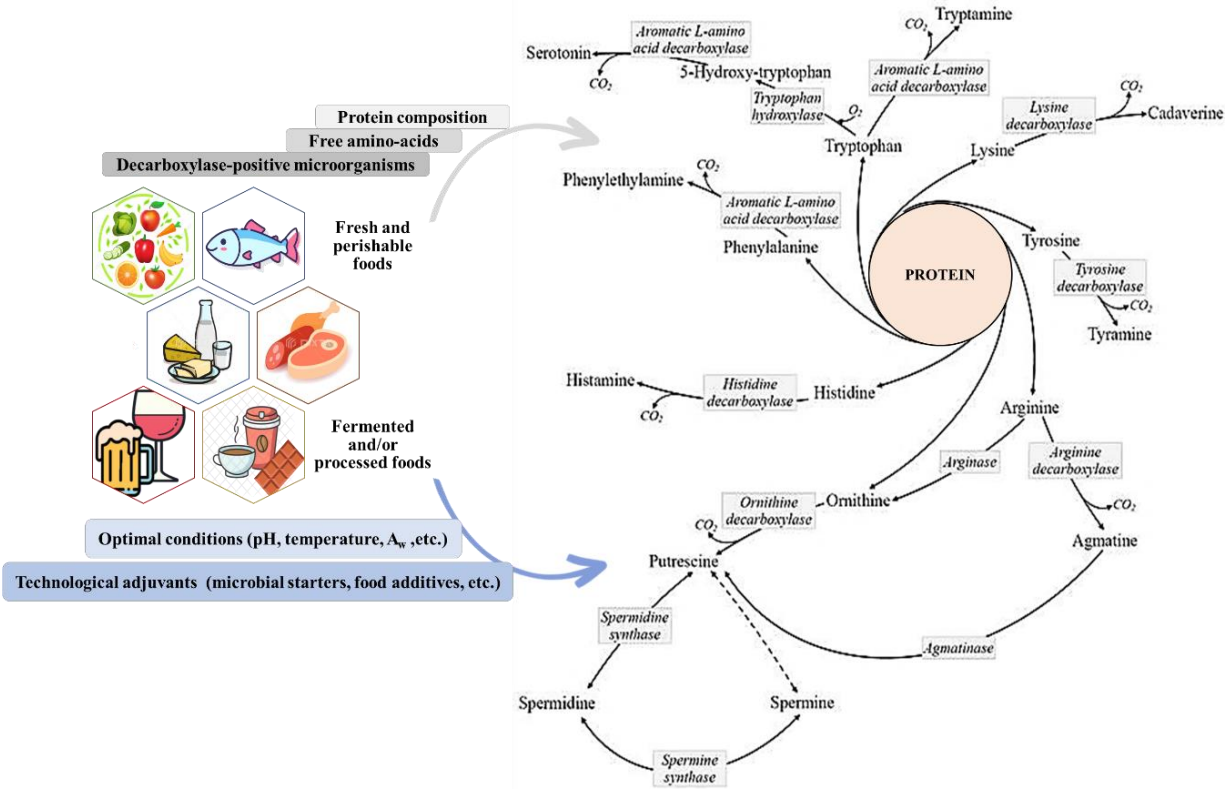


Figure 1.21. Biogenic amines synthesis pathways in foods. Source: author's elaboration adapted from (Erdag et al., 2018).

In eukaryotic cells, the biosynthesis of BAs has a key role as precursors for the synthesis of hormones, alkaloids, nucleic acids, and proteins (Kalac et al., 2005). Some biogenic amines, such as serotonin, play an important role as neurotransmitters, while the polyamines putrescine, spermine, and spermidine have an essential role in cell function, covering important physiological processes, such as the regulation of nucleic acid and protein synthesis, as well as the stabilization of cell membranes. They also act as secondary messengers involved in cell metabolism, growth, and renewal (Bardòcz, 1995). Albeit, BAs have important physiological roles, their excessive intake, through

contaminated food, could have toxic effect on human body, thus involving symptoms that are similar to those of food poisoning: nausea, gastric disturbances, sweating, heart palpitations, respiratory distress, and psychoactive effects Table 1.5. highlights the main physiological and toxicological effects of biogenic amines on human health. However, the toxic effects of BAs are dose-dependent, and the severity of the toxicity response is also influenced by individual tolerance to these compounds (Vinci et al., 2021). In physiological conditions, amines can be metabolized by three different enzymes: *i.* diamine oxidase (DAO), *ii.* monoamine oxidase (MAO), and *iii.* histamine-N-methyltransferase (HMT), which is present in the gastrointestinal tract of mammals, showing the potential of oxidizing the amino groups ($-NH_2$) of BAs. This system can degrade BAs amounts ingested through daily food consumption.

Table 1.5. Physiological and pathological effects of BAs on human health.

Source: (Vinci et al., 2021).

Biogenic Amine	Physiological Effects	Pathological Effects
Histamine	Release of adrenaline and noradrenaline Allergic processes, Stimulation of the smooth muscles of the uterus, intestine, and respiratory tract, Stimulation of sensory and motor neurons Control of gastric secretion	Allergic reaction, also known as *"Scombrotoxin Fish Poisoning"* (nausea, burning in the mouth, flushing of the face and body, abdominal cramps, diarrhea, swelling of the face and tongue)
Tyramine	Peripheral vascularization Increase in cardiac output Increased lacrimation and salivation Increased breathing Increased blood sugar levels Noradrenaline release of the sympathetic nervous system Migraine	High blood pressure, Rapid heart rate, Tremors, Seizures, Hyperthermia
Putrescine	Hypotension Bradycardia Lockjaw Extremity paralysis	Cytotoxicity, Rule in tumor growth, Enhancement of the toxicity of other amines
Cadaverine		
ß- Phenylethylamine	Noradrenaline release of the sympathetic nervous Increase in blood pressure Migraine	Migraine
Serotonin	Modulation of anger, aggression, mood and sexuality, appetite Physiological homeostasis Muscle contraction Blood pressure regulation	Altered behavior and neurochemical activities, cognitive decline, muscular inflammation, and immune activation
Tryptamine	Increase in blood pressure	Relaxations, Mild euphoria, Hallucinogens
Spermidine		Acute decrease in blood

Spermine	Hypotension, Bradycardia	pressure, Respiratory symptoms, Nephrotoxicity, carcinogenesis, tumor invasion, and metastasis, Enhancement of the toxicity of other amines

The acceptable daily intake of BA for the human body is not yet known, as the toxic effects of individual BA are related and enhanced by their co-occurrence in food products. Based on this, EFSA (European Food Safety Authority) has focused on food products with the highest risk of contamination by biogenic amines. Currently, European legislation sets maximum acceptable concentration limits for histamine in fish and fish products only (Reg. EU no. 1019/2013), whereas in other foods there are only proposed or recommended limits (Table 1.6.), and only a few European countries (Germany, France, the Netherlands, Belgium and Austria) have adopted this legislation, accordingly.

Table 1.6. National and International legislation of limits the concentration for histamine in fish and fishery products. Source: author's elaboration

Regulation	**Food products**	**Limits (mg·Kg^{-1})**	**Reference**
Regulation (EU) No 1019/2013 amending **Annex I to Regulation (EC) No 2073/2005**	Fish, except those that have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine	200–400	(European Commission, 2005), (European Commission, 2013)
	Fish from fish species associated with a high amount of histidine (*Scombridae, Clupeidae, Engraulidae, Coryphenidae, Pomatomidae*, and *Scombreresosidae* species)	100–200	
	Fish derivatives (i.e., sauce) produced by fermentation of fishery products	400	
US Food and Drug Administration **(USFDA) 2005**	Fresh or fresh-frozen fish	50–500	(US Food and Drug Administration (USFDA)., 2005)
Food Standards Australia New Zealand Act **(FSANZ) 2014**	Fish and fishery products (*Combridae, Coryphaenidae, Pomatomidae, Carangidae, Clupeiformes, Clupeidae, Engraulidae, Beloniformes, Scomberesocidae* species)	200	(Food Standards Australia New Zealand (FSANZ), 2014)
Codex Alimentarius, 2003	Fish and fishery products (*Clupeidae,Scrombridae, Scromberesocidae, Pomatomidae, and Coryphaenidae* species)	100	(Codex Alimentarius Commission, 2003)

1.6.2 Free Fatty Acids

Fatty acids (FAs) are the components that distinguish different types of lipids, from triglycerides to complex lipids. These compounds are characterized by the presence of a long carbon chain, which is referred to as an aliphatic chain, with a carboxyl group (–COOH), and a methyl group ($–CH_3$). FAs are naturally occurring compounds synthesised through the condensation of malonyl-coenzyme A groups, by the action of a multienzyme complex called *'fatty acid synthetase'*. Based on the carbon chain length, FAs can be classified into *i.* short-chain fatty acids, which have fewer than 14 carbons in the aliphatic chain, and *ii.* medium- and long-chain fatty acids, which have more than 14 carbons (Philip et al., 2015). It is worth noting that FAs of biological interest are carboxylic acids with an even number of carbon atoms (mostly between 4 and 26).

The carbon chain can be in linear, branched, or cyclic form depending on the number of double bonds therein. Depending on the number of double bonds, fatty acids are classified into: *i.* saturated fatty acids (if they have no double bonds); *ii.* monounsaturated fatty acids (if they have only one double bond); and *iii.* polyunsaturated fatty acids (if they have two or more double bonds). Table 1.7. shows the main fatty acids (saturated and unsaturated), their chemical characteristics, and related food sources (Cabras, P., and Martelli, A., 2004).

Among the polyunsaturated fatty acids, linoleic (ω-6) and α-linolenic (ω-3) acids, are of great nutritional relevance, thus being abundant in vegetables and animal oils. In particular, ω-6 linoleic acid (LA) is particularly found in vegetable oils, such as flaxseeds, soybeans (14.25–53.23 g/100g), and oil seeds, i.e., hempseed, chia, corn (5.84–33.76 g/100g) (Saini et al., 2018). While among FAs belonging to the ω-3 series, the most abundant are: *alpha-linolenic* acid (ALA), mostly found in vegetable oils (1.16–53-37 g/100g); *ii.* eicosapentaenoic acid (EPA) in fish oils, mainly cod liver, sardine and salmon-based oils (6.27−13.20 g/100g), and fishery products, such as herring, salmon, and caviar (0.86–2.74 g/100g); docosapentaenoic acid (DHA), found mainly in fish oils, and fish products ranged within 4.21−18.23 g/100 g, and 0.94–3.80 g/100 g, respectively (Saini et al., 2018).

Table 1.7. Chemical structure of fatty acids and main food sources. Source: author's elaboration adapted from (Cabras, P., and Martelli, A., 2004).

Common name	IUPAC nomenclature	Carbon chain abbreviation	Food sources
Saturated Fatty Acids			
Butyric Acid	Butanoic Acid	C 4:0	Milk fat
Caproic Acid	Esanoic Acid	C 6:0	Milk fat, coconut oil, palm kernel oil
Caprylic Acid	Octanoic Acid	C 8:0	
Capric Acid	Decanoic Acid	C 10:0	
Lauric Acid	Dodecanoic Acid	C 12:0	*Lauraceae* seeds, coconut and palm kernel oils (40-50%)
Myristic Acid	Tetradecanoic Acid	C 14:0	All vegetable and animal oils and fats, notably milk (8-12%), coconut and palm kernel (15-30%), *Myristicaceae* (70-80%)
Palmitic Acid	Esadecanoic Acid	C16:0	All vegetable and animal oils and fats, mainly lard (10%), palm (30-50%), cocoa (25%)
Stearic Acid	Ottadecanoic Acid	C 18:0	All vegetable and animal oils and fats, mainly suet (20%), lard (10%), cocoa (35%), and vegetable oils (1-5%)
Arachidic Acid	Icosanoic Acid	C 20:0	Low amounts in all animal oils and fats, peanut oil (1-2%)
Beenic Acid	Docosanoic Acid	C 22:0	
Lignoceric Acid	Tetracosanoic Acid	C 24:0	
Monounsaturated Fatty Acids (MUFAs)			
Lauroleic Acid	(Z)-Dodeca-9-Enoic Acid	C 12:1 N-3	Milk
Myristoleic Acid	(Z)-Tetradeca-9-Enoic Acid	C 14:1 N-5	
Palmitoleic Acid	(Z)-Sadeca-9-Enoic Acid	C 16:1 N-7	All vegetable and animal fats
Oleic Acid	(Z)-Octadeca-9-Enoic Acid	C 18:1 N-9	All vegetable oils and fats, olive oil (59-83%), seed oil (40-70%)
Polyunsaturated Fatty Acids (PUFAs)			
Linoleic Acid (ω-6)	(9z,12z)-Octadeca-9,12-Dienoic acid	18:2n-6	Vegetable oils, mainly sunflower oil and maize oil
α-Linolenic Acid (ω-3)	(9z,12z,15z)-Octadeca-9,12,15-Trienoic acid	18:3n-3	Fish and fish oils, vegetable oils, mainly sunflower, soybean, and rapeseed oils
γ-Linolenic Acid	(6z,9z,12z)-Octadeca-6,9,12-Trienoic acid	18:3n-6	Fish oils, *Borrago officinalis* seed oil, *Oenothera biennis* seed oil
Arachidonic Acid	(5z,8z,11z,14z)-Icosa-5,8,11,14-Tetraenoic acid	20:4n-6	Fish oils
Eicosapentaenoic Acid (EPA)	(5z,8z,11z,14z,17z)-Icosa-5,8,11,14,17-Pentaenoic acid	20:5n-3	Fish oils, and seafood
Docosapentaenoic Acid (DHA)	(7z,10z,13z,16z,19z)-Docosa-7,10,13,16,19-Pentaenoic acid	22:5n-3	

The fatty acids ω-6 and ω-3 are referred to as *essential fatty acids* (EFAs) as they cannot be produced within the human body. As a direct result of the competing roles of ω-6 and ω-3 EFAs in the modulation and synthesis of anti-inflammatory and inflammatory eicosanoids, a balanced intake of these EFAs is necessary to avoid chronic diseases, while maintaining good health. In these regards, the recommended dietary ratio of ω-6/ω-3 FAs to achieve health benefits is 1:1-2:1, thus representing 0.1% of total daily caloric consumption (Attia et al., 2022). Among all fatty acids, EFAs have an essential functioning role for the human body, notably acting to resolve inflammation and prevent infection, cancer prevention, brain development, obesity, diabetes, and cardiovascular disease (CVD) prevention. (Saini et al., 2018).

In food, FAs are often bonded to a glycerol molecule ($C_3H_8O_3$), thus forming glycerol esters, also called triglycerides or triacylglycerols. In these molecules, the aliphatic chains of three different FAs replace the hydroxyl groups of the glycerol molecule, thus releasing free fatty acids (FFAs).

The concentration of FFAs can be considered as a quality marker for the evaluation of food shelf-life since it increases the acidity of food. This phenomenon, called "*off-flavoring"*, is favoured by moisture and the combined action of light and the enzyme lipase, also leading to the off-flavor formation in food products. A further phenomenon that occurs in FAs is ketone rancidity, a process of an enzymatic nature that causes β-oxidation of FAs with the formation of the corresponding β-keto acid, which in turn undergoes decarboxylation, leading to the formation of a methyl ketone (Nurulain, et al., 2021). Another food-borne degradation process in lipids is oxidative rancidity, in this case, due to the action of light, heat, or highly oxidizing environments. Oxidative rancidity involves an early initiation phase in which the formation of radicals occurs, a propagation phase, and a final termination phase in which the radicals are locked into stable chemical forms. This process is favoured in unsaturated or polyunsaturated fatty acids as the presence of double bonds stabilizes the radical, thus favoring initiation and propagation reactions. The co-presence of antioxidant species in the food is therefore important, as they are able to intervene especially in the phase of radical initiation by slowing it down, to prevent the oxidative rancidity process (Zhang et al., 2019).

1.6.3 Phenolic compounds

Polyphenols constitute one of the most representative and widespread groups of phytochemicals, with more than 8,000 phenolic structures currently identified in plants and plant-based foods (Tsao, 2010). This heterogeneous group of high molecular weight-compounds are chemically characterized by an aromatic ring with at least one hydroxyl group and their structure can vary from simple molecules, i.e., phenolic acids, to highly polymerized compounds, such as tannins.

Polyphenols can be classified according to their origin, their biological functions as well as their chemical structure (Tsao, 2010). In particular, the latter is the most widely adopted classification in literature, according to which polyphenols can be classified into two major groups: flavonoids and non-flavonoids (Belščak-Cvitanović et al., 2018). Figure 1.22. shows polyphenols classification according to their chemical structure (Câmara et al., 2021).

Flavonoids represent the major phenolic class, which is very abundant in plant-based foods, (i.e., fruits, vegetables, chocolate, tea, wine, coffee, etc.). In general, these compounds have a common basic structure of diphenylpropanes (C6-C3-C6), which consists of three benzene rings: rings A and B connected by a 3-carbon bridge, to form an oxygenated heterocycle (ring C) (Câmara et al., 2021). Based on the heterogeneity in the heterocyclic ring, flavonoids can be divided into six major subclasses including flavonols, flavones, isoflavones, flavanols, anthocyanidins, and flavanones (Durazzo et al., 2019). Among flavanols, it is worth mentioning the monomer form (catechins), which are predominant in plant-based foods. They are most commonly abundant in fermented beverages and foods, particularly green tea (rich in epigallocatechin gallate) (EGCG), or cocoa (rich in epicatechin), as well as in fruits, (i.e., berries, apples), and some nuts. Catechins have received attention for their potential health benefits due to their antioxidant and anti-inflammatory properties, as well as neuroprotective effects and cardiovascular disease prevention (Durazzo et al., 2019).

Anthocyanidins are another important group of flavonoids that are responsible for the red, and blue colors of flowers, fruits, vegetables, and certain varieties of grains, such as black rice. They are also abundant in red wines, where are most commonly found as cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin in the glycoside forms (Boris et al., 2022).

It has been a long time considering that polyphenol components in wine could largely contribute to the health-promoting effects of human organisms, thus exerting prevention

of oxidative reactions, inflammatory processes, degenerative pathophysiological states in adults, and cardiovascular diseases. In particular, the health effects of grapes and related products are mostly related to the grape richness in bioactive molecules, including flavonoid compounds, among which anthocyanidins are one of the major contributors.

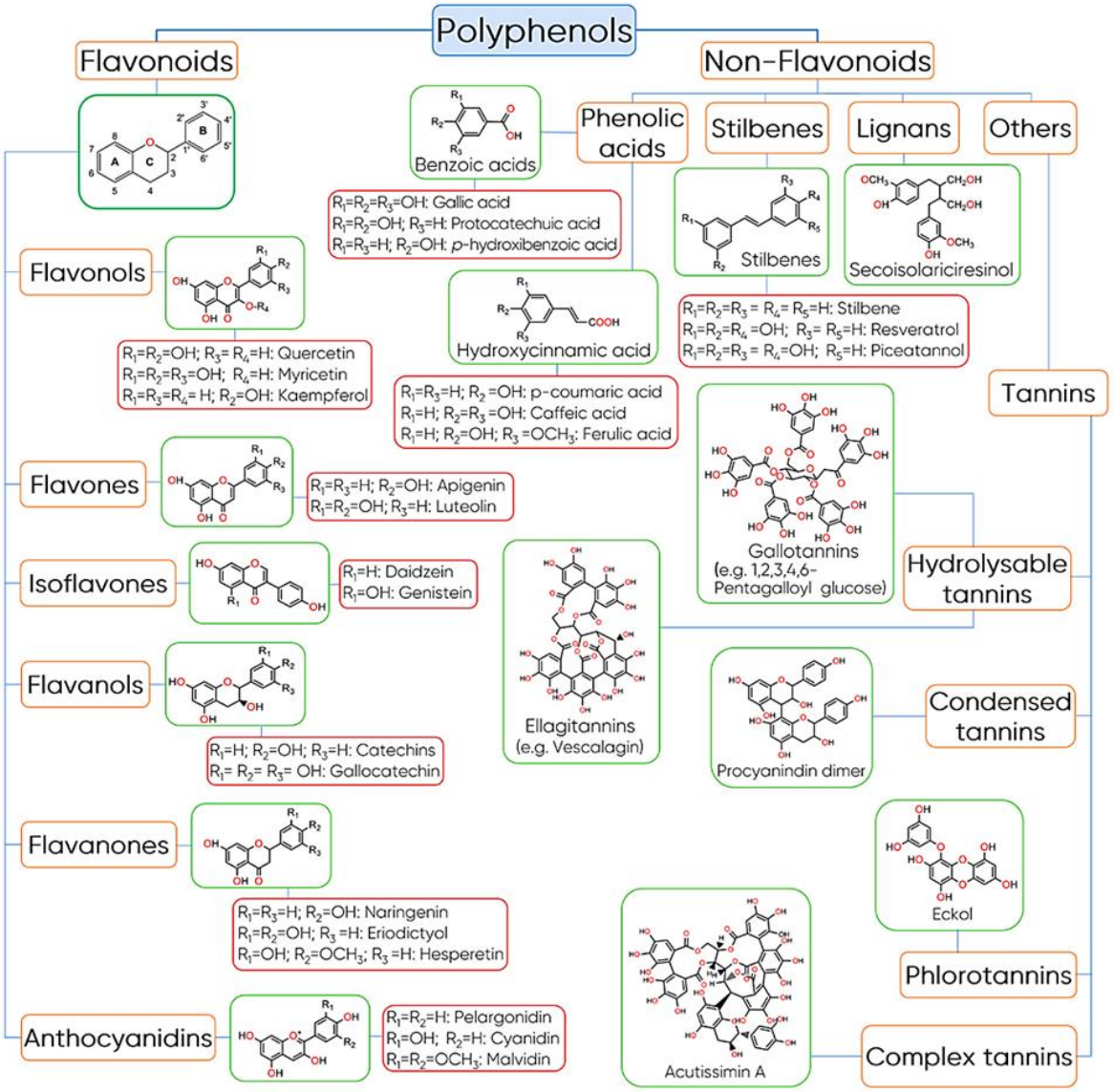


Figure 1.22. Polyphenols classification and related chemical structure. Source: (Câmara et al., 2021).

Regarding non-flavonoid compounds, this group includes phenolic acids, stilbenes, lignans, and tannins (Fig. 1.22.). The latter represents a large group of complex phenolic biomolecules, naturally synthesized by a wide range of plants. Based on their chemical structure, tannins are divided into four main categories: hydrolyzable tannins, which are further subdivided into gallotannins and ellagitannins, condensed tannins, phlorotannins,

and complex tannins. In general, they are responsible for the astringent and bitter taste of foods such as wine, chocolate, coffee, and tea, also exerting beneficial effects on human health, in terms of cancer and cardiovascular disease (CVD) prevention, antioxidant and anti-inflammatory properties (Boris et al., 2022).

Nevertheless, considering the health-damaging properties of tannins, there is a trade-off between limiting tannin consumption in individuals at risk of iron-deficiency anemia (IDA), and the potential health benefits of tannin-rich diets. Tannins can inhibit the absorption of non-heme iron, so there has been an increased effort in food systems and agriculture practices to reduce tannin content in grains, legumes, and vegetable foods (Webb et al., 2011), (Delimont et al., 2017). However, studies suggest that the correlation between tannin consumption and IDA is related to both the frequency of consumption and the type of tannins consumed. Evidence from the literature shows that long-term consumption may have a different impact on iron status than that predicted by studies on single meals. Furthermore, iron bioavailability studies using condensed tannins, which are more commonly consumed, may better predict iron bioavailability during meals (Delimont et al., 2017). Polyphenols are not only valuable compounds for human health but also for their technological value. Food production processes, in fact, greatly influence the composition of polyphenols in the final product. Indeed, these compounds generally degrade upon light exposure, temperature, or due to the co-presence of enzymes in the food. In addition, polyphenols can be oxidized, with the formation of quinones, in which hydroxyl groups (-OH) are transformed into carbonyl groups (-C=O), these transformations can lead to sensory changes in food composition and properties. In these regards, polyphenols can be considered as food quality markers, that can provide information on the chemical changes that can occur in food along the production chain.

1.7 Analytical methods for the analysis of bioactive compounds in food matrices

In the ever-evolving landscape of agri-food production and consumption, the assurance of product quality and safety remains a paramount concern for both consumers and producers. In this context, the analytical determination of bioactive compounds is particularly important for both quality and safety assessment of fresh and processed food products. In general, the process of determining target analytes consists of a series of interconnected steps: *i.* sampling, *ii.* sample preparation, extraction, and purification of the extract (which in some cases may be conducted simultaneously with the extraction), and possible concentration or dilution of the extract, *iii.* analytical determination, and *iv.*

results interpretation, all converging into the analytical procedure, as indicated in Figure 1.23. It is worth noting that the choice of analytical procedure depends on the specific target bioactive compound, its chemical properties, and the complexity of the food matrix. (Câmara et al., 2021).

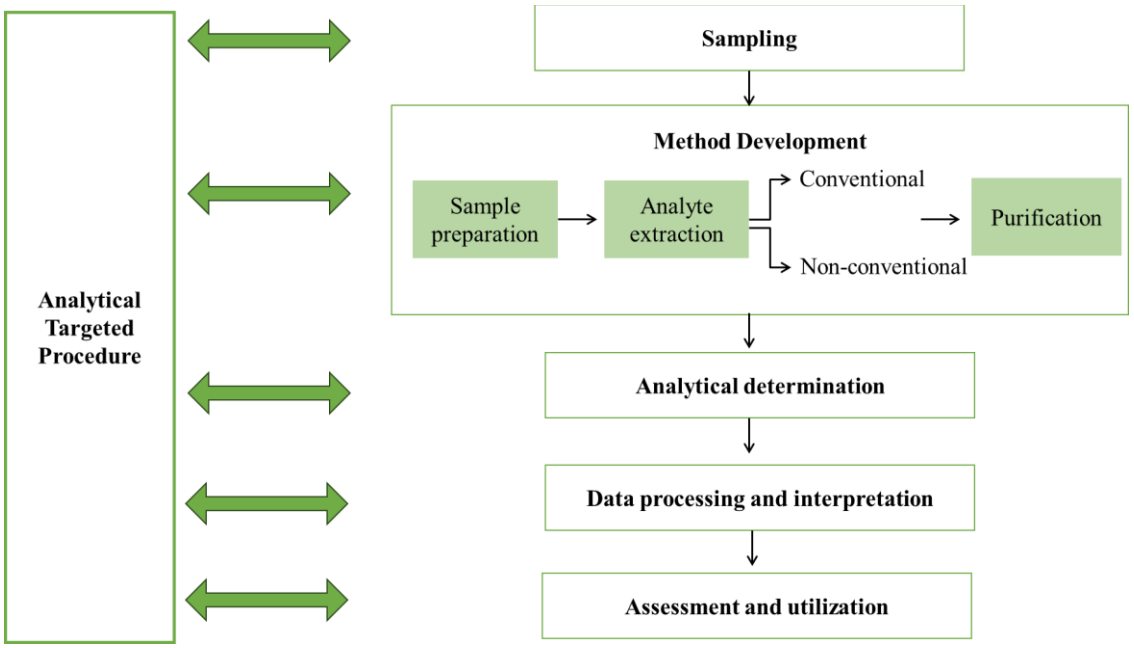


Figure 1.23. Main steps of the analytical procedure. Source: author's elaboration

Hence, to make the field of research selective, the current state of the art concerning the analytical determination of biogenic amines, polyphenols, and fatty acids in food matrices is presented in the following sections, as well as target analytes, that the research project focused on.

1.7.1 Analytical determination of Biogenic Amines

The determination of BAs is mainly carried out as a result of their potential toxicity for human health, as well as these compounds can be used as food quality indicators (Biogenic Amines Quality Index; BAQI and Biogenic Amines Index; BAI) (Cheng et al., 2016) (Ruiz-Capillas et al., 2019). Indeed, the major applications of BAs analysis include *i.* quality control of raw materials, intermediate and finished products (product quality marker); *ii.* monitoring of fermentation processes, control of food processing (process quality marker); and, *iii.* storage and shelf-life control (food spoilage marker) (Vinci et al., 2002);(Ruiz-Capillas et al., 2019).

The analytical determination of biogenic amines in food matrices generally involves

three steps, *i.* sample preparation and purification; *ii.* derivatization procedure, and *iii.* instrumental analysis for quantitative determination. In detail:

1. In the first phase of the analytical method - the so-called *'clean-up'* phase - extraction is performed, generally in an acidic solution, which is necessary to extract the compounds of interest from the rest of the matrix and to minimize the amount of co-extracted interfering substances. In general, extraction represents a critical step in the process, as it negatively affects the quantitative recovery of BAs (Alberto et al., 2002). BAs' extraction from a *'solid'* food matrix is generally solid-liquid extraction (SLE) and is preliminary to the homogenization step in the presence of an acid solution, which may consist of perchloric acid ($HClO_4$), trichloroacetic acid (TCA) and hydrochloric acid (HCl), at different molar ratios. Moreover, methanol and other organic solvents can also be used for extraction as a substitute for the acid solution (Alberto et al., 2022).

   Following, the purification step, which is performed to reduce the amount of co-extracted interferents, according to two processes: liquid-liquid extraction (LLE) or solid-liquid phase extraction (SPE). In the case of LLE, organic solvent mixtures are added to remove hydrophilic interferents; the disadvantages of this method are the use of organic solvent, the possible formation of emulsions, and the difficult automation of the process (Gianotti et al., 2008)., SPE is performed using suitable cartridges characterized by specific adsorbent solids, that allow the extract to be concentrated beside of being purified, simultaneously. In these regards, the advantages that favour this method over LLE, consist of reduced sample handling and a considerable reduction in organic solvent use (Pena-Gallego et al., 2009). A derivatization process is required whenever spectrophotometric detectors are used since not all BAs have the necessary chromophore group to enable their detection. The derivatization reaction, occurring between the amine groups ($-NH_2$) of the BAs and the marker reagents, can be performed before (pre-column methods) or after (post-column methods) chromatographic analysis. Although pre-column methods are more widely used, they are more susceptible to the various matrix components, than post-column methods (Önal, A., 2007). The most commonly used derivatizing agent is dansyl-chloride (DNS-Cl), dabsyl-chloride (DABS-Cl), benzoyl-chloride, and ortho-phthaladehyde (OPA). Among these derivatives, DNS-Cl is employed most frequently as, unlike the others, it is a non-specific compound reacting with all

BAs types (primary, secondary, tertiary) to form stable derivatives, as well as interferent molecules (i.e., phenols, aliphatic alcohols, and sugars) (Önal, 2007). Although the dansylation reaction offers stable products, it requires long reaction times and also requires heating of the samples. An example of a reaction between amines and DNS-Cl is shown in Figure 1.24.

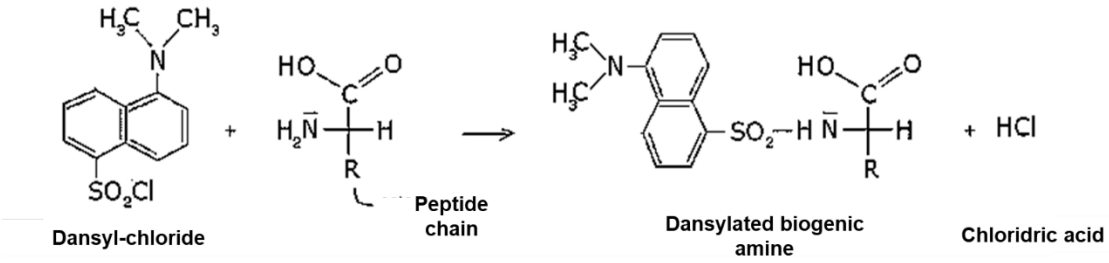


Figure 1.24. Derivatization reaction of biogenic amines

2. For the quantitative determination of BAs in food matrices, the most commonly used methods are chromatographic separation techniques, including thin layer chromatography (TLC), gas chromatography (GC), and high or ultra-high performance liquid chromatography (HPLC and UHPLC) (Önal et al., 2013). Each separation process is followed by the detection of analytes using systems chosen 'ad hoc' according to the nature of the sample and the type of analysis to be performed. Different types of detectors can be employed in LC, the most common being the UV-Vis (ultraviolet-visible) and FLD (spectrofluorimetric) detectors. In addition, other methods used for BAs detection are mass spectrometry (MS), thermal conductivity detector (TCD), and conductivity detector. The most widely used technique for the qualitative-quantitative determination of biogenic amines in food is High-Performance Liquid Chromatography (HPLC), which involves the use of a reversed-phase C-18 column (Önal et al., 2013). Furthermore, this method has been designated by Regulation (EU) No 1019 of 23 October 2013 as a standard method for the analysis of BAs in fish and fermenting and processed fish products (Duflos et al., 2019). The second analytical method used is gas chromatography (GC), generally coupled with mass spectrometry (MS). Through these hyphenated techniques, BAs are first extracted in a methanol solution, then injected into a column and subsequently detected without the need for derivatization (Jain et al., 2018). However, this technique is adopted to perform the analysis of fairly volatile and

thermostable amines (Önal, A., 2007). Recently, excellent results in the determination of BAs in food matrices have been obtained using UHPLC (Ultra High-Performance Liquid Chromatography), which operates according to the same principles as HPLC, but with higher pressures that considerably shorten analysis times. The only disadvantage lies in the excessive instrumentation costs (Yu-jia et al., 2019).

1.7.2 Analytical determination of Free Fatty Acids

The determination of free fatty acids (FFAs) content is an important analysis to assess both the quality of raw materials, as well as their degradation during storage and throughout the shelf-life of different food matrices. In particular, FFAs content can be indexed for the saturation degree of the dietary oil sources and fatty acids absorption (Rodriguez-Sanchez et al., 2021). According to the findings of Rodriguez-Sanchez et al., (2021), including fat by-products rich in FFAs ($\leq$35%) (i.e., oils) does not have a negative impact on the FAs absorption in the gastro-intestinal tract. In addition, the oxidation processes (i.e., oxidative rancidity, *off-flavouring*, etc.) caused by light, heat, or highly oxidizing agents**,** may influence quality and sensory attributes in food products.

The analytical determination of free fatty acids involves extraction from the food matrix by liquid-liquid extraction (LLE) or solid-phase extraction (SLE) techniques. Currently, different types of organic solvents are used for extraction: chloroform ($CHCl_3$), n-hexane (C_6H_{14}), n-heptane (C_7H_{16}), petroleum ether (C_6H_{14}), or chloroform/methanol (MeOH) solution. One of the most common and widely used techniques is the Bligh and Dyer (B&D). The B&D method has been considered the standard method for the determination of total lipids in biological tissues such as microorganisms (Manirakiza et al., 2002). In this technique, methanol, chloroform, and water are added to the sample in a two-step extraction and, after phase separation, lipids are quantified in the chloroform phase; while phenolic compounds in methanol fraction.

Nowadays, alternative techniques involving reduced use of organic solvents, such as microwave or supercritical fluid extraction, are preferred for the extraction of FFAs, as they are more favourable both in terms of reliability, extraction time, and environmental impact (Medina et al., 2015). Frequently, to determine the total concentration of FFAs in foodstuffs before the extraction procedure, lipase enzymes are used to break the ester bond between the fatty acid and glycerol, thus releasing all the FAs present in the sample examined (Kwon et al., 1986).

For the analytical determination of FFAs in food matrices, different methods are currently available, including volumetric titration, high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and analysis by gas chromatography (GC) (Bernárdez et al., 2005) (Klampfl et al., 2000) (Olmo-García et al., 2018). To date, chromatographic methods are the most widely used, in particular gas chromatographic methods. Two different types of detectors are mainly coupled to GC techniques: the mass spectrometry (MS) detector and the flame ionization detector (FID). The latter detector is one of the most widely and commonly used detectors for FFAs determination, because of its sensitivity and resolution. However, other interferent compounds apart from FFA can also detected by the FID detector.

Meanwhile, mass spectrometry (MS) detectors tend to thermally degrade samples before they can be detected (Olmo-García et al., 2018). However, although determination by GC-FID enables better accuracy of results, samples with this detection procedure must be esterified, as gas chromatography allows the detection of volatile analytes.

Another widely adopted method for determining FFAs is high-performance liquid chromatography (HPLC), since it has a higher selectivity than other methods, and can detect an increased number of analytes. However, this method of analysis is highly expensive and involves a lengthy procedure in sample preparation as, to detect free fatty acids, these need to be derivatized, with different types of derivatizing agents (methyl iodide, 2- hydrazinoquinoline, 2-(4-nitro) phenyl-1-H-phenanthrene imidazole, etc.) (Nurulain et al., 2021); (Ciano et al., 2023).

1.7.3 Analytical determination of Polyphenols

The determination of polyphenols in food products is relevant both for the health-promoting properties of these compounds (i.e., anti-inflammatory, antioxidant, anti-bacterial, etc.) as well as for food quality assessment. The production and processing of foodstuffs greatly affect the composition of polyphenols (Giacometti et al., 2015). These BCs indeed tend to be easily degraded by extrinsic factors, such as exposure to light and heat, humidity, temperature, and the co-presence of micro-organisms in food (i.e., yeasts, lactic acid bacteria, molds, etc.) (do Carmo Brito et al., 2017), thus undergoing complex biochemical reactions particularly valuable for the organoleptic, and sensory characteristics of food.

The analytical procedures for the determination of polyphenols in food matrices are performed following extraction and purification of the analyte of interest. Polyphenol

extraction can be carried out using polar solvents, such as water, hot water, methanol, methanol/formic acid, methanol/water/acetic acid, or formic acid solutions in different molar ratios. This procedure can be performed by liquid-liquid extraction (LLE) or by counter-current chromatography (CCC). A further extraction method applied for the determination of polyphenols is Solid Phase Extraction (SPE), which is mainly carried out on absorbent C-18 cartridges (López-Fernández et al., 2020). Additionally, state-of-the-art modern techniques highlight ultrasonic extraction, microwave-assisted extraction, extraction using supercritical CO_2, extraction using pressurized liquids, etc. (Fanali et al., 2018). The extraction conditions (temperature, extraction time, solvent-solute ratio, solvent molar ratios, concentrations, etc.) should be optimized for each food matrix considered. In case a sample has low amounts of polyphenols, it can be concentrated through ultrafiltration techniques. The application of spectrophotometric assays based on the absorbance variation of polyphenols after a reaction with oxidizing reagents, such as Folin-Ciocâlteu reagent, and ABTS and DPPH assays, are the most widely used methods for the cumulative determination of polyphenols in food matrices and antioxidant capacity, respectively (Vinci et al., 2022). For the qualitative and quantitative determination of individual polyphenol species, the most commonly used techniques are ultra and/or high-performance liquid chromatography (UHPLC, HPLC) or, in some cases, capillary electrophoresis, which, as mentioned above, are techniques based on the separation of molecules based on their interaction with a stationary phase or an electric field. Also, gas chromatography (GC) coupled to mass spectrometry (MS), and supercritical fluid chromatography (SFC) has been widely employed (Qureshi et al., 2019). These qualitative and quantitative analyses provide more specific and detailed information than spectrophotometric methods.

Before chromatographic separation, pre-treatment and purification steps are required to remove possible interferent molecules. For this purpose, specific adsorbent resins or semi-preparative columns can be used to be applied before the HPLC column. The most popular cleaning technique, however, involves the use of SPE (Solid-Phase Extraction) cartridges, which, through the use of fiber and small solvent volumes, can retain interferents and purify and concentrate the analyte in the sample.

To ensure high chromatographic resolution, several experimental parameters must be optimized, such as gradient elution, mobile phase composition, stationary phase, and column temperature. Elution is generally performed with a binary solvent system, using methanol (MeOH) or acetonitrile (ACN) (pure or acidified) as an organic solvent, and

acidified water (with formic or acetic acids at percentages between 0.05–5%, *v/v*) as polar solvent. Acidification is necessary to minimize peak tailing, which suppresses the ionization of phenolic hydroxyl groups (López-Fernández et al., 2020); (Girelli et al., 2015). The detection of polyphenols is carried out by combining LC with different types of detectors, including the photodiode array detector (PDA), the fluorescence detector (FD), and MS. Among these, the PDA is widely used due to its robustness and low price, for the identification of phenolic compounds (detected at λ= 240–285 nm), as well as flavones and flavonols (λ= 350–365 nm), and anthocyanins (λ= 460–560 nm) (Girelli et al., 2015). Despite the higher selectivity and sensitivity (and therefore lower detection limits) of those obtained with PDA, FD is less used in the analysis of polyphenols since it requires the existence of fluorescence, a phenomenon that only occurs in certain types of molecules, such as procyanidins, and free catechins and epicatechins. The best performance is usually obtained using MS detectors with electrospray ionization in positive or negative ion mode.

CHAPTER II

KNOWLEDGE OF STATE-OF-THE-ART AND CHALLENGES FOR THE AGRI-FOOD SECTOR IN ADDRESSING SUSTAINABILITY AND FOOD QUALITY ISSUES

In the coming decades, feeding the expanded global population nutritiously and sustainably is a forthcoming challenge requiring substantial improvements to the global food system worldwide. In particular, it mainly concerns climate-resilient food production with the same or fewer resources and wastes less, thus guaranteeing food availability, access, use, and quality. After an overview of the current situation of the global and national situation about the several pathways toward sustainable food production and related environmental impacts, food quality and green technologies for the analysis of BCs in food products and by-products could represent a key issue to prevent further environmental implications. In this regard, this section will concern the state of the art and main challenges for the agri-food sector in answering sustainability and food quality issues, giving an insight into sustainable technologies for the analysis of bioactive compounds in agri-food products focusing on both analytical parameters, in terms of extraction yield, purification, and sustainability concerns.

2.1 Pathways towards sustainable food production

In response to environmental concerns and the need for long-term food security, it is important to promote actions for sustainable food production, involving the interconnection of multiple aspects related to human health, the ecosystem, and the socio-economic context (Fig. 2.1.). Climate change is expected to adversely affect diets, nutrition, and health in the present and future through impacts on the quantity, quality (nutrient content), diversity, safety and accessibility of food produced. Assessing the issue of nutrition and health for the sustainability of food production, the actions mainly aim at shifts towards sustainable healthy diets that protect both human and planet health from ongoing challenges. The focus of policies to shift consumption towards healthy and

sustainable diets should include: *i)* increasing availability, access, and affordability of nutritious foods; *ii)* discouraging excessive consumption of meat-based products (particularly red and processed meats); and *iii)* encouraging demand for fruits, vegetables, as well as alternative protein sources (i.e., legumes, nuts, etc.) as nutritious and healthy foods, for both human health and environment. For this purpose, the EAT-Lancet Commission has recommended a 'benchmark healthy diet' that includes an abundance of vegetable food, diverse plant sources of protein and other essential nutrients, low consumption of animal-based foods, and limited amounts of refined grains and ultra-processed foods (Willett et al., 2019). Adopting this sustainable dietary choice as a reference would help to prevent an estimated 11 million premature deaths per year among adults and, when combined with sustainable production practices and reductions in food loss and waste, keep food production within safe planetary limits. Concerning the nutritional adequacy assessment on two examples of dietary plans in Italy (one from the EAT-IT dietary pattern and one based on the Italian Dietary Guidelines - IDG) proposed by Tucci et al., (2021) highlighted some potential issues related to the frequency of consumption of some foods/food classes and/or the intake of specific nutrients (Tucci et al., 2021), in particular, in the amount of fruits and vegetables provided, chicken meat, fish, eggs, and dairy products, which was on average 35% lower in the EAT-IT compared to the IDG dietary plans. The study highlighted lowest environmental impact was obtained with a partial substitution of food with a high ecological footprint, thus confirming the previously existing literature results (Collins et al., 2007), and also by re-enforcing the development of specific food-based dietary guidelines and policies as a support for consumers to tailor their dietary habits to achieve a more sustainable dietary pattern promoting the use of alternative sources, that is, simultaneously, respectful of local culture and tradition. In these regards, the possibility of improving the adoption of eating habits in consumers in line with these guidelines could also be facilitated by implementing new products that can include such foods, and target different consumer groups, such as the younger consumers who are very often those that are less adherent to healthy dietary patterns. A key factor is certainly consumer information, education, awareness-raising, and orientation, which should be included in all comprehensive strategies aimed at shifting consumer demand toward sustainable food consumption.

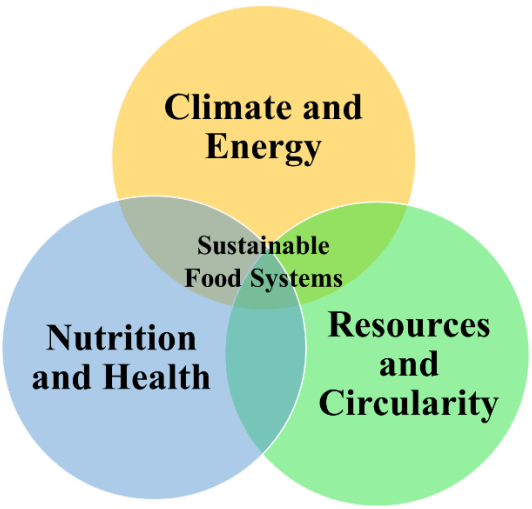


Figure 2.1. Interconnection of key-issues for sustainable food systems. Source: author's elaboration

As well as food labeling and certifications (i.e., organic, Fair Trade, and Rainforest Alliance) can also support consumers in their efforts to make healthy and sustainable food choices (Poore et al., 2018).

Considering climate and energy concerns, the sustainability of agri-food systems is implemented by promoting an enabling environment for accelerated clean energy development, and climate resilience. Globally, the energy sector accounts for almost three-quarters of total GHGs, thus being responsible for the majority of climate-altering impacts on rural livelihoods, including growing water, energy, food insecurity, and environmental depletion. According to a recent report from the Intergovernmental Panel on Climate Change (IPCC), annual investments of USD 2.4 trillion in energy production systems are needed to limit global warming to 1.5 °C (IPCC, 2023). In this context, the prompt of clean energy innovations adapted to rural areas, including agriculture and small businesses, is increasingly spreading. One major innovation is small-scale solar technologies that can pump water both for domestic use and for irrigation, storage and, transportation to support agri-food production. These advances in the cold chain can improve productivity and food safety and extend the shelf-life of perishable and high-value foods such as dairy products, eggs, and green leafy vegetables, through monitoring storage temperatures. In addition, decentralized or distributed renewable energy (DRE) systems could represent a clean energy solution for rural areas. In particular, these systems can provide locally generated electricity for farm production and drinking water systems, thus being also integrated with inputs from multiple electricity sources, (i.e., solar photovoltaic panels, micro-hydropower systems, biomass energy, and diesel

generators)(Vezzoli et al., 2018). These local-level investments would support the de-carbonization of fossil fuel polluting uses, by increasing the production of cleaner energy and improving energy efficiency. In these regards, it is worth noting the importance of considering potential synergies or trade-offs of local economies with either natural resources (i.e., water and land use) or other sectors and actors, such as food production, to avoid unnecessary costs and environmental damage. For example, the installation of large fields of solar panels can compete with agricultural landscapes or natural habitats, thus affecting food security, livelihoods, and biodiversity. Therefore, appropriate design of policies, institutions, and governance systems at all scales for clean energy production can contribute to land-related adaptation and mitigation while facilitating the pursuit of climate-adaptive development pathways and local economies, thus ensuring food security, and expanding rural employment. This represents one of the main challenges proposed by the European Commission Decision C (2023) 2178 of March 2023 in the Horizon Europe Work Program 2023-2024 on Climate, Energy, and Mobility, within the framework of cross-sectoral solutions for the climate transition (EC, 2023).

Another relevant factor in developing sustainable agri-food systems could also consist in enhancing productivity and sustainability among food value chains, for the circularity of resources. Climate change can be considered as a multiplier threat, that is expected to reshape value chains (from small farmers to urban consumers) through increased potential economic changes, and the likelihood of shocks that will result in the risk of conflicts and food insecurity (Burke et al., 2018).

Focusing on agricultural production, gradual changes in precipitation patterns, temperatures, and humidity levels will affect the whole food production chain, in particular increasing post-harvest losses. Recent studies in literature suggest that post-harvest losses already average around 14% of the total potential harvest, but vary substantially between crop types and regions (FAO, 2019); (Delgado et al., 2021). Albeit most of these estimates refer to non-perishable crops, among perishable crops (i.e., grains, fruits, and vegetables) the losses may be higher. The effects of climate change on food spoilage could make, in fact, perishables and their associated micronutrients even more scarce thus leading to an overall nutritional quality loss. In addition to the increasing scarcity of these foods, food-borne pathogens (i.e., *Salmonella spp*.) in animal products, are likely to become more prevalent (Delgado et al., 2021).

In this context, agrifood value chains must adapt to climate change by reorganizing both upstream and downstream value chains to reduce related GHGs. Focusing on

preventing food losses and waste (FLW), possible solutions mostly rely on avoiding food spoilage in the value chain midstream of perishable and non-perishable products, by monitoring various stages along the food supply chain. According to the findings of Moraes et al., (2021), the practices and methods to prevent or minimize FLW, particularly concerns:

1. Storage: adopting strategies to *i)* improve manufacturing processes to reintegrate products within the production line; *ii)* stock monitoring and rotation policies, and *iii*) time–temperature indicator.
2. Demand control: related to food consumer practices as *i)* storage in appropriate packaging; *ii)* cooking the right amount, and *iii)* monitoring surplus food.
3. Logistics: relies on adopting *i)* intelligent food logistics*; ii)* food waste tracking and analytics, and *iii)* reducing food distance transport.
4. Selling: which considers the possibility of *i)* minimizing portion size and service; *ii)* adopting smaller packages for smaller portion sizes; iii) promoting marketing of end-of-day sales; as well as *iv)* dynamic shelf-life (Moraes et al., 2021).

In addition, implementing actions and programs in agricultural research, education, policies, donations, reuse, and support activities, were identified as highly effective pathways to both reducing FLW and addressing climate change impacts on food systems.

Following the circular economy framework proposed by Teigiserova et al., (2019) for the FLW in the food supply chain (FSC) (Figure 2.2.), closing the loop for food waste and surplus involves all levels of the FSC (Teigiserova et al., 2019). In particular, by distinguishing between inedible and edible fractions for End-of-Life (EoL) options, it is shown that edible streams, when entering the recycling model (at all stages of production), could represent a potential resource to be reused throughout production. The pre-condition for edible streams to enter this framework is that prevention of becoming food surplus failed. From this perspective, the reuse of edible surplus food is proposed mainly in the consumption and serving stage (i.e., food banks), in the retail stage, and also in the consumption and re-distribution stage (redistribution of catering leftovers). Whereas, inedible food waste (i.e., agricultural production and subsequent activities), is expected to be reused for animal feed, thus representing an opportunity for nutrient and energy recovery.

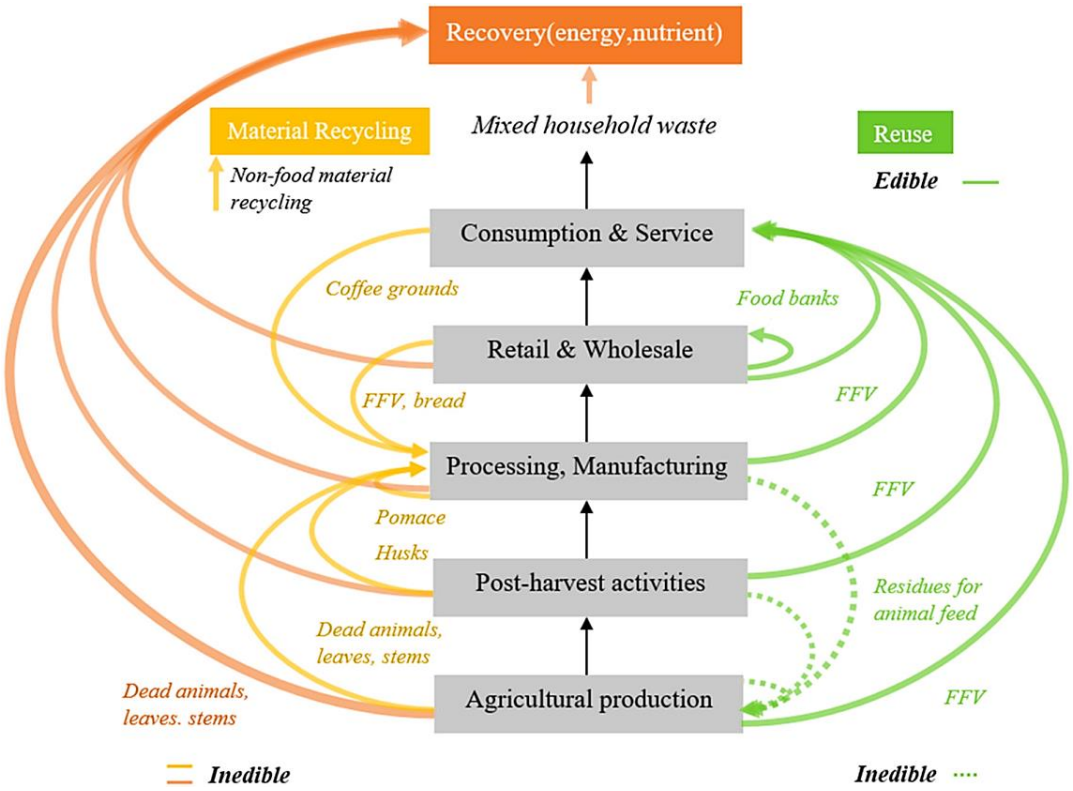


Figure 2.2. The circular economy framework of Food Losses and Waste in the food supply chain (FSC), proposed by (Teigiserova et al., 2019). FFV: fresh fruits and vegetables. Dotted lines: inedible for humans but edible by animals. Only recycling within the food supply chain is explicitly shown, but recycling may also occur for non-food applications.

All other non-edible inputs (i.e., husks, pomace, etc.) are generated and recirculated through all stages of the FSs, where recirculation options that involve material recycling (yellow streams) take precedence over those that go directly to nutrient and energy recovery (orange streams). For example, tomato processing waste can be used as a food additive to extend the shelf life of foodstuffs, through the extraction of bioactive compounds (i.e., carotenoids). In this framework, linear economy models reconfigure from a circular economy perspective with increasingly innovative solutions in the valorization of food by-products; this would allow re-valuating agrifood by-products as "*second raw materials*" for new markets, thus limiting overproduction while extending food shelf-life. In these regards, it would be of the greatest importance the possibility of extracting bioactive compounds that are potentially valuable for other production chains, to valorize food products and their by-products through the circularity of production.

2.2 Sustainable technologies for the recovery of bioactive compounds from agri-food products and by-products

Nowadays, increased consumer awareness regarding the potential health benefits of their eating habits has led to an increased demand for foods containing bioactive compounds (BCs). In particular, polyphenols and antioxidant compounds gained great interest due to their potential positive effects on human health, such as anti-cancer, antioxidant, and anti-inflammatory activity (Durazzo et al., 2019). Moreover, the growing sustainability and environmental consciousness have also promoted the need to replace conventional solvents with eco-friendly, harmless, and non-toxic extraction BCs in food. Therefore, food research focuses both on identifying new and sustainable sources of BCs (including food processing by-products), while optimizing extraction and purification methods that enable their sustainable recovery from food matrix, leading to their possible reuse for the development of innovative health-promoting functional foods (Manuela et al., 2020).

The first phase in the analysis and recovery of BCs in food is the extraction of target analytes. In this phase, the use of conventional organic solvents (e.g., methanol, ethyl acetate, n-hexane, etc.) is widespread as an efficient extraction solvent for BCs; however, its significant impact on the environment, operator safety, and high toxicity are not neglected.

The concept of Green Chemistry (GCy) developed in the ever-evolving landscape of sustainability issues, as well as a natural evolution of pollution prevention initiatives. In the mid-20th century, following several pollution-related disasters, many governments began to regulate the generation and disposal of waste and industrial emissions. In the 1970s, the United States established the Environmental Protection Agency (EPA) to protect human health and the environment by setting and enforcing environmental standards. In this context, GCy moves forward, creating a new reality for chemistry and engineering by requiring chemists and engineers to design chemicals, chemical processes, and commercial products to avoid, at a minimum, the creation of toxic substances and waste. Developed, for the first time, by Paul Anastas and John Warner in 1998, the 12 principles are mainly based on the minimization or non-use of toxic solvents in the chemical processes and analyses, as well as on the non-generation of residues resulting from these processes (Anastas & Warner, 1998). These main goals were further implemented by Galuszka et al., in 2013, to better incorporate GCy into analytical procedures, in the so-called Green Analytical Chemistry (Gałuszka et al., 2013).

Prompted by these concepts, conventional methods have been joined or replaced by new and emerging extraction techniques and methodologies (i.e., supercritical fluids, Ionic Liquids (ILs), Deep Eutectic Solvents, supramolecular liquids, etc.) focusing on both analytical parameters, in terms of extraction yield, purification as well as sustainability concerns.

Most of the prominent emerging extraction techniques include microextraction techniques (μETs), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE), as well as supercritical (SFE) and subcritical fluid extraction (SbCE), electrotechnologies-based extraction such as pulsed electric fields (PEF) and high-voltage electric discharge (HVED), and nano sorbent-based extraction techniques (Câmara et al., 2021). These sorbents and solvent-based microextraction techniques have several advantages, such as simplicity, versatility, and high extraction efficiency, as well as environmental compatibility. In addition, they are applicable to a wide range of sectors, including pharmaceuticals, clinical, nutraceuticals, agriculture, industrial, etc. (Pereira et al., 2019). Polyphenols, in particular, represent the most target analytes, being extracted with microextraction techniques, besides other molecules of nutritional interest, such as vitamins, tocopherols, steroids, fatty acids, etc. (Câmara et al., 2021).

Considering the target analytes, it is worth emphasizing that the concentration, diversity, and bioactivity of the extracted BCs are related to the extraction technique, while the choice of technique is determined by food matrix composition, as well as target analytes, procedure cost, and operation efficiency. In some cases, as the extraction is neither selective nor specific, additional steps are required to remove potential compounds, including pigments, waxes, glycosides, fats, and sugars, which may affect instrumental analysis. For this purpose, the aforementioned SPE and dispersive SPE (d-SPE) are the most commonly used clean-up procedures (Pena-Gallego et al., 2009).

In recent years, ultrasound-assisted extraction (UAE) has been one of the most widely used sustainable extraction techniques. UAE involves the use of US radiation in various devices, such as sono-reactors, probes, water baths, etc. Ultrasound applications, characterized by sound power (W), sound intensity (W/m^2), or sound energy density (W/m^3), may be subdivided based on their intensity/frequency ratio *i.* high-intensity low frequency (20 kHz ≤ f ≤ 100 kHz), that do not alter the physical or chemical properties of the material; and *ii.* low-intensity high frequency (f > 100 kHz), that generates intense pressures and temperature gradient due to the disruption effect within the matrix. Figure

2.3. shows a schematization of the UAE method.

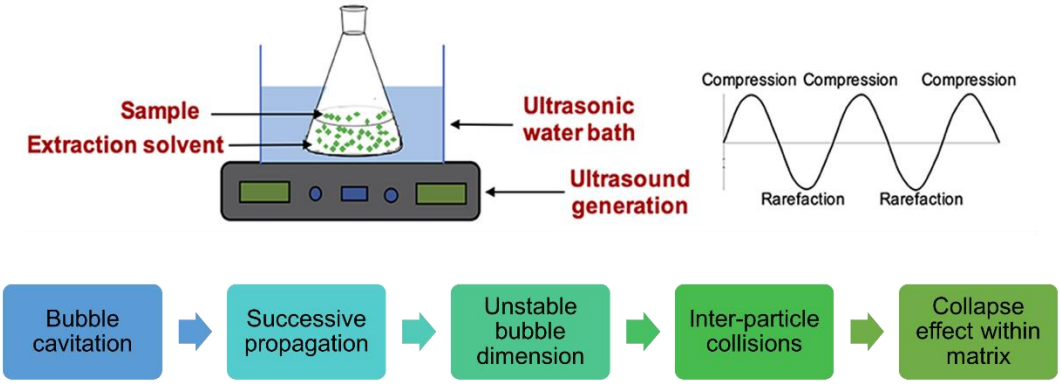


Figure 2.3. Schematic representation of UAE method and main characteristics. Source: author's elaboration adapted from (Rahman, 2021).

This simplifies the extraction of BCs from food samples due to the changes produced in the matrix cell wall by bubble cavitation, resulting in improved recovery of target analytes as well as increased extraction yield, reduction of extraction time and solvent use (Bakirtzi et al., 2016); (Kim et al., 2023).

2.2.1 Deep Eutectic Solvents (DESs) as sustainable extraction solvents for food bioactive compounds

One of the promising green solvents for the extraction of BCs from food matrices are Deep Eutectic Solvents (DESs). DESs consist of a mixture of a halide salt, which acts as a hydrogen-bond acceptor (HBA), and a hydrogen bond donor (HBD), which can be sugars, amines, amino acids, organic acids, or alcohol. Abbott et al., (2003) introduced DESs as a system formed by two or more Lewis or Brønsted-Lowry acids and bases that when mixed at a specific molar ratio, have a lower melting point than the individual substances, thus allowing the formation of a clear solution, which is used as an extracting solvent (Abbott et al., 2003).

Choline chloride (ChCl), a quaternary ammonium salt, has always been the most used HBA for the formation of DESs, as well as betaine which recently emerged as HBA, due to its cost-effectiveness and less toxic effects than ChCl (Chianioti et al., 2018). Figure 2.4. shows the chemical structures of commonly used HBAs and HBDs for the preparation of DESs. Recently, some DES mixtures have been obtained by combining natural molecules, i.e., primary metabolites and bio-renewable materials such as amino acids, sugars, and polyols (Fig. 2.4.), thus referring to as "Natural Deep Eutectic Solvents"

or NADES (Manuela et al., 2020).

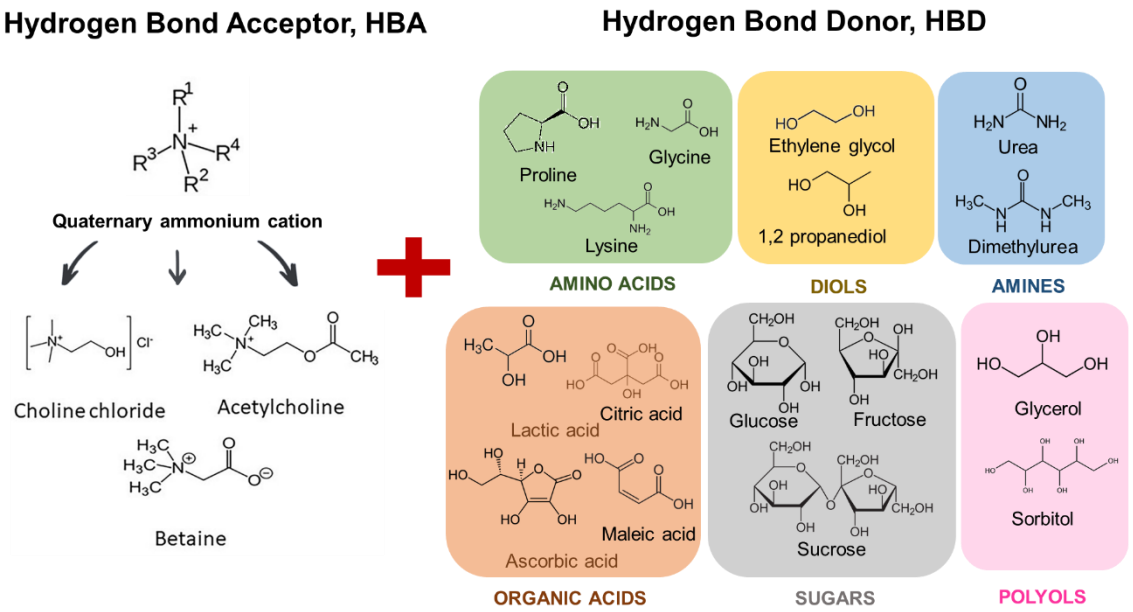


Figure 2.4. Chemical structures of most commonly used HBA and HBD compounds for DES preparation. Source: author's elaboration

The physicochemical properties of DES, in terms of density, viscosity, melting point, and conductivity are strictly linked to the chemical structure of DES. In particular, the type of HBD, as well as the type of salt used, and their related molar ratios are the most influencing factors for the density, surface tension, and viscosity of DESs. In general, DESs' viscosity is higher than other molecular solvents or ILs (>100 centipoise, cP), due to the presence of an extensive hydrogen bond network as well as other interactions (i.e., van der Waals and electrostatic forces) that restricts the mobility of free species inside DES, thus increasing its viscosity. However, it is worth noting that the excessive addition of water (≥ 40%, *w/v*) may lead to the weakening of the hydrogen-bonding interactions between DES constituents, thus influencing viscosity (Dai et al., 2015). Bajkacz and Adamek (2017), evaluated the water content in NADES (10–75%, *w/w*) for the micro-extraction of flavonoids from plant matrices. According to the authors, the extraction yield increases with the increase in water content up to 30%. For water concentrations of 75%, a dilution was obtained so high as to weaken the hydrogen bonds, reducing the solvation capacity of the DESs or NADESs (Benvenutti et al., 2019).

The viscosity of DESs is also associated with the free volume principle, the so-called *"hole theory"*, according to which the holes' highly diluted limits ion mobility (Abbott et al., 2006). To that end, the preparation of DESs with small cations of HBD is

recommended to obtain DESs with low viscosity. While, the viscosity of DESs tends to decrease as their temperature increases, thus being inversely related to temperature. For example, the DES of ChCl:ethylene glycol (1:4) showed lower viscosity than the DES of ChCl:sorbitol (1:1), i.e., 19 cP versus 12,730 cP, respectively, at 20 °C (Zhang et al., 2012). In line with the viscosity trend, the surface tension of DES exhibited a linear correlation with temperature, with a decrease in surface tension as temperature increases. In general, DESs are reported to have a higher surface tension than most conventional solvents (Abbott et al., 2006). It is assumed that the excessive addition of ChCl to glycerol may reduce the strength of the intermolecular hydrogen bond network of glycerol, resulting in a decrease in the surface tension of the DES ChCl:glycerol (Zhang et al., 2012).

Another important physicochemical property of DES is density, which mainly depends on the different molecular compositions. In general, the densities of both DESs are even greater than those of individual starting materials; based on the aforementioned hole theory, the HBD-HBA mixture results in an increase in the density of DESs over the densities of the individual starting components (Abbott et al., 2006) (Zhang et al., 2012). In these regards, the molar ratios of HBD-HBA components have major effects in modulating the densities of DESs. Considering the polarities, DESs, and NADES are generally defined as "hydrophilic" due to their high electronegativity and ability to form hydrogen bonds through dipole-dipole interactions. Hence, they are usually comparable with polar solvents, although some mixtures, such as citric acid and methanol, show polar and non-polar properties to each other (Benvenutti et al., 2019). These solvents can be prepared in three different ways involving specific operating conditions in terms of heating time, temperature, etc., depending on the composition of the analytes.

i. Heating and stirring method: HBA-HBD pairs are placed in a closed bottle and heated to 60 °C under magnetic agitation until a clear liquid is formed. It is worth noting that, when ChCl based-DES are mixed with carboxylic acids such as HBD, heating is not recommended to avoid the formation of impurities, such as esters; instead, it is preferable to grind the DES pairs in a mortar, at room temperature until the liquid solvent is formed (Ruesgas-Ramón et al., 2017).

ii. Evaporation method: The pairs are dissolved in water and evaporated at 50 °C using a rotary evaporator. The resulting liquid is placed in a desiccator with silica gel until it reaches a constant weight.

iii. Freeze-drying method: based on the freeze-drying of a mixture of the aqueous

solutions of the individual DES-pairs.

Both DESs and NADES have been employed in several fields of biotechnologies, and the chemical industry, among which food matrix analysis are prevalent applications. In particular, the extraction and separation techniques using DESs and NADES-based compounds have been successfully implemented to extract a wide range of bioactive compounds (i.e., phenolic acids, flavonoids, polyphenols, saponins, etc.) from several types of food matrices and natural sources.

To conduct bibliographical research on the current DESs application for food BCs extraction, the scientific database Scopus® was used, using for the investigated systems representative keywords, as *"Deep Eutectic Solvents"* AND *"Food"*, or *"Deep Eutectic Solvents"* AND *"Bioactive Compounds".* In addition, considering the research focus, only studies on food products and by-products t were considered; otherwise, the other exploring plant-derived products, essential oils, and flowers were not included. In this way, about 781 were found to be published from 2014 to June 2023. Table 2.1. summarizes the state of art about the application of DESs in the extraction of BCs from different foodstuffs. Several combinations of DES have been tested for the extraction of bioactive compounds from different plants and foodstuffs, thus covering a wide range of phytochemicals, mostly polyphenols such as flavonoids, phenolic acids, anthocyanins and alkaloids (Zainal-Abidin et al., 2017). It is worth mentioning that the majority of these applications were not developed as analytical tools before analysis, rather they were optimized as extraction solvents to achieve greener separations of analytes of interest.

Flavonoids are among the most studied compounds to be extracted with DESs, in particular, lactic acid and choline-chloride based – DESs are the most commonly used HBA pairs, generally mixed with organic acids (i.e., citric acid, oxalic acid, etc.), sugars (i.e., glucose), and diols (i.e., ethylene glycol) as extractant solvents. Extracts obtained with DES or NADES are mainly tested by functional analysis for bioactivity and subsequently characterized by chromatographic techniques (HPLC-UV/PDA/MS) or NMR spectroscopy. As regards the bioactivity tests reported for DES extracts, antioxidant activity assays (TPC, DPPH, and FRAP) are the most widely used to assess the phenolic content and antioxidant capacity of DES extracts, given their higher affinity for phenolic compounds. Barbieri et al. (2020) demonstrated that chloride-based DESs can be used as green solvents for the extraction and stabilization of phenolic compounds from *Rosmarinus officinalis L.* (Barbieri et al., 2020). The ability of DESs to stabilize phenolic compounds can be explained by intermolecular interactions, mainly due to hydrogen

bonds between the phenolic acids present in rosemary extracts and the solvent. This interaction reduces oxidative degradation by reducing oxygen contact at the DES-air surface.

Concerning the extraction methods mostly employed in literature, ultrasound-assisted solid-liquid extraction (UAE) is the commonly used extraction procedure. UAE is an energetic extraction procedure that can easily disrupt the intracellular structure, thereby releasing bioactive compounds. This represents a simple and reliable method that offers the advantage of being inexpensive and more efficient, with reduced extraction time, low solvent consumption, and low process temperature compared to conventional extraction methods (Bakirtzi et al., 2016); (Kim et al., 2023). Microwave-assisted extraction (MAE) has also been used for the DES extraction of active compounds (Panić et al., 2019); nevertheless, this technique is not recommended for the extraction of unstable and volatile substances, because it may cause local over-heating, as well as requiring expensive equipment or high energy demand.

Agricultural by-products became an important source of bioactive compounds enabling their potential utilization in the food industry, to be applied in food fortification and nutraceuticals, for cosmetic or pharmacological uses (Manuela et al., 2020). In this context, it is worth mentioning the possibility of extracting bioactive compounds using NADES as a unique advantage both for the sustainable recovery of BCs from agri-food by-products, as well as for the synthesis of natural-based solvents. This characteristic allows the extracted BCs to be administered directly without the need to evaporate the residual solvent, due to the lower or absent toxicity and higher biocompatibility than chemically synthesized DES or conventional solvents.

Table 2.1. Literature overview of DES extraction of BCs from food matrices. Source: author's elaboration

Bioactive compounds	DES (Molar Ratio; Water content, %)	Temperature (° C) × incubation time (min)	Method of extraction	Food Sample	References
Total polyphenols	Lactic acid:ChCl (3:1; 20%, *v/v*)	80 °C × 90 min	UAE	Fennel	(Bakirtzi et al., 2016)
Total polyphenols	Lactic acid:sodium acetate (1:3; 20%, *v/v*)	80 °C × 90 min	UAE	Fennel	
Total polyphenols	Lactic acid:ChCl (3:1; 20%, *v/v*)	80 °C × 90 min	UAE	Mint	
Total polyphenols	Lactic acid:sodium acetate (1:3; 20%, *v/v*)	80 °C × 90 min	UAE	Mint	
Total flavonoids	Lactic acid:ammonium acetate (3:1; 20%, *v/v*)	80 °C × 90 min	UAE	Mint	
Chlorogenic acid, morine, luteolin, coumaric acid, ferulic acid, rutin, myricetin, quercitin, apigenin	ChCl:p-toluene sulfonic acid (1:2; -)	80 °C × 90 min	SLE and UAE	Goji (*Lycium barbarum* spp.) fruits	(Ali et al., 2019)
Oleacein	ChCl:1,2-propanediol (1:1; -)	40 °C × 60 min	Heating and stirring	Virgin olive oil	(García et al., 2016)
Oleochantal	ChCl:lactic acid (1:2; -)	40 °C × 60 min	Heating and stirring	Virgin olive oil	
Total phenolic acids	Lactic acid:glucose:water (6:1:6; -)	N/A	Agitation	Virgin olive oil	(Paradiso et al., 2016)
Hydroxytyrosol, tyrosol, oleuropein aglycon, oleuropein, aglycon isomer, lygstroside, aglycon	Betaine:glycerol (1:2; 30%, *v/v*)	25 °C × 20 min	UAE	Virgin olive oil	(Fanali et al., 2020)
Total polyphenols, gallic acid, protocatechuic acid, (+) catechin, (–) epicatechin, epicatechin 3-o-gallate, quercetin, myricetin, kaempferol	ChCl:lactic acid (1:2; -)	80 °C × 30 min	UAE	Hazelnuts skins	(Fanali et al., 2018)
Total polyphenols, 5-p-coumaroylquinic acid, quinolactone, $-O- caffeoylquinic acid, 4,5-dicaffeoylquinic acid	Betaine:triethylene glycol (1:2; 30%, *v/v*)	80 °C × 30 min	UAE	Spent coffee ground	(Fanali et al., 2020)
Total phenolics, total procyanidins, catechin, protocatechuic acid, epicatechin, procyanidins B1 and B2	Betaine:glucose (1:1; 30%, *v/v*)	60 °C × 50 min	Heating and stirring	Cocoa by-products	(Manuela et al., 2020)

Rosmarinic acid, caffeic acid, 7-ethylrosmanol, rutin, naringin, ferulic acid	Glycerol:ChCl (1:2; -; 10% (*w*/*w*) water); lactic acid:ChCl (1:3; -; 10% (*w*/*w*) water); 1,2-propanediol:ChCl (1:2; -; 10% (*w*/*w*) water); oxalic acid:ChCl (1:1; -; 10% (*w*/*w*) water)	50 °C × 60 min	UAE	*Rosmarinus officinalis L.*	(Barbieri et al., 2020)
2 phenolic acids, 2 phenolic alcohols, vanillin (phenolic aldehyde), 11 flavonoids, and pinoresinol	ChCl:citric acid (2:1; 30%, *v*/*v*)	50 °C × 120 min	UAE/MAE	Grape and olive pomace	(Panić et al., 2019)
4 phenolic acids, 2 phenolic alcohols, 6 flavonoids, oleuropein, cinnamic acid	Lactic acid:glucose (5:1; 15%, *v*/*v*)	40–80 °C × 60 min	Heating and stirring	Olive, onion, tomato, and pear food by-products	(Fernández et al., 2018)
Total polyphenols content, punicalagin content, and ellagic acid content	ChCl:OA (1:1; 30%, *v*/*v*); ChCl:LA (1:1; 30%, *v*/*v*); ChCl:U (1:2; 30%, *v*/*v*); ChCl:Gly (1:2; 30%, *v*/*v*); ChCl:CA (2:1; 30%, *v*/*v*)	80 °C	UAE	Pomegranate peels	(Kim et al., 2023)

UAE: Ultrasound-Assisted Extraction; MAE: Microwave-Assisted Extraction; ChCl: Choline chloride; ChCl-OA mixture of choline chloride and oxalic acid, ChCl-LA mixture of choline chloride and lactic acid, ChCl-U mixture of choline chloride and urea, ChCl-Gly mixture of choline chloride and glycerine, ChCl-CA mixture of choline chloride and citric acid; N/A: not available.

2.3 Application of Life Cycle approaches for the sustainable recovery of bioactive compounds from agri-food products

In the last decades, different emerging technologies have been introduced in the field of chemical extraction processes, commonly considered *green* extraction processes. In particular, nowadays, sustainable applications for the analysis of BCs from agri-food products are focused on analyzing the environmental performances of the process, identifying hotspots, or comparing different scenarios and operational conditions in the context of an eco-design approach (Panzella et al., 2020). In these regards, Life Cycle based-approaches can be considered as useful tools for a multi-criteria optimization of the chemical process based on its environmental performances. In the scientific literature, the application fields of LCA for the comparative study of the environmental performance associated with chemical extraction processes from food matrices is mainly related to the extraction processes of bioactive compounds from food waste and microalgae (Santiago et al., 2021). In the study of Santiago et al., (2021), LCA methodology has been applied to evaluate the environmental performance of three scenarios (such as Soxhlet, pressurized liquid, and supercritical fluid extractions, all of them using ethanol as the extracting agent), for the valorization of *Asparagus officinalis L*. by-products (stalks) to extract rutin as target product and digestate under a biorefinery approach. The main findings of the study indicate that, among the different methods for rutin extraction, the PLE method is the most environmentally friendly. However, when considering the main allocation processes (mass and economic) in reporting results, it is noticeable that these affect the final result variably, and when using an economic allocation approach, the PLE-based scenario shows the best environmental performance profile. In contrast, when mass allocation is applied, this scenario presents the worst environmental profile for all impact categories (Santiago et al., 2021). However, when calculating the total environmental impacts, it is an important consideration to select the allocation method applied considering the case study complexity. In these regards, the LCA methodology allows for the establishment of a comparative framework that can be a useful tool for identifying the advantages and disadvantages of each selected technique, as well as the possibility of identifying the operational parameters on which to act in the short and medium term.

As regards alternative solvents used for the extraction of BCs from agri-food products, Zapata-Boada et al., (2023) applied LCA methodology to assess for the first time the environmental sustainability of algae biodiesel production, comparing the performance of three alternative organic solvents (limonene, ethyl *tert*-butyl ether (ETBE), and

cyclohexane) to the conventional solvent, hexane, for lipid extraction (Zapata-Boada et al., 2023). A "*cradle to grave*" LCA was performed to determine the environmental impacts at the midpoint level, through the ReCiPe method, thus highlighting hexane exhibited the lowest environmental impacts in 11 out of 19 categories, including climate change and energy demand, even though it is regarded as a hazardous solvent. Limonene, on the other hand, decreased the impacts in most of the remaining categories, given its low volatility, and non-hazardous nature. In these regards, the iterative integration of process design and optimization with LCA analysis could be meaningful for the development of greener and more sustainable alternatives to conventional ones. Among alternative technologies for the extraction of BCs from food matrices, Nutrizio et al., (2022) explore the use of high-voltage electrical discharges (HVED) for the extraction of phenolic compounds from oregano assessing both analytical parameters as well as environmental sustainability. The life cycle assessment (LCA) of HVED was evaluated in comparison with conventional and non-thermal extraction methods (i.e., infusion and maceration), thus showing that HVED technology exhibits the best extraction performances both in terms of extraction yield (2%–34% higher yield of phenolic compounds compared to infusion and 6%–91% higher yield compared to maceration), as well as environmental sustainability (global warming potential, ranged from 1.13 to 4.64 kg CO_2 eq for HVED samples, compared to 53.69 kg CO_2 attributable to conventional infusion treatments) (Nutrizio et al., 2022).

Furthermore, from the literature review on environmental assessment of extraction processes, the number of studies focusing on LCA of green extraction methods (i.e., UAE, MAE, DES, etc.) is still lacking. The study of Vauchel et al., (2018) conducted a comparative LCA of UAE of polyphenols from chicory by-products (i.e., grounds) under different operational conditions, in terms of temperature, water content, ethanol (EtOH) content, and extraction time. The main findings of the research highlight that increased temperature (over 60 °C) resulted in a hotspot leading to huge GHG emissions in all impact categories investigated, thus denoting 20%-80% higher results than extraction processes performed at temperatures of 20 °C (Vauchel et al., 2018). In addition, the use of ethanol as a solvent implied a huge increase in impacts, in particular on toxicity effects on human health (92%), terrestrial and freshwater eutrophication (90-95%), as well as abiotic resources, such as land use (98%), and water consumption (99.5%). In this framework, it is worth mentioning the importance of including environmental impact criteria as well as productivity and economic criteria while optimizing processes, in the

view of tending to more sustainable solutions.

Otherwise, there are no studies investigating both analytical parameters and environmental performances of green extraction processes carried out with DES for bioactive compound analysis in food matrices. In these regards, The Ph.D. research project conducted a research article on the environmental sustainability assessment through LCA testing Deep Eutectic Solvents concerning conventional extraction techniques for bioactive compounds from dark chocolate samples.

2.4 The importance of integrating sustainability assessment tools in chemical processes

The analysis of LCA applications on chemical extraction processes, reveals the importance of integrating sustainability assessment tools in the chemical industry to contribute to a fair transition towards greater economic, environmental, and social sustainability. In these regards, the main focus of Green Chemistry (GCy) is to increase the performance and value of chemical processes while preserving human health and the environment (Anastas & Warner,1998) (Manley et al., 2008). Furthermore, the possibility of developing green processes, for example through the use of safer solvents and auxiliaries, reduction of reaction derivatives, and energy and raw materials efficiency, joined with environmental life cycle assessment would result in the reduction of risks associated with products and process design directly at their source, thus representing real-time analysis for pollution prevention. Therefore, it is possible to highlight a strong relationship between GCy and sustainability to address the challenge of sustainable development on a global scale. In this sense, combined GCy and LCA, also referred to as *"Sustainable Chemistry"*, ensures a safer and greener use of chemical processes both for human health and the environment (Blum et al., 2017). Figure 2.5. shows the strict relationship within the use of life cycle-based approaches and green chemistry processes for a multi-dimensional perspective (social, environmental, and economic) of food sustainability. As afore-mentioned, the implementation of GCy principles and LCA analysis in chemical and industrial processing would represent a benefit both from: *i.* an economic perspective, feasible through the reduction of waste and recovery of fossil and renewable resources resulting in a profit reduction; *ii.* a social perspective, according to the Triple-Bottom-Line (TBL) model for pursuing both business objectives (i.e., better performances, improved competitiveness, etc.), and to preserve human health; *iii.* environmental perspective, including the reduction or removal of waste, hazardous

materials, and the use of renewable resources (Manley et al., 2008), (Silvestri et al., 2021).

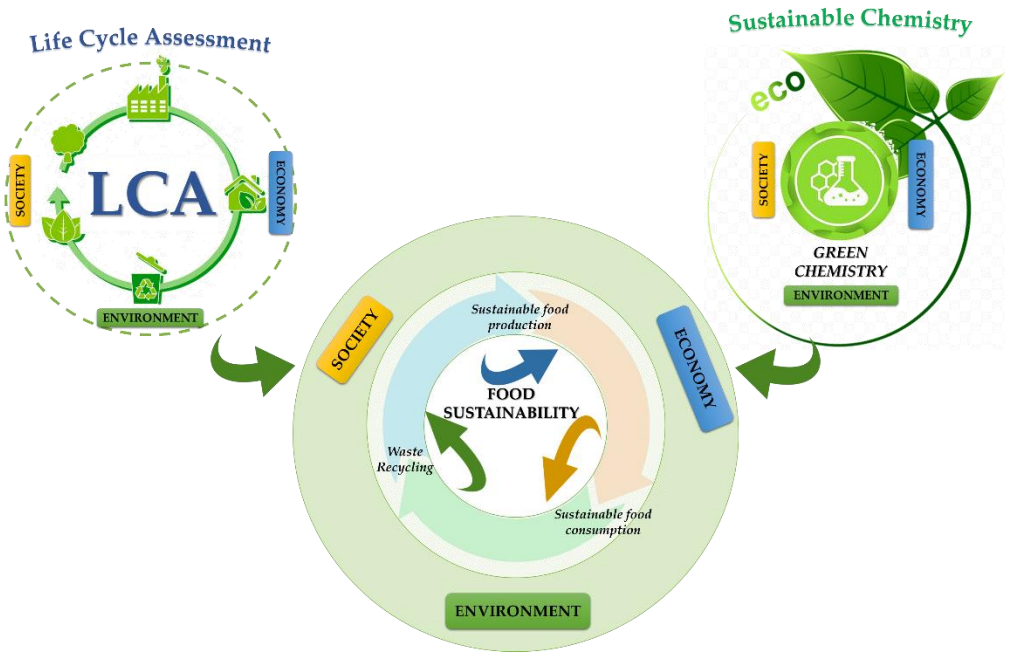


Figure 2.5. Framework of food sustainability in the context of Green Chemistry and Life Cycle Assessment. Source: author's elaboration.

In addition, another important linkage to remark is between food sustainability and the circular economy (CE) concept. According to Linder (2017), GCy can be the primary driver of CE actions, thus denoting its key role in driving society towards a sustainable future, embracing all three dimensions of TBL (Linder et al., 2017). Sheldon (2017) demonstrated that both sustainable production and the circular economy perspective can be achieved through chemical production based on bio-based raw materials, as well as through waste reduction and more efficient use of energy. In this context, the close relationship between GCy and the circular economy is very significant, and LCA is an indispensable tool to achieve sustainability in the food sector based on the three dimensions of TBL. In this context the possibility of valorizing agri-food processing by-products by means of greener, cost-effective, and sustainable technologies could result in environmental, human health, and economic benefits (Panzella et al., 2020). Building on the previous considerations, it is possible to approach CE as a macrosystem able to create economic, social, and environmental values, thus promoting the reuse and recycling of new or "secondary raw" materials without producing waste. In such a macrosystem, the Sustainable Chemistry subsystem, through the principles of GCy, combined with Life Cycle Assessment (LCA), contributes to the design, production, and re-use of materials in manufacturing processes.

CHAPTER III

EXPERIMENTAL SECTION

The Ph.D. research project was developed through the study of food quality, sustainability, and safety aspects in several food matrices, namely samples from the ancient "Senatore Cappelli" durum wheat chain (grains, flour, pasta, and husks), wines, psychoactive foods and beverages (tea, coffee, and chocolate), olive oil, and mushrooms. During the Ph.D. course, the main results of the proposed research project were the object of no. 11 publications in scientific International Journals. Table 3.1. reported reports all publications grouped by macro-area: Sustainability (S), Food Quality (FQ), and Food Safety (FS). In the following section, the published editorial versions (PDF original copy, and DOI addresses) were attached.

In addition, alongside the activities conducted within the proposed research project, collateral research studies on different areas and topics, mainly concerning environmental and human health aspects, were carried out, resulting in several publications in scientific International Journals, participation in national and international conferences, and book chapters, all listed in "*Other publications*" section.

Table 3.1. Main research articles in the proposed subject area. Articles are grouped by research macro-area: Sustainability (S), Food Quality (FQ), and Food Safety (FS).

Authors	Title	Year	Journal	DOI	Research macro-area
Vinci, G., **Prencipe, S. A.**, Pucinischi, L., Perrotta, F., & Ruggeri, M.	Sustainability assessment of waste and wastewater recovery for edible mushroom production through an integrated nexus. A case study in Lazio.	2023	Science of The Total Environment, 166044	10.1016/j.scitotenv.2023.166044	**S**
Vinci G., Maddaloni L., **Prencipe S.A.,** Orlandini E. Sambucci M.	Simple and reliable eco-extraction of bioactive compounds from dark chocolate by Deep Eutectic Solvents. A sustainable study.	2023	International Journal of Food Science & Technology, 58(7), 4051-4065	10.1111/ijfs.16315	**S, FQ**
Vinci, G., **Prencipe, S. A.**, Armeli, F., & Businaro, R.	A Multimethodological Approach for the Valorization of "Senatore Cappelli" Wheat Milling By-Products as a Source of Bioactive Compounds and Nutraceutical Activity	2023	International Journal of Environmental Research and Public Health, 20(6), 5057	10.3390/ijerph20065057	**S, FQ, FS**
Gobbi L., Maddaloni L., **Prencipe S.A.**, Vinci G.	Bioactive Compounds in Different Coffee Beverages for Quality and Sustainability Assessment.	2023	Beverages, 1(9), 1-18	10.3390/beverages9010003	**S, FQ, FS**
Restuccia D., **Prencipe S.A.**, Ruggeri M., Spizzirri U.G.	Sustainability Assessment of Different Extra Virgin Olive Oil Extraction Methods through a Life Cycle Thinking Approach: Challenges and Opportunities in the Elaio-Technical Sector.	2022	Sustainability, *14*(23), 15674	10.3390/su142315674	**S**

Vinci, G., **Prencipe, S.,** Abbafati, A., Filippi, M.	Environmental Impact Assessment of an Organic Wine Production in Central Italy: Case Study from Lazio	2022	Sustainability, *14*(22), 15483	10.3390/su142215483	**S**
Vinci, G., D'Ascenzo, F., Maddaloni, L., **Prencipe, S.A**., Tiradritti, M.	The influence of green and black tea infusion parameters on total polyphenol content and antioxidant activity by ABTS and DPPH assays.	2022	Beverages (8:2, 18)	10.3390/beverages8020018	**FQ**
Prencipe S.A., Maddaloni L., Ruggieri R., Vieri S., Vinci G.	Quality markers evaluation in chocolates with different cocoa content.	2022	International Journal of Health Sciences 6(S6), 7762-7773	10.53730/ijhs.v6nS6.11071	**FQ, FS**
Gobbi L., Maddaloni L., **Prencipe S.A**., Vinci G.	Evaluation of biogenic amines, phenolic and antioxidant compounds in "Senatore Cappelli" durum wheat products.	2022	International Journal of Health Sciences 6(S6), 7774–7784	10.53730/ijhs.v6nS6.11095	**FQ, FS**
Vinci G., Maddaloni L.; **Prencipe, S. A**.; Ruggieri R.	Natural Contaminants in Wines: Determination of Biogenic Amines by Chromatographic Techniques.	2021	International Journal of Environmental Research and Public Health, 18(19), 10159	10.3390/ijerph181910159	**FQ, FS**
Vinci, G., Gobbi, L., Maddaloni, L., **Prencipe, S.A**.	Simple, reliable determination of biogenic amines in Italian red wines. Direct analysis of underivatized biogenic amines by LC-ESI-MS.	2021	Journal of Advanced Mass Spectroscopy, 1(1), 1-6	JAMS/article/view/26	**FQ, FS**

3.1 Research article no. 1

Title: Sustainability assessment of waste and wastewater recovery for edible mushroom production through an integrated nexus. A case study in Lazio.

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Authors: Vinci, G., **Prencipe, S. A.**, Pucinischi, L., Perrotta, F., & Ruggeri, M.

Research macro-area: **Sustainability (S)**

Graphical Abstract:

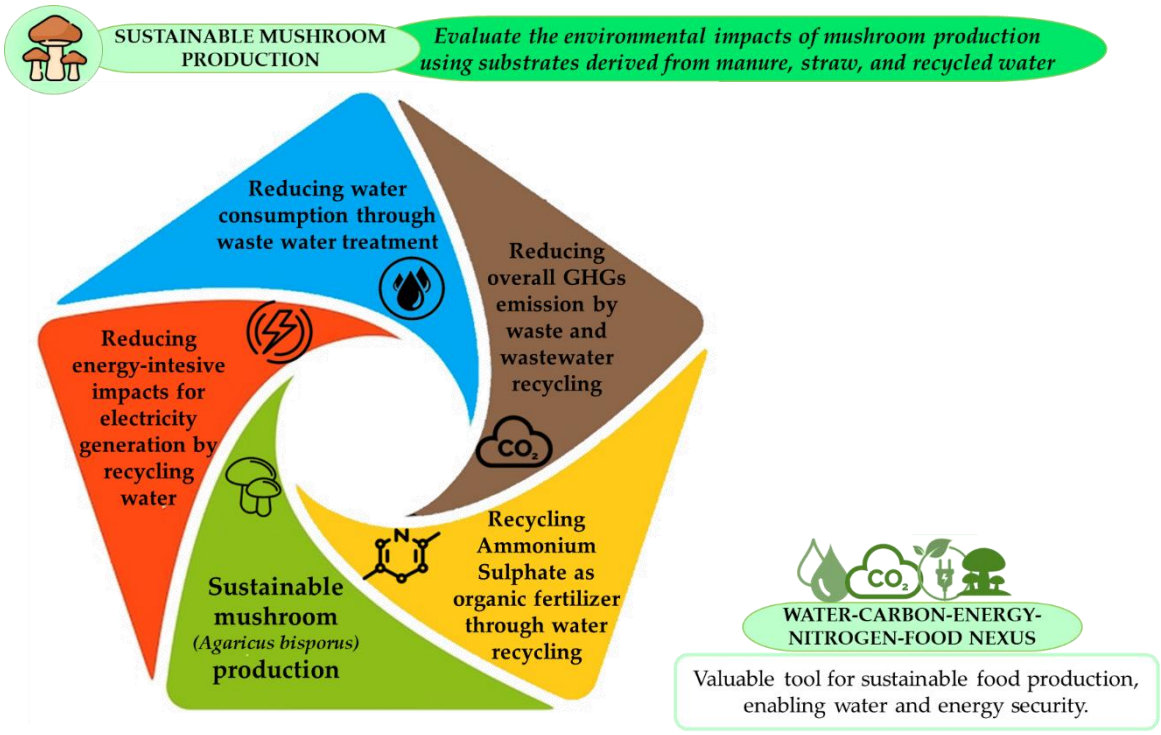

Figure 3.1. Graphical Abstract of experimental study no. 1. Source: (Vinci G, Prencipe SA, Pucinischi L, Perrotta F, Ruggeri M., 2023)

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Sustainability assessment of waste and wastewater recovery for edible mushroom production through an integrated nexus. A case study in Lazio

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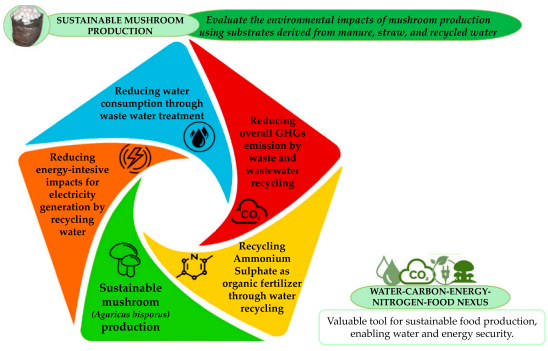
HIGHLIGHTS

- A Life cycle assessment of fungal production from recycled materials is studied.
- The water-energy-nitrogen-carbon-food nexus is considered.
- The production of 1 kg of mushrooms emits about 2.28 kg CO2 eq.
- Wastewater recovery reduces environmental impacts without compromising water and energy security.

GRAPHICAL ABSTRACT



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ABSTRACT

With a global population of eight billion people, improving the sustainability and nutritional quality of diets has become critical. Mushrooms offer a promising solution because of their nutritional value and ability to be grown from agricultural residues, in line with the circular economy. This study, therefore, focuses on assessing the environmental compatibility of Agaricus bisporus mushroom production in Italy, the world's third largest per capita consumer, by using a Life Cycle Assessment (LCA) and an integrated Water-Energy-Nitrogen-Carbon-Food (WENCF) nexus analysis. The LCA results reveal that for a functional unit of 23,000 kg of the substrate, the production process emits 2.55×10^4 kg of CO_2 eq. Sensitivity analysis shows that changing input quantities can reduce environmental impacts by about 5 %. In addition, one scenario evaluates the environmental effects of recycling resources by introducing water and ammonium sulfate from scratch instead of continuous recycling, along with water purification. The study shows that sustainable food production can mitigate resource depletion, climate-altering emissions, and intersectoral competition. Using agro residues for mushroom cultivation and optimizing resource management contribute to environmental sustainability. This approach could not only improve the resilience and efficiency of the food system but could also improve the sustainability of diets. In conclusion, this study highlights the importance of adopting sustainable and circular approaches in mushroom production to address global challenges related to food sustainability.

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1. Introduction

The agri-food sector contributes around 17.3 billion tons of carbon dioxide (CO_2) each year, accounting for 35 % of anthropogenic greenhouse gas (GHGs) emissions (Xu et al., 2021). However, these rates will most probably increase even more, in part due to the continuing growth of the population, which reached 8 billion in November 2022 and is estimated to reach 9 billion by 2037. This would result in an increasing demand for food (+40 %) (Gouel and Guimbard, 2019), leading to greater pressure on natural resources. One solution to these challenges could be providing more food with fewer inputs for its production, as well as reducing unavoidable food waste, developing circular economy (CE) systems, and reusing and recycling organic waste materials (Grimm and Wösten, 2018). CE, whose relevance in current research and policies is demonstrated by its central role in the European Green Deal and cities' action plans (Mairie de Paris, 2017; European Commission, 2020), calls for a move away from linear economy models and focuses on closing the circuits of raw materials, energy sources, and nutrients. Through this philosophy, it might be possible to optimize and reduce the production of raw materials by replacing them with waste products and consequently lower polluting emissions by avoiding waste and making products more competitive.

In the agri-food sector, due to the high amount of organic waste it produces, CE could be implemented quite efficiently, as some organic agro-wastes (livestock manure and lignocellulosic biomass) can be bioconverted for food production, through their use as organic fertilizers and soil improvers, instead of synthetic ones (Bai et al., 2018; Grimm and Wösten, 2018). One sector that could particularly benefit from the bioconversion of agro-residues such as straws, leaves, stems, bagasse, and manure, is the production of mushrooms. This is because, although they normally grow in the wild on moist, nutrient-rich forest soils, they can also be grown effectively on organic waste and plant-derived materials, such as spent wood from various agro-industrial waste products containing lignocellulose and/or in association with manure (Dorr et al., 2021). Currently, among the approximately 2000 edible mushroom species in the world (Soto, 2019), 85 % of the total production is covered by five species: *Lentinus edodes* (Shiitake), *Pleurotus ostreatus* (Oyster), *Boletus edulis* (Porcini), *Agaricus bisporus* (Champignon) and *Flammulina velutipes* (Royse et al., 2017). These five species contribute about $16.7 billion (Research and Market, 2021) to the mushroom market, by global production of about 11.8 million tons (Faostat, 2021), +57 % within 2010–2020 (Faostat, 2021), thus demonstrating their considerable importance. In particular, this is due to increased awareness about their nutritional qualities, such as crude fiber (19 % of total dry weight), vitamins (A, C, E, K), carbohydrates and minerals (potassium and selenium), antioxidant compounds (flavonoids, tannins, etc.), monounsaturated fatty acids (ω-3 and ω-6, etc.) and their protein value making them are a good plant source of essential amino acids (Cheung, 2010; Valverde et al., 2015; Thakur, 2020). Furthermore, the cultivation of edible mushrooms offers an opportunity to address the issue of unsustainable disposal or burning of agro-residues like manure and agricultural biomass (Chen et al., 2022). By integrating mushroom cultivation, we can achieve multiple benefits simultaneously, including the recovery of raw materials, waste reduction, and the bioconversion of these residues into high-quality agro-food products. Therefore, mushroom cultivation could be a potential alternative for balancing nutritional deficiencies and food insecurity, as well as tackling the decline of natural resources, and climate change. In addition, since a fungal spore germinates, the fungus is highly dependent on water for its growth (which is needed for all stages of its life cycle, even because fungi consist of about 90 % water and degrade organic matter by secreting enzymes, which need water to break down the substrate) (Herman and Bleichrodt, 2022). Then, in a CE context, proper water resource management must be included to avoid the multiple impacts associated with its extraction (Tarpani and Azapagic, 2023) and preserve the conservation of that resource. In this regard, a widely used and valid tool for studying the environmental compatibility of products or processes is the Life Cycle Assessment (LCA), especially in the agri-food sector (Zingale et al., 2022). In recent years few LCA studies have been conducted in relation to mushroom production, mostly related to the cultivation of *Agaricus bisporus*. Starting with the pioneering work of Gunady et al. (2012), showing that for every kg of *Agaricus bisporus*, 2.76 kg CO_2 eq is produced. Dissimilar results from the study by Leiva et al. (2015) (Spain), who, for 1 kg of *Agaricus bisporus* found about 4.41 kg CO_2 eq. Or even Robinson et al. (2019) studied the production of 1 kg of *Agaricus bisporus* in the U.S. from compost, showing how the results ranged from 2.13 to 2.19 kg CO_2 eq. Until the contribution of Dorr et al. (2021), who analyze the environmental impacts of a circular mushroom farm in France, showing how 1 kg of *Pleurotus ostreatus* emits about 2.99 to 3.18 kg CO_2 eq. Differences in results are a function of variability in processes, background inventory assumptions, and the methodological approach underlying LCA studies. But also, background systems, cultural practices, and soil and climate conditions. In addition to *Agaricus bisporus* and *Pleurotus ostreatus*, an additional study related to *Shiitake* mushroom cultivation (Rungnapa Tongpool and Pongpat, 2013) in Thailand was found in the literature. The main finding made by the two authors is that 1 kg of mushrooms emits about 1.87 kg CO_2 eq, although *Shiitakes* are cultivated differently than *Agaricus*. The literature related to the analysis of the environmental compatibility of mushroom production is rather modest and covers only a few countries, including the USA, Australia, China and Spain. However, no study has focused on identifying impacts on the mushroom production process in Italy, which on the basis of market research appears to be the world's third largest consumer per capita (4.87 kg per person) (Research and Market, 2021). Considering the nutritional qualities of mushrooms and given the great opportunities their production offers to close the cycles of matter and energy it may be important to lead efforts to increase their consumption as part of a healthier and more sustainable diet. Therefore, to help fill knowledge gaps on the environmental impact of the CE and mushroom cultivation, a Life Cycle Thinking (LCT) approach was used in this research to assess the sustainability of mushroom production. The study involved the Italian company "*Funghitex S.S*," which is active in the production of substrates for growing *Champignon* located in Giulianello (Latina). The aim of the study is: i) to quantify the impacts of this type of activity; ii) to identify the most impactful production steps, and iii) to investigate CE aspects of the company and adaptable improvement opportunities. Consistently with the twofold objectives of this study, LCA was coupled with a Water-Energy-Nitrogen-Carbon-Food Nexus analysis (ISO, 2006a, 2006b; Frischknecht et al., 2004; IPPC, 2006; Aldaya et al., 2011). The research was complemented by a sensitivity analysis that was carried out with the aim to identify some possible scenarios for reducing environmental impact in *Agaricus bisporus* production and in the field of agriculture and food. Our study aims to contribute to the existing body of knowledge in different ways. First, by exploring the environmental impacts of mushroom production using specific substrates derived from manure, straw, and recycled water. This approach is in line with the principles of waste reduction, resource efficiency, and sustainable practices. By focusing on this particular production method, we aim to highlight the potential of using agricultural by-products and recycled water as inputs for mushroom cultivation, thus promoting the principles of the circular economy. Furthermore, another highlight lies in the possibility of proposing sustainable food production from recycled raw materials in an integrated nexus perspective, in which energy use and water system were continuously interchanged, thus generating a reduction in total CF and GHG emissions. The results could then provide knowledge to the scientific community, practitioners, policymakers, and other stakeholders involved in mushroom production and sustainability, contributing to a broader understanding of the environmental impacts associated with different production methods and promoting informed decision-making for more sustainable practices. Finally, although the study focuses on specific case studies, the results and methodologies could provide valuable insights for similar production systems. The

challenges faced by the mushroom industry in terms of waste management, resource efficiency, and greenhouse gas emissions are not unique to our case study but are relevant to many regions globally.

2. Materials and methods

2.1. Case study description

Funghitex operates in the production of substrates for the cultivation of Champignon in Giulianello (41°40′20.648″ N, 12°52′59.499″ E) (Lazio, Italy). It produces almost exclusively with raw materials from previous agricultural production of other neighboring farms (i.e., manure and straw) according to a logic of industrial symbiosis, while water and ammonium sulfate are continually recycled and reintroduced into subsequent production cycles, as shown in S1 (paragraph 1.1) (Supplementary). The case study of Funghitex was selected for three orders of reasons: first, as a matter of relevance to the research objectives and the specific focus of the study. Indeed, the selected case study provided an opportunity to evaluate the environmental performances of mushroom production using substrates derived from manure, straw, and recycled water. This specific production method was considered attractive because of its potential for waste reduction, resource efficiency, and sustainable practices. Next, because of the question of data availability. In particular, the availability of comprehensive information for this case study has a key role in its selection, as access to detailed data on inputs, processes, and emissions from the company allowed for in-depth analysis and accurate assessment of the environmental impacts associated with mushroom production. And finally, as a matter of feasibility since data accessibility and cooperation from the company made it possible to conduct the study in an acceptable time frame.

2.2. Life cycle assessment

2.2.1. Goal and scope

The study aimed to analyze the resources consumed and substances emitted to produce the substrate for mushroom cultivation through different quantities of raw materials used. The FU is 23,000 kg of finished bulk product, i.e., the quantity of a mushroom cultivation room. The system boundaries considered the entire substrate production process, from the procurement of raw materials to the finished product (Fig. 1).

2.2.2. Life cycle inventory (LCI)

All inventory data are primary (2018 production) and described in Table 1 and Table A1 (Supplementary). In this study, the current model used by the company was selected as it represents the standard operational practices implemented in their Agaricus bisporus mushroom production. The production data used in the analysis was obtained by considering average operational parameters, including input quantities, energy consumption, and waste generation, based on the company's historical records and production logs.

As for water, its management follows the principle of reuse. Specifically, there are four water collection tanks and three silos intended for recovery in the processing cycle. All tanks are covered and equipped with both an aeration system to avoid anaerobic phenomena and an air intake system sent to the Scrubber treatment system. Water used in the production process is classified into black and clean water (Fig. 2). The first is the wastewater from the leaching of material within the production areas, from the slab, from the collection at the bottom of the production areas of rainwater, and from the occasional washing of the yards and the trucks (Fig. 2A). Clean water comes from the company's three wells and is stored in silos, and used to cool the hatchery unit, control temperature, toilets, and wash yards and trucks (Fig. 2B). Rainwater, on the other hand, is stored in three cisterns and flowed into special channels.

All water is sent to the production cycle and continuously recycled. A total of 23,000 kg of substrate is obtained from the production process, and transportation was also considered for the study (Table 1). Data were modeled through databases in SimaPro 9.2.2. and adapted to Italian conditions.

2.2.3. Allocation procedures

Impact allocation to define the percentage contribution of the environmental burden to each processing by-product according to the simple cut-off method (Ekvall and Tillman, 1997), also known as one of the most used methods for modeling recycling process in LCA and recommended by the international system for Environmental Product Declarations (EPD).

The method is applicable when the environmental impacts of recycling are lower than the combined impacts of virgin material production and final waste management and thus promotes the use of recycled materials throughout the life cycle. This cut-off method means the LCA does not include activities that are avoided due to, for example, the recovery of materials or energy in waste-management processes. In particular, the method gives incentives to use recycled material as long

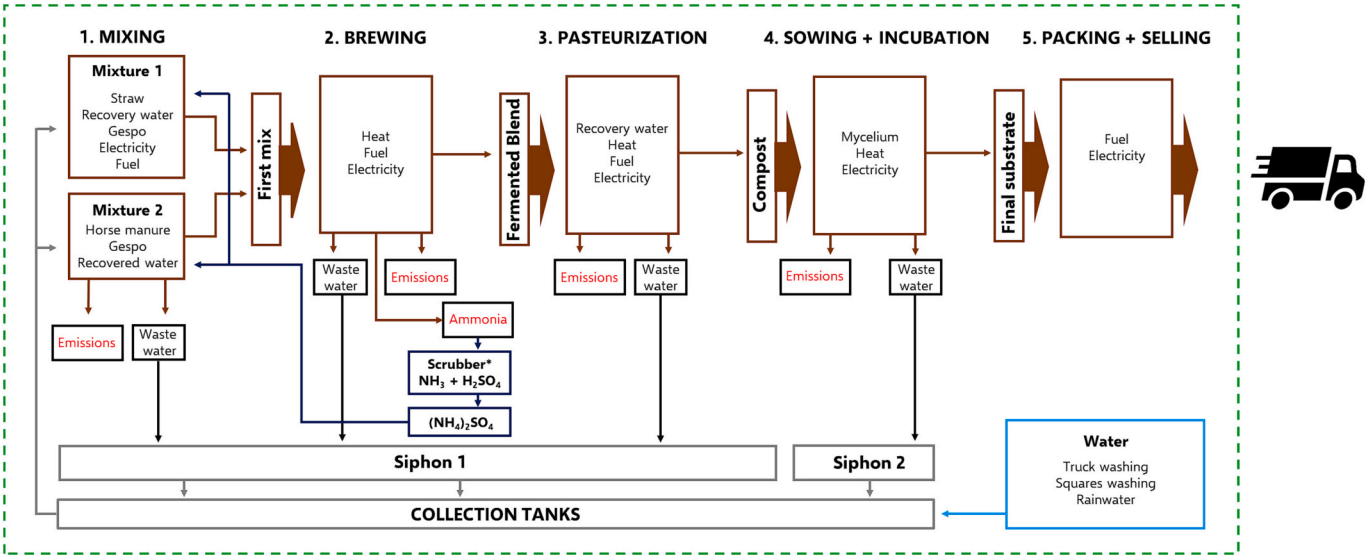

Fig. 1. System boundaries of mushroom production.

Table 1
LCI of the production process of Funghitex.

Input/Output	Unit	Quantity	Provenance	Means of transportation	Tkm	Source
Input						
Horse manure	kg	18,000	Lazio, Tuscany, Campania	Truck	90	Agribalyse
Wheat straw		3500	Puglia		300	
Poultry manure		2700	Campania, Molise	Tanker truck	250	Ecoinvent v3.8
Agricultural Gypsum (Calcium Sulfate)		1200	Tuscany		296	
Ammonium sulfate (solid)		800	Lazio	Truck	93	WFLDB
Mycelium (*Agaricus bisporus*)		980	France		1600	
Ammonium sulfate (liquid)		0.11	By-product	Production Plant	–	–
Diesel	m^3	50				Ecoinvent v3.8
Water		168.3				–
Electricity	kWh	2016				Ecoinvent v3.8
Output						
Substrate	kg	23,000				
Atmospherical emissions						
CO_2	kg CO_2 eq	22,822				IPPC, 2006
CH_4		2425				
N_2O		261				
SF_6		18.5				
FCs		0.9				
Emissions to water (Freshwater)						
Phosphate	kg P eq	0.3736				IPPC, 2006
Phosphorus		0.0379				
Emissions to water (marine)						
Ammonia	kg N eq	0.0565				IPPC, 2006
Ammonium, ion		0.0449				
Nitrate		0.6469				
Nitrite		0.0003				
Nitrogen		0.0067				

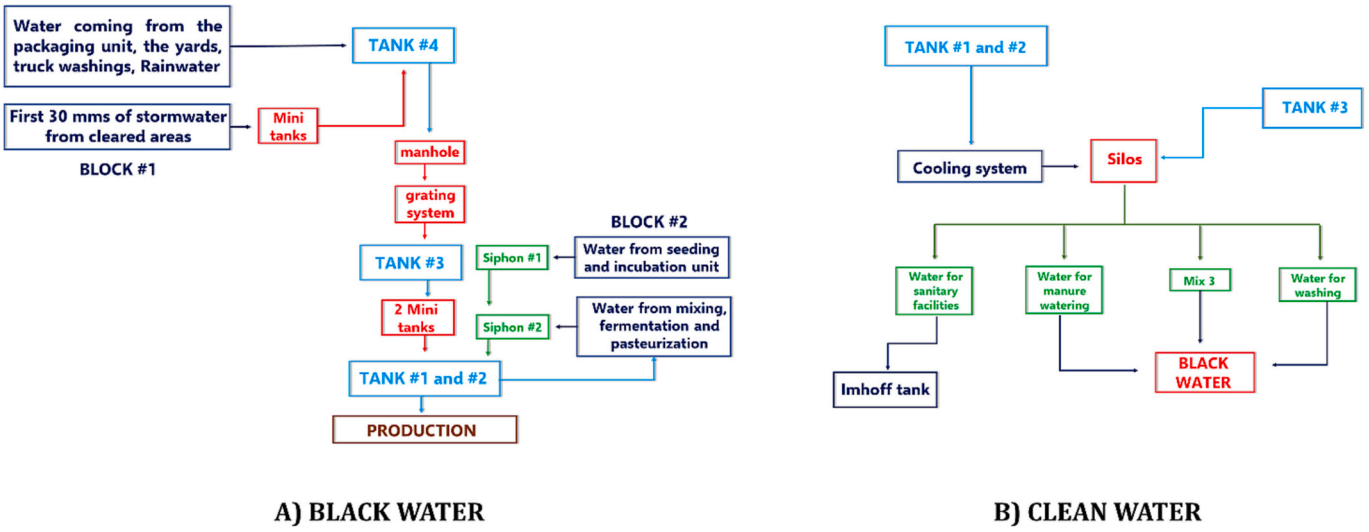

Fig. 2. Description of the cycle of Funghitex black water (A) and clean water (B).

as the recycling has less environmental impact than virgin materials production (EV > ER). Furthermore, the recycling process of a product after use is emphasized when the final disposal has a negative net impact on the environment (ED > 0). According to Ekvall et al. (2020), each product should be assigned all the environmental impacts (E) directly attributable to the production process, as in Eq. (1):

$$E = (1–R_1) \times E_V + R_1 \times E_R + (1–R_2) \times E_D \quad (1)$$

where R_1 is the share of recycled material in the product; R_2 is the rate of recycling of material after use in the product, and E_V, E_R, and E_D correspond to the environmental burdens of the virgin raw material.

2.2.4. *Life cycle impact assessment (LCIA)*

To ensure the robustness of our study, we followed the ReCiPe 2016 MidPoint (I), a recognized LCA methodology that provides a systematic and transparent approach for selecting impact categories. SimaPro 9.2.2. software was used, and the 18 impact categories were grouped into four macro areas.

i. *Atmospheric Effects*: Global Warming Potential (GWP); Stratospheric Ozone Depletion (SOD); Ionizing radiation (IR); Ozone Formation, Human Health (OFHH); Fine Particulate Matter Formation (FPMP); Ozone formation, Terrestrial ecosystems (OFTE); Terrestrial acidification Potential (TAP),
ii. *Eutrophication*: Freshwater Eutrophication Potential (FEP) and Marine Eutrophication Potential (MEP),
iii. *Toxicity*: Terrestrial Ecotoxicity (TEC); Freshwater Ecotoxicity (FEC); Marine Ecotoxicity (MEC); Human Carcinogenic Toxicity (HCT); Human Non-Carcinogenic Toxicity (HNCT),
iv. *Abiotic Resources*: Land Use (LU); Mineral Resources Scarcity (MRS); Fossil Resources Scarcity (FRS), Water Consumption (WC).

We considered several factors when selecting impact categories, including scientific relevance, stakeholder concerns, and environmental context. The ReCiPe 2016 MidPoint (I) was chosen and preferred over other calculation methods such as ILCD 2011, CML 2001, or TRACI because having eighteen impact categories (compared to 16 in ILCD 2011 Midpoint, 15 in IMPACT 2002 +, 11 in CML -IA Baseline, and 9 in TRACI) it can provide more comprehensive, articulate, and specific results on the environmental impacts of mushroom production. Therefore, ReCiPe could provide a broader picture with a greater degree of detail on the environmental impacts of production. In adopting the ReCiPe method, we aimed to capture a full range of potential impacts associated with mushroom production, although some impact categories might seem less obvious in an internal context. For example, marine eutrophication and marine ecotoxicity may seem far removed from our LCA concerns, but in conducting our LCA study, we aimed to take a holistic approach that considers direct and indirect contributions from specific processes or inputs within the life cycle of mushroom production. While our focus was on direct environmental impacts, we recognize that these impacts can have downstream consequences, and for this reason, it may be important to consider a broader environmental context.

2.2.5. Sensitivity analysis (SA)

Because it is not regulated by ISO, SA is an optional step, deferred to the voluntariness of the authors, who create alternative scenarios to demonstrate possible examples of improvement (Ferretti et al., 2016). However, because SA measures how variability in inventory data can affect results, it could be useful because it improves model prediction by qualitatively and quantitatively studying the study's response to varying input variables. Moreover, although interpretation is voluntary, it remains a key step in LCA because, in addition to ensuring the reliability and robustness of the study, the transition to more sustainable and circular economy models requires various hotspot improvement and management options, which can be examined precisely through an SA. Therefore, based on these assumptions, SA was conducted to assess how, by changing certain input parameters, the company's environmental performance may change. Specifically, three scenarios were created:

1. Scenario 1 (S1) (2018): The process with the initial inputs.
2. Scenario 2 (S2) (2019): horse manure, compared with S1, increased by +11 %, while wheat straw, poultry manure, and agricultural gypsum decreased by −9 %, −11 %, and − 20 %, respectively. Solid ammonium sulfate increased by +13 %. Net electricity consumption decreased by −13 %.
3. Scenario 3 (S3) (2020): horse manure, compared with S2 increased by +5 %, while wheat straw decreased by −13 %. Poultry manure, agricultural gypsum, and solid ammonium sulfate increased by +17 %, +20 %, and + 22 % respectively. Electricity consumption decreased by −7 %.
4. Scenario 4 (S4) (2021): horse manure increased compared to S3 by +15 %, while wheat straw was reduced by −29 %. Poultry manure was unchanged, while calcium sulfate (−13 %) and solid ammonium sulfate (−36 %) decreased. In contrast to S2, liquid ammonium sulfate increased by +45 %. In the latter case, mycelium was also reduced by −8 % as well as electricity by −2 %.

The four scenarios were established through a combination of expert knowledge and model simulations. Expert knowledge from on-farm experts helped determine changes in quantity based on the cost of raw materials used, thus preferring to increase manure and reduce straw, keeping the final quality of the compost unaltered. Then, to verify the environmental as well as economic feasibility of this change, experimental tests and simulations were done using Simapro 9.5 software, which allowed the data collected on resource use and emission factors to be entered, thus calculating environmental footprints. Therefore, the scenarios were first modeled based on production costs, which allowed the amount of raw material to be determined, which was then verified from an environmental perspective arriving at the various production scenarios over the years.

2.3. Water-energy-nitrogen-carbon-food nexus

Subsequently, an alternative scenario was created, in which the company, instead of continuously recycling water and ammonium sulfate, adds them from time to time. In addition, it was also assumed that the wastewater, instead of being fed back into the production cycle, is treated in a wastewater treatment system. So, to highlight potential synergies and identify critical hotspots in the mushroom production system a Water-Energy-Nitrogen-Carbon-Food nexus was assessed. The objective was to quantify the potential savings of resources and emissions, thus identifying possible interactions to improve energy, water, food, and environmental issues.

2.3.1. Carbon footprint (CF)

Next, to estimate how much the company could save in GHG emissions from the use of recycled water and ammonium sulfate, an additional scenario was created in which these two inputs were replaced with non-recycled inputs, and the CF was calculated according to Forster and Artaxo (2007) as in Eq. (2)

$$CF = \sum G.G_i \times k_i \tag{2}$$

where G.G.i is the amount of GHG produced and k_i is the CO_2 equivalent coefficient for that gas.

2.3.2. Water footprint (WF)

WF was calculated based on the business water footprint model proposed by Aldaya et al. (2011). This methodology has been chosen because in this way, as opposed to focusing on water use in business operations, taking into consideration the entire supply chain could explore far larger water footprints than the normal operational water footprint. WF is the total volume of freshwater used directly or indirectly for an industrial production expressed in m^3. It is calculated as the sum of the operational (direct) WF (the volume of freshwater consumed or polluted due to business operations), and the supply chain (indirect) WF (the volume of freshwater consumed or polluted to produce all goods and services that constitute inputs, as shown in Eq. (3).

$$WF_{bus\ [u]} = WF_{bus,oper} + WF_{bus,sup} \tag{3}$$

where $WF_{bus\ [u]}$ is the water footprint of the business unit, $WF_{bus,oper}$ is the operational water footprint of that unit (water incorporated into the product, water consumed or polluted through a process), and $WF_{bus,sup}$ is the supply chain water footprint (water footprint of product ingredients purchased by the company, water footprint of other elements of the company for product processing, water footprint of materials and energy for general use). Therefore, to calculate the WF associated with output production, assuming a schematization of the enterprise into multiple business units through which the process is articulated, the total water

footprint ($WF_{bus,tot}$) is calculated by aggregating the WFs of the various business processes. To avoid double counting, virtual water flows between the various business units within the enterprise must be subtracted. The calculation of the output water footprint is shown in Eq. (4).

$$WF_{bus,tot} = \sum_{u} WF_{bus\ [u]} - \sum_{u}\sum_{p} \left(WF_{prod}[u,p] \times Pk[u,p]\right) \quad (4)$$

where $\sum_{u} WF_{bus\ [u]}$ is the WF of business unit u, calculated as in Eq. (3), $WF_{prod}[u,p]$ represents the product WF of product p exiting business unit u and $Pk[u,p]$ represents the volume of product output p from business unit u. The calculations were performed with SimaPro Software 9.2.2.

2.3.3. Energy footprint (EF)

In this study EF was calculated based on the Cumulative Energy Demand (CED) approach, which is an impact indicator that expresses the consumption of fossil energy, from hard coal, lignite, natural gas and crude oil during the entire product life cycle (Boldrin et al., 2022). In this study, the authors calculated it according to Frischknecht et al. (2004), using the 'Cumulative Energy Demand' method (v 1.11) described in Ecoinvent database v. 3.8. Specifically, the energy removed from nature was calculated for each input involved in the production process and multiplied by each production factor. The method was used because, as stated by Huijbregts et al. (2006), it considers the energy consumed directly and indirectly for each step in the life cycle of a process, distinguishing between renewable and nonrenewable energy, which in this study were summed and considered total energy.

2.3.4. Nitrogen footprint (NF)

During the mushroom fermentation phase, ammonia-rich air is released, the emission of which is prohibited by Legislative Decree 152/06. Thus, to comply with these obligations, the air is captured by extraction systems and transferred into the Scrubber working with sulfuric acid (H_2SO_4). This system converts the ammonia (NH_3) into ammonium sulphate ($(NH_4)_2SO_4$), as follows (Eq. (5)):

$$2NH_3 + H_2O + H_2SO_4 \rightarrow (NH_4)_2SO_4 + H_2O \quad (5)$$

In this study, NF was used to assess the total N emissions and related losses along the ammonium sulfate production process for mushroom production (Eq. (6))

$$NF = \frac{NEtotal}{U} \quad (6)$$

where NF is nitrogen footprint (kg N eq), NE_{total} is the total amount of N emissions throughout the entire process of mushroom production from cradle to gate, and U is the functional unit, considering Eqs. (7)–(9).

$$NE_{total} = NE_{inputs} + NVNH_3 \quad (7)$$

$$NE_{inputs} = \Sigma\ NE_{straw} + NE_{manure} \times U \quad (8)$$

where NE_{inputs} is to the cumulative amount of N emissions associated using straw and manure as input per unit of the process (U). The nitrogen emissions include the volatilization loss (NV) of NH_3 (kg N eq), mainly allocated to the use of fertilizers (49 %), manure distribution (30 %), livestock and liquid manure storage (21 %). The NV_{NH3} was calculated as in Eq. (9) from Arunrat et al. (2022).

$$NV_{NH3} = N \times \varphi \times \frac{17}{14} \times 0.833 \quad (9)$$

where N corresponds to the pure amount of nitrogen emissions (kg N eq), φ is the coefficient of NH_3 volatilization loss (0.338), $\frac{17}{14}$ corresponds to the molecular weight ratios of NH_3/N, and 0.833 is the eutrophication potential factor of NH_3 (kg N eq).

3. Results and discussions

Considering LCIA results (Table 2), diesel showed the highest environmental values (50 m^3/FU mainly for heating production departments and operating mechanical shovels), which causes major impacts in 16 out of 18 categories. It mainly affects:

- For 98 % and 97 % on IR and FRS, respectively;
- For 92 % of HCT;
- For 91 % of SOD, FMPF, TAP, and FEC;
- For 90 % of MEC.

For the remaining impact categories, such as GWP, OFHH, OFTE, FEP, TEC, HCNT, and MRS, diesel impacts ranged between 46 and 82 %. The only two categories in which diesel does not have higher impacts than the others are MEP and WC. In both cases, wheat straw is responsible for 50 % of the total impacts. Also, mycelium showed about 38.3 % of the MEP impacts. It is worth noting also the FEP category, where ammonium sulfate accounts for 36 % of the total impacts (1.50×10^{-1} kg P eq out of 4.20×10^{-1} kg P eq total). As for IR, for which diesel fuel has the greatest relative impact (98 % of total IR), it is produced by mining and offshore oil and gas platforms (Chambers et al., 2008). Diesel has also a significant impact on the category of Human Carcinogenic Toxicity, primarily due to the release and inhalation of heavy metals from exhaust gases during combustion (Mohammadi et al., 2019). Additionally, poor waste disposal practices during the oil mining phase can lead to heavy metals entering groundwater near the mining sites (Wang et al., 2005), subsequently accumulating in human organisms. Likewise, heavy metals released from the diesel process/combustion, also create damage to ecosystems, as they are emitted into water and air, and this is reflected in a high value (91 % of the total impact) in FEC (Costa-Böddeker et al., 2020), confirming the value for HCT. Values around 91 %, are related to SOD (4.20×10^{-2} kg CFC11 eq), FPMF (5.78×10^{1} kg $PM_{2.5}$ eq), and TAP (1.71×10^{-2} kg SO_2 eq), which express the release of GHGs.

These values can be attributed to the fact that fossil fuels used emit GHGs such as CO, CO_2, SO_2, NO_x (Dincer and Ratlamwala, 2013). Although trichlorofluoromethane has been chosen as a reference substance for SOD in LCA studies, the Montreal Protocol prohibits its production. Therefore, the values related to SOD, although expressed in CFC_{11} eq, most likely refer to NO_x emissions (one of the main causes of ozone depletion) due to diesel combustion, precisely because CFCs and other ozone-depleting gases (among which NO_x is not included) have been banned by the Montreal Protocol (Ahove and Bankole, 2018) and currently their concentrations in the atmosphere have been reduced. Regarding MEC, diesel accounts for 90 % (1.73×10^{-2} kg 1.4-DCB), very similar percentages to FEC (91 %) although the values are more than three times as high. Again, as with MEC, probably particularly affecting this impact category is the release of heavy metals in wastewater because of the oil extraction and diesel refining process (Pulles et al., 2012). Concerning GWP, OFTE, OFHH, FEP, TEC, HCNT, MRS, diesel affects values between 69 and 82 %. In GWP, 79 % of the contribution is from the direct combustion of diesel in the production process (Ahove and Bankole, 2018), and this is also confirmed by the fact that 11 % is also due to the transportation of the various inputs, which is done by endothermic-engine vehicles. Finally, an additional 6 % is due to the ammonium sulfate production process, which involves the synthesis of pure NH_3 and sulfuric acid at 60 °C. In the case of OFTE and OFHH, they are both expressed as NO_x eq, which is produced during diesel combustion, and in these two categories, as with GWP, these data are confirmed by the transport phase (15 % for OFTE and 14 % for OFHH) during which NO_x is produced. In the case of TEC and HNCT, again diesel is the main culprit, causing the emission of 6.75×10^4, and 3.43×10^3 kg 1.4-DCB eq, respectively due to heavy metals released during the combustion process. For TEC, an additional 9 % is generated from the ammonium sulfate production and an additional 9 % from input

Table 2
Results from life cycle impact assessment.

Impact categories	Unit	Mycelium		Horse Manure	Poultry Manure	Agricultural Gypsum (Calcium Sulfate)		Diesel		Ammonium Sulfate		Wheat Straw		Electricity		Transport		Total
		Value	%			Value	%	Value	%	Value	%	Value	%	Value	%	Value	%	
Atmospheric effects																		
GWP	kg CO_2 eq	1.01×10^{2}	0.40 %	–	–	8.98×10^{0}	0.04 %	2.01×10^{4}	79 %	1.60×10^{3}	6 %	2.07×10^{2}	1 %	8.67×10^{2}	3 %	2.68×10^{3}	11 %	2.55×10^{4}
SOD	kg CFC11 eq	8.07×10^{-4}	2.10 %	–	–	8.52×10^{-6}	0.02 %	3.78×10^{-2}	91 %	4.97×10^{-4}	1 %	1.24×10^{-3}	3 %	6.66×10^{-4}	2 %	1.00×10^{-3}	1 %	4.17×10^{-2}
IR	kBq Co-60 eq	5.59×10^{-1}	0.10 %	–	–	9.04×10^{-2}	0.01 %	1.08×10^{3}	98 %	6.12×10^{0}	1 %	5.14×10^{-1}	0 %	6.71×10^{0}	1 %	3.26×10^{0}	0 %	1.09×10^{3}
OFHH	kg NO_x eq	4.91×10^{-1}	0.50 %	–	–	9.60×10^{-2}	0.09 %	8.58×10^{1}	81 %	2.33×10^{0}	2 %	4.09×10^{-1}	0 %	1.56×10^{0}	1 %	1.54×10^{1}	15 %	1.06×10^{2}
FPMP	kg $PM_{2.5}$ eq	1.90×10^{-1}	0.30 %	–	–	1.17×10^{-1}	0.19 %	5.78×10^{1}	91 %	1.75×10^{0}	3 %	2.97×10^{-1}	0 %	9.34×10^{-1}	1 %	2.08×10^{0}	3 %	6.31×10^{1}
OFTE	kg NOx eq	4.99×10^{-1}	0.40 %	–	–	9.77×10^{-2}	0.09 %	9.10×10^{1}	82 %	2.42×10^{0}	2 %	4.17×10^{-1}	0 %	1.59×10^{0}	1 %	1.55×10^{1}	14 %	1.11×10^{2}
TAP	kg SO_2 eq	5.20×10^{-1}	0.30 %	–	–	5.75×10^{-2}	0.03 %	1.71×10^{2}	91 %	4.54×10^{0}	2 %	1.24×10^{0}	1 %	2.72×10^{0}	1 %	6.87×10^{0}	4 %	1.87×10^{2}
Eutrophication																		
FEP	kg P eq	1.26×10^{-2}	3.00 %	–	–	1.05×10^{-4}	0.02 %	1.95×10^{-1}	46 %	1.52×10^{-1}	36 %	3.47×10^{-2}	8 %	2.59×10^{-2}	6 %	1.00×10^{-3}	0 %	4.22×10^{-1}
MEP	kg N eq	2.68×10^{-1}	38.30 %	–	–	4.15×10^{-5}	0.01 %	7.22×10^{-2}	10 %	5.63×10^{-3}	1 %	3.40×10^{-1}	49 %	9.34×10^{-3}	1 %	4.00×10^{-3}	1 %	6.99×10^{-1}
Toxicity																		
TEC	kg 1,4-DCB	2.56×10^{2}	0.30 %	–	–	1.08×10^{2}	0.13 %	6.75×10^{4}	80 %	7.90×10^{3}	9 %	4.24×10^{2}	1 %	1.02×10^{3}	1 %	7.54×10^{3}	9 %	8.47×10^{4}
FEC	kg 1,4-DCB	2.23×10^{-1}	0.40 %	–	–	2.02×10^{-2}	0.04 %	5.05×10^{1}	91 %	6.90×10^{-1}	1 %	3.96×10^{-1}	1 %	3.78×10^{-1}	1 %	3.57×10^{0}	6 %	5.57×10^{1}
MEC	kg 1,4-DCB	3.28×10^{-1}	0.20 %	–	–	8.64×10^{-2}	0.04 %	1.73×10^{2}	90 %	6.63×10^{0}	3 %	5.76×10^{-1}	0 %	1.41×10^{0}	1 %	1.01×10^{1}	5 %	1.92×10^{2}
HCT	kg 1,4-DCB	6.89×10^{-1}	0.20 %	–	–	2.09×10^{-1}	0.06 %	3.12×10^{2}	92 %	1.36×10^{1}	4 %	1.04×10^{0}	0 %	1.27×10^{1}	4 %	5.50×10^{-1}	0 %	3.41×10^{2}
HNCT	kg 1,4-DCB	2.49×10^{2}	5.60 %	–	–	2.18×10^{0}	0.05 %	3.43×10^{3}	77 %	4.12×10^{2}	9 %	3.45×10^{1}	1 %	1.56×10^{2}	3 %	1.70×10^{2}	4 %	4.46×10^{3}
Abiotic resources																		
LU	m^2a crop eq	1.64×10^{2}	14.60 %	–	–	7.10×10^{-1}	0.06 %	3.92×10^{2}	35 %	6.57×10^{1}	6 %	3.12×10^{2}	28 %	1.92×10^{2}	17 %	–	0 %	1.13×10^{3}
MRS	kg Cu eq	4.80×10^{-1}	1.00 %	–	–	3.42×10^{0}	6.92 %	3.41×10^{1}	69 %	9.01×10^{0}	18 %	6.20×10^{-1}	1 %	1.63×10^{0}	3 %	9.00×10^{-2}	0 %	4.94×10^{1}
FRS	kg oil eq	2.44×10^{1}	0.00 %	–	–	2.91×10^{0}	0.01 %	4.85×10^{4}	97 %	5.31×10^{2}	1 %	2.60×10^{1}	0 %	2.68×10^{2}	1 %	8.22×10^{2}	2 %	5.02×10^{4}
WC	m^3	7.22×10^{0}	7.10 %	–	–	3.47×10^{-2}	0.03 %	2.11×10^{1}	21 %	5.74×10^{0}	6 %	5.06×10^{1}	50 %	1.70×10^{1}	17 %	2.40×10^{-1}	0 %	1.02×10^{2}

transport. Also, for HNCT 9 % (4.12×10^2 kg 1,4-DCB) is generated from the ammonium sulfate production process while a 4 % (1.70×10^2 kg 1.4-DCB) from transportation and a 3 % (1.56×10^2 kg 1.4-DCB) from electricity. Among these data, it is interesting to note how ammonium sulfate affects TEC and HNCT, thus showing adverse effects on ecosystems and human health. In fact, in the first case, although ammonium sulfate, is not harmful to aquatic fauna in the long term, it may be harmful to fish in the short term. In the second case, on the other hand, several studies, have reported that the effects of ammonium sulfate inhalation could include noncancer effects such as asthma (de Vooght et al., 2010), inflammation (Last et al., 1982), and damage to reproductive functions (Bae et al., 2020). Finally, the last category in which diesel has the greatest impact is MRS (69 %) but it is noteworthy that ammonium sulfate also has a nonnegligible impact (9.01×10^0 kg Cu eq, or 18 % of total MRS). MRS is most likely due to the depletion of fossil resources used for diesel and ammonium sulfate production, as this could lead to a general increase in prices, which results in a consequent increase in the extraction of mineral raw materials. Finally, in FEP, MEP, and WC, a major contribution is made by Ammonium Sulfate (36 % in FEP), Mycelium and Wheat Straw (38.3 % and 49 % in MEP), and Wheat (50 % in WC), respectively. Especially, in the case of FEP, 36 % of the contribution (1.50×10^{-1} kg P eq) comes from ammonium sulfate. This is because its production generates a large amount of wastewater with a high concentration of ammonia nitrogen, which, although it can be used as a nutrient of microalgae, high levels could inhibit their growth, as also noted by Guo et al. (2021) and Qin et al. (2021). Regarding MEP, the contribution to the impact categories is divided between Mycelium (2.70×10^{-1} kg N eq, or 38.3 % of the total) and Wheat Straw (3.40×10^{-1} kg N eq, or 49 %). In the former case, the impacts are probably due to the mycelium production that begins with the selection of the growth medium (or coating layer), which is generally represented by sorghum, wheat, or rye seeds (Leiva et al., 2015). Indeed, the physical structure of the seeds is a favorable element for the growth and development of mycelium as a lignocellulosic source. Therefore, MEP for mycelium production could be affected by rye preparation, which requires fertilizers. Likewise, even in the case of straw production, MEP is most likely influenced by the fertilizers used in its formation. Again, for reasons related to grain growth, straw also affects the WC (50 % of the total impacts). Regarding the latter impact category, a residual share of impacts is related to diesel (2.12×10^1 m^3 of water, or 21 % of total WC) and electricity mix production (1.70×10^1 m^3, or 17 %). In this regard, literature studies (Wang et al., 2018) show how some of the depletion of water resources in the electric mix production chain could also be due to the water supply for the cooling towers of power plants, which are fueled by fossil fuels.

3.1. Sensitivity analysis

Since the amount of diesel cannot be changed for production reasons (i.e., drive of machinery), the improvement options focused especially on the use of manure, wheat straw, calcium, ammonium sulfate, and electricity (Table 3), thus indicating three alternative improvement options.

The results of the SA are expressed in Fig. 3. Regarding atmospheric effects (Fig. 3A), the preferred option is S4, as it induces improvements in all 7 impact categories. For example, compared with S1, GWP goes from 2.55×10^4 to 2.50×10^4, or SOD goes from 4.17×10^2 to 4.15×10^2, in both cases with a − 2 % reduction, while for the remaining there is a − 1 % reduction. The observed improvements in environmental performance are most likely attributed to the reduction in electricity consumption from fossil fuels, achieved through the installation of photovoltaic panels, as well as the decreased use of mycelium. Regarding eutrophication and toxicity on the other hand (Fig. 3B), even in these cases, S4 is the preferred option over the other three. In this case, these reductions are most likely due to a lower use of straw, the amount of which was almost halved in three years (−43 %) as well as due to a reduction in the mycelium. As for toxicity, on the other hand, again there are reductions, although more pronounced, between S1 and S4, reductions that are −2 % (TEC), −1 % (FEC, MEC and HCT) and − 3 % (HNCT). Finally, even in the case of abiotic resource depletion, S4 is the preferred option over the other three (Fig. 3C), with lower land consumption (from 1.13×10^3 to 9.31×10^2 m^2a) due to less area for straw cultivation, which therefore also induces water savings of −26 % (from 1.02×10^2 to 7.55×10^2 m^3). The preferred option is S4, as it induces the greatest reduction in impacts compared to the other three. Therefore, the company was able to devise a new recipe, which involves a lower consumption of straw that is balanced by a higher consumption of horse manure, which turns out to have a minimal environmental impact. To ensure product quality, however, it was necessary to keep the compost structure unchanged, and it was, therefore, essential to also change the amounts of poultry manure and gypsum.

The company has also recently installed meters for greater control over electricity consumption as well as photovoltaic systems, so as to reduce electricity consumption, significantly lowering the production of CO_2, resulting in economic and environmental benefits.

Table 3
Variation of input parameters for sensitivity analysis.

Input/Output	Unit	(S1) 2018	(S2) 2019	(S3) 2020	(S4) 2021
Input					
Horse manure	kg	18,000	20,000	20,900	24,000
Wheat straw		3500	3200	2800	2000
Poultry manure		2700	2400	2800	2800
Agricultural Gypsum (Calcium Sulfate)		1200	960	1150	1000
Ammonium sulfate (solid)		800	900	1100	700
Ammonium sulfate (liquid)	m^3	0.11	0.11	0.11	0.16
Mycelium (*Agaricus bisporus*)	kg	980	980	980	900
Diesel	m^3	50	50	50	50
Electricity	kWh	2016	1761	1633	1600
Water	m^3	168	168	168	168
Output					
Substrate	kg	23,000	23,000	23,000	23,000

3.2. Comparison with other LCAs for mushrooms production

The results of our study show that a 23,000 kg substrate emits about 25,049 kg CO_2 eq, and since the yield is 11,000 kg of mushrooms per substrate, it is possible to consider how 1 kg of mushrooms emits 2.28 kg CO_2 eq. In order to assess the validity and consistency of our findings, we conducted a comparative analysis by examining data from other studies that investigated the environmental impacts of various mushroom species. It is worth noting that the existing literature on the assessment of the environmental impacts of mushroom production is relatively limited, and only a few studies have been previously published. Therefore, it was possible to compare and verify our results with a small number of articles available in the literature. For instance, Gunady et al. (2012) examined the carbon emissions of *Agaricus bisporus* in Australia and reported emissions of around 2.72 kg of CO_2 eq per 1 kg of mushrooms. Similarly, Leiva et al. (2015) found emissions of approximately 4.41 kg of CO_2 equivalent per 1 kg of *Agaricus bisporus* in their study. Furthermore, Robinson et al. (2019) investigated the carbon footprint of *Agaricus bisporus* and reported a range of emissions between 2.13 kg and 2.19 kg of CO_2 eq per 1 kg of mushrooms. Additionally, Dorr et al. (2021) explored the emissions of *Pleurotus* and found a range of 2.99 kg to 3.18 kg of CO_2 eq per 1 kg of mushrooms. Comparing our study results with these findings, it is possible to note how our emission estimates for mushroom production are in line with the previous body of research. In these regards, it is worth highlighting those differences in the farm-

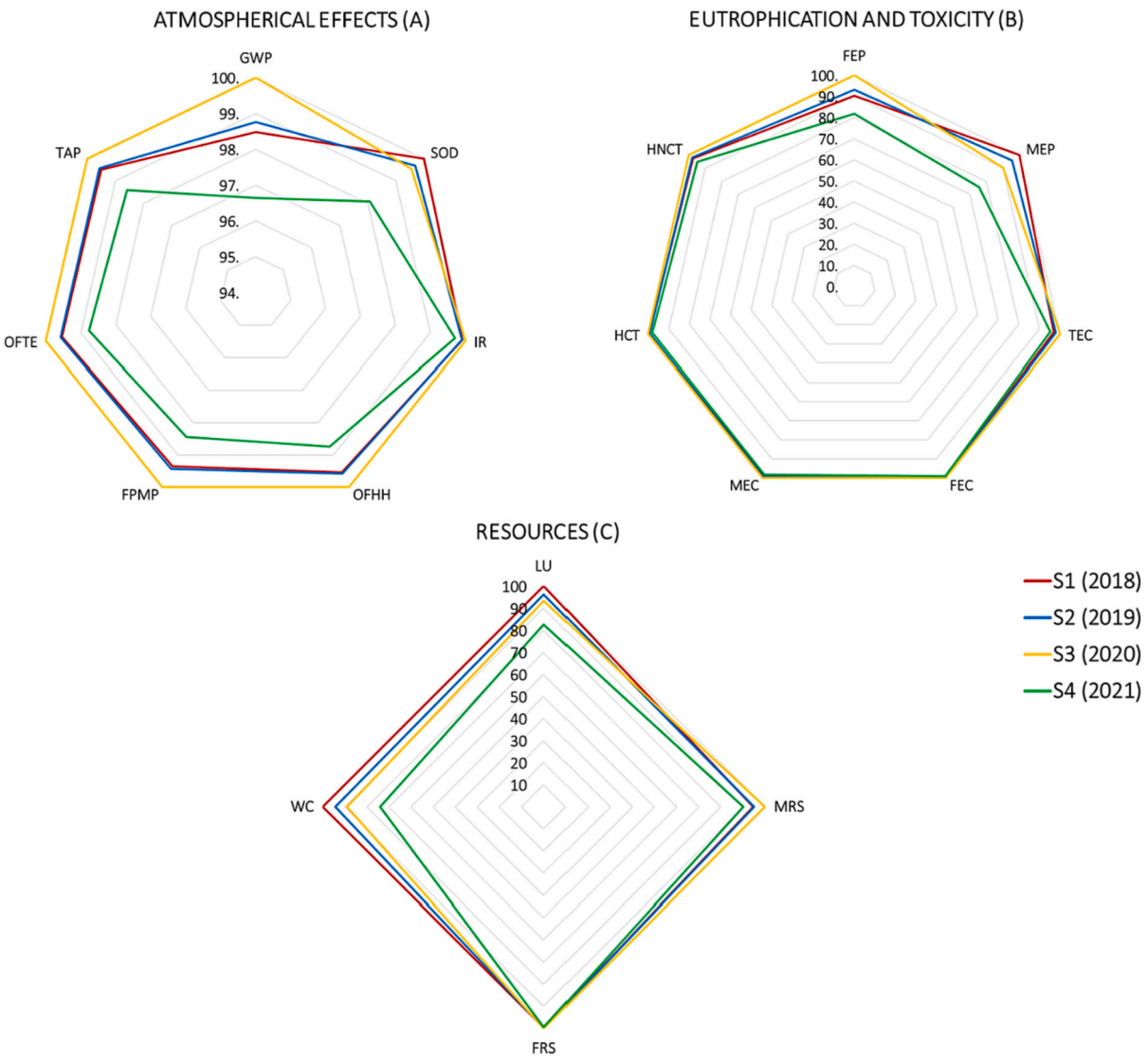

Fig. 3. Sensitivity analysis results.

specific cultivation practices, substrate materials as well as climate conditions, background systems, and modeling choices can represent a source of variability in LCA results. Despite these variations, the overall range of emissions per kilogram of mushrooms remains relatively similar. These results, therefore, are consistent with and reinforce the findings of previous research, arriving at comparable estimates of emissions for mushroom production, contributing to a more comprehensive understanding of the environmental impact of this industry. In addition, it is also important to underline that also pedo-climatic conditions (especially in terms of temperature and humidity) were regarded as key meteorological variables for growing straw mushrooms. In particular, the study of Robinson et al. (2019) observed a relation between GWP intensity and regional variance, mainly influenced by grid electricity generation, fuel, and upstream water consumption. Different requirements in terms of energy demand in certain climate zones could lead to an increase in greenhouse gas emissions, as well as fuel consumption for transport and logistics. For example, considering different energy mixes at the regional level between literature cited countries for mushroom production, mainly Spain, France, Australia, and the U.S.A., it was possible to highlight that regions consuming more renewables (such as Spain: 28 %, and Australia: 29 %) and less coal resulted into lower GWP per megajoule generated (Gunady et al., 2012; Leiva et al., 2015). In this sense, renewable energy technologies, particularly solar and wind power, have a key role in the reduction of GWP, when used as an alternative to conventional fossil fuel-based energy sources. The energy use for the Funghitex production process proves to be markedly lower (0.087 kWh/kg mushrooms) compared to other studies in the literature, on a per kilogram of mushroom basis, reporting energy requirements ranging from 0.26 kWh to 0.56 kWh/kg mushrooms (Robinson et al., 2019; Leiva et al., 2015). The differences observed in emissions between our study and other research are mainly due to two factors: i) different modeling choices, such as data sources, and the selection of GWP conversion factors used for carbon accounting, which lead each study to make specific assumptions or use different methodologies to assess environmental impacts, generating variations in the reported results; ii) regional and geographic factors, which affect the final energy mix. Regions with greater integration of renewables and less reliance on coal-based electricity generally induce a lower GWP per megajoule generated, resulting in lower overall environmental impacts. In Robinson et al. (2019), for example, the location of the production site influences the electricity mix. Producing within a western rather than an eastern region allows more or less renewable energy produced on-site through a biomass gasification unit to be used, thus helping to reduce the electricity demand of the grid, in turn reducing emissions. The above factors, therefore, help to better understand the nuances in emission estimates across studies and regions, emphasizing the importance of

adopting location-specific data, uniform calculation methodologies, and considering regional energy mixes. Furthermore, the availability and use of renewable energy sources (i.e., solar and wind energy), could positively influence the mitigation of impacts associated with GWP. In this sense, Italy has been actively promoting the use of renewables to reduce its reliance on fossil fuels, and in 2021, the Italian electricity system relied on 42.3 % of on-site renewable energy production (GSE, 2021), representing the third-largest producer of renewable energy in Europe. However, the results of our study could also change by considering regional differences in terms of on-site renewable energy production. For example, it is useful to note that northern Italy generally receives less solar irradiance (about 3.6 k kWh/m^2/day) than central and southern Italy (about 5.4 k kWh/m^2/day) (ENEA, 2023), mainly due to higher latitude and the presence of mountainous terrain that can cause shading and reduce available sunlight. Therefore, considering the above, taking into consideration a production of 416 kWh of electricity from photovoltaics by the company in question, should it be in northern Italy it could benefit from slightly less irradiation (by 10–20 % less), producing solar energy of about 332–374 kWh. Conversely, if the Funghitex farming company would be in southern Italy, it could receive slightly more irradiance (by 10–20 % more), producing an energy of 457–499 kWh. In both cases then, there would be an increase or decrease in the amount of net energy, as shown in Table 4, which would affect, albeit slightly, the number of emissions generated (Table 4).

Therefore, by selecting a suitable location with optimal solar irradiation, mushroom farms could maximize their potential for on-site renewable energy production. This can lead to greater energy self-sufficiency and less dependence on external energy sources, which often have associated environmental impacts. However, it is important to note that the solar irradiance values presented are estimates and may vary depending on the specific location and prevailing weather conditions. Factors such as latitude, cloud cover, and shading from surrounding structures or topography can influence the actual solar energy received. Therefore, it is important for mushroom farm operators to conduct site-specific assessments and consider local climate data to accurately determine the expected solar irradiance.

3.3. *WENCF nexus: recycling scenario vs non-recycling scenario*

To quantify the savings in resources and avoided emissions, an additional scenario (S5) was created in which water and ammonium sulfate are added from time to time, assuming also that the same amount of water that is used as input in S4 is purified in S5 as it should actually be. This scenario contrasts with S4, where water and ammonium sulfate are constantly recycled and re-injected. The WENCF nexus between S4 and S5 was then calculated (Fig. 4).

In S4 (Fig. 4A), through wastewater recovery, it might be possible to reduce the overall WF of the entire production process, which is associated with the consequent reduction of the energy used for its extraction as well as the total CF and Nitrogen emissions. This could be attributable to the following reasons.

1. First, the water supply chain is highly energy intensive and has a high environmental impact due to the process of generating electricity (Gilron, 2014), which is required for its extraction (wells), treatment (processes and sludge removal), transmission and distribution, cooling and heating of power towers, resulting in climate-changing emissions (0.3–0.7 kg CO_2 per 1 kWh of energy) (Alresheedi et al., 2022). Moreover, in S5, if the water was not recycled and used as a production input, it would have to be purified as prescribed by legislative decrees 152/1999, 152/2006, and Law 167/2017, and the most surprising finding in our study is about the CF of the final process, which increases from 2.43×10^4 kg CO_2 eq in S4 to 1.46×10^6 kg CO_2 in S5. This is because, as also reported in literature (Alresheedi et al., 2022; Cieri et al., 2022; Zhang and Liu, 2022), wastewater treatment is also associated with high energy and water consumption, leading to a significant amount of GHG emissions. Within this study, on the other hand, a more sustainable wastewater recycling approach is shown to reduce water withdrawals from natural water systems as well as their pollution, due to indiscriminate discharge of untreated wastewater, as shown by WF (7.83×10^1 m^3 in S4 vs 1.76×10^2 m^3 in S5, with a reduction of −46 %). Consequently, direct emissions (generated at wastewater collection and discharge points) and indirect emissions (electricity consumption, use and transfer of chemicals during in-process sludge treatment) are reduced, avoiding at least 1.44×10^6 kg CO_2 eq per FU, recycling water for food production and saving energy (−1.64 MJ eq) for water extraction.
2. Secondly, water recycling also induces the recycling of ammonium sulfate, which is commercially used as fertilizer (21 % N and 24 % S). In the case of the company in question, ammonium sulfate is obtained by the direct reaction between H_2SO_4 and NH_4, the latter being retained and converted by means of a scrubber. Without in-house water recycling (S5), it would need to be supplied externally, whereas, through water recycling (S4), there is a reduction in WF, as well as in energy and climate-changing emissions. in fact, by recycling $(NH_4)_2SO_4$, a dispersion of nitrogen (5.29 × 10–1 kg N eq for S4 vs 7.57×10^{-1} kg N eq for S5, with an N reduction of −43 %) and ammonia into the air would be avoided, also in view of the fact that atmospheric NH_3 levels in Italy were 334.59 kt in 2020, 95 % of which came from the agricultural sector (ISPRA, 2021). By recycling ammonium sulfate instead, the company could contribute to achieving the targets set by the NEC Directive (2016/2284), namely −5 % reduction in ammonia emissions.

Therefore, the results of the scenario analysis show how a circular approach to wastewater reuse could potentially be effective in promoting sustainable resource use, thus establishing an interesting nexus. In fact, it is shown how for food production through wastewater recycling, it might be possible to reduce water to produce energy and to produce food, as well as to reduce energy to produce water and food, ultimately reducing energy use by the water system and water use by the energy system for food production, associated with a reduction in total CF and emissions. It is therefore shown how the recovery of wastewater that is destined for food production could be a way to maximize its economic, environmental, and social value, oriented towards the need to manage water resources in a circular way, rather than transforming it entirely into drinking water. Instead, through a recycling approach, it might be possible to reduce the generation of energy, the use of water that is destined for other food production, as well as to recover ammonium sulfate, thus providing a useful way to contribute to food security, without compromising water and energy security.

Table 4
Energy and GWP changes by irradiance in southern and northern Italy.

Location	Required energy	Energy from solar	Net energy	GWP (FU)
Central Italy	2016 kWh	416 kWh	1600 kWh	25,049 kg CO_2 eq
North Italy		332–374 kWh	1684–1642 kWh	25,067–25,085 kg CO_2 eq
South Italy		457–499 kWh	1559–1517 kWh	25,013–25,031 kg CO_2 eq

4. Conclusions, limitations, and future perspectives

This study assessed the environmental compatibility of *Agaricus bisporus* production in central Italy through an integrated Water-Energy-Nitrogen-Carbon-Food (WENCF) footprint assessment, considering an LCA approach. The results of the analysis show that, per functional unit (23,000 kg of the substrate), diesel impacted more in 16 of the 18 categories considered, highlighting IR and FRS as the largest contributors.

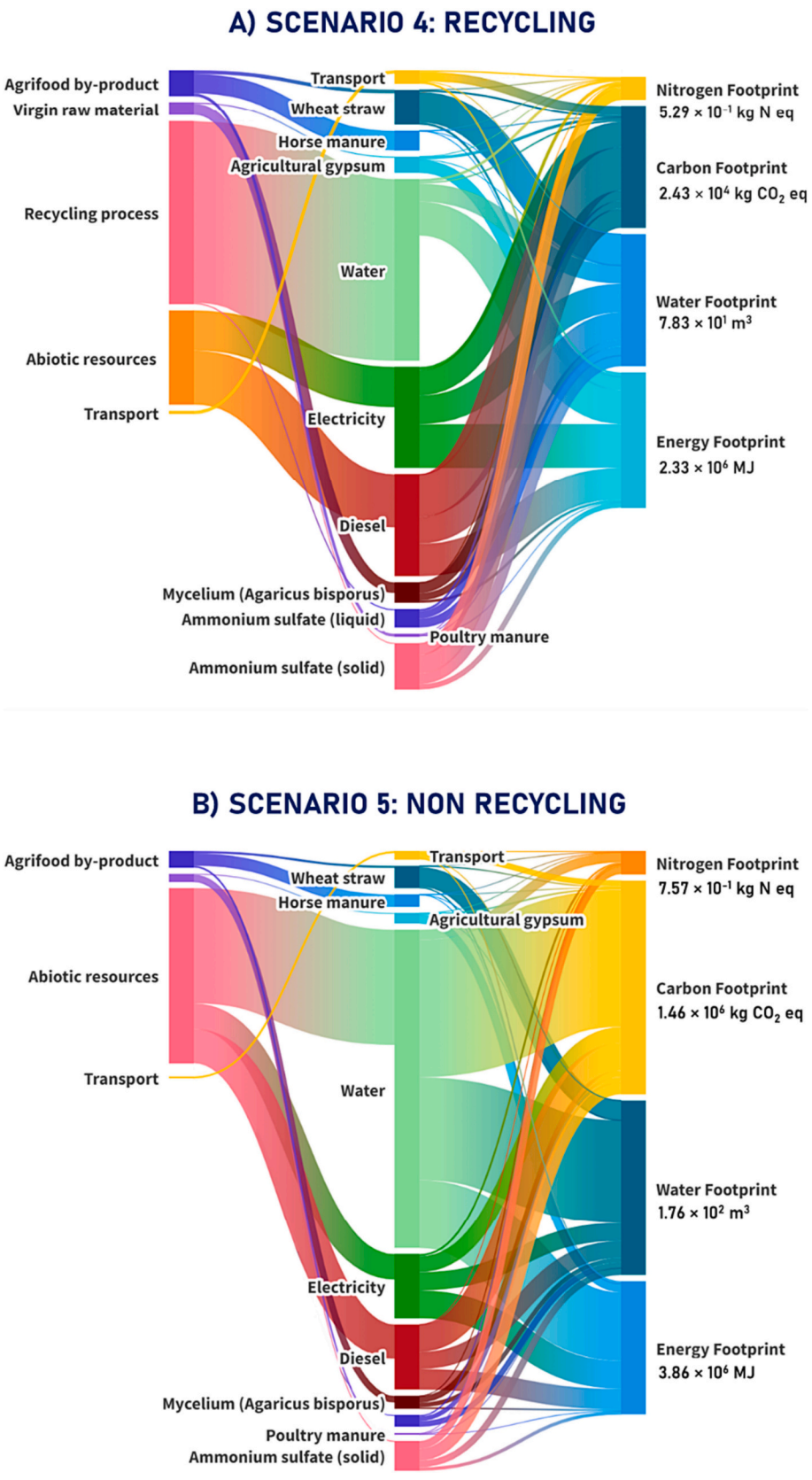

Fig. 4. Difference between the two scenarios. S4: Recycled water and ammonium sulfate; S5: Non-recycled water and ammonium sulfate.

The amounts of straw and ammonium sulfate also showed higher impacts on WC (50 %), LU (28 %) for straw and FEP (36 %), and MRS (18 %) for ammonium sulfate. Considering the environmental responses of mushroom production, a sensitivity analysis was carried out for four scenarios, thus showing that lower consumption of straw (−43 %), agricultural gypsum (−17 %), solid mycelium (−13 %), ammonium sulfate (−8 %) and electricity from fossil fuels (−21 %), offset by higher consumption of horse manure (+33 %) and poultry manure (+4 %), could allow the farm to obtain the same quality of mushrooms, balancing the final compost structure. This resulted in a decrease in environmental impacts of −5 % on average, with values ranging from a low of −1 % to a high of −26 %. Finally, to evaluate the environmental effects of the water and ammonium sulfate recycling strategy, an additional scenario (S5) was created in which these two elements, instead of being continuously recycled, are externally supplied each time ex novo for each production cycle, including that water is dispersed into the environment and purified. Thus, the holistic water-energy-nitrogen-carbon-food nexus was considered. The results show that the recovery strategy (S4) could lead to a reduction of WF by −56 %, which induces a reduction of EF by −40 % and NF by −30 %. Thus, there were three main contributions of the study: it is shown how sustainable food production can result from efficiency improvements within the system rather than from further integration of circular principles. A comprehensive life cycle inventory of mushroom production in Italy is provided so that it can also be used for other LCA studies. In particular, understanding these results could help create useful comparisons to reduce the impacts of the agri-food system. Finally, recommendations are provided to suggest where improvements can be made within the commercial mushroom production system, from cradle to gate. These recommendations include reducing the consumption of straw, gypsum, mycelium, ammonium sulfate, and energy, as well as increasing manure, also being able to open paths of industrial symbiosis in this way. However, it is important to note that although this case study provides valuable insights, its selection is not intended to represent a comprehensive example of mushroom production. In this regard, it is indeed important to recognize the limitations and scope of the study. Indeed, the case study represents a specific production system and may not necessarily be representative of all mushroom production practices worldwide. The findings and conclusions of this study contribute to the existing body of knowledge on environmental impacts in mushroom production, but further research is needed to capture the diversity of production systems in different regions and countries.

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CRediT authorship contribution statement

Giuliana Vinci: Conceptualization, Methodology, Software.

Marco Ruggeri and Sabrina Antonia Prencipe: Data curation, Writing- Original draft preparation.

Federica Perrotta: Visualization, Investigation.

Luigi Pucinischi: Supervision.

Giuliana Vinci: Software, Validation.

Marco Ruggeri and Sabrina Antonia Prencipe: Writing- Reviewing and Editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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3.2 Research article no. 2

Title: Simple and reliable eco-extraction of bioactive compounds from dark chocolate by Deep Eutectic Solvents. A sustainable study.

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Authors: Vinci G., Maddaloni L., **Prencipe S.A.**, Orlandini E. Sambucci M.

Research macro-area: **Sustainability (S), Food Quality (FQ)**

Graphical Abstract:

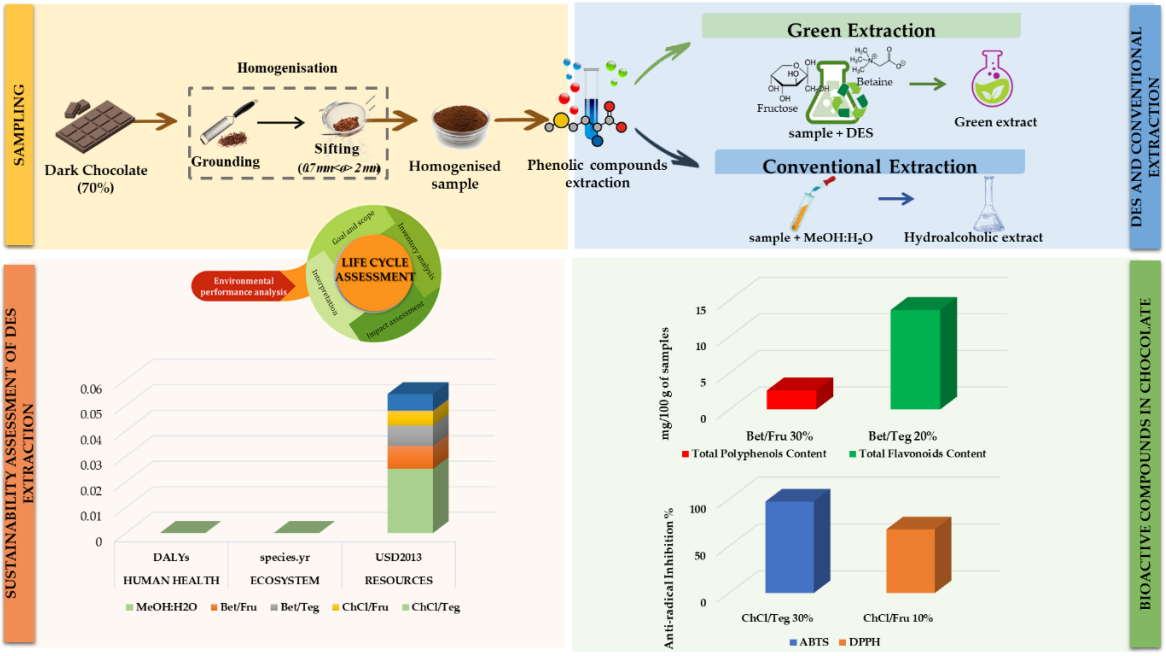

Figure 3.2. Graphical abstract of experimental study no. 2. Source: author's elaboration

International Journal of Food Science +Technology

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Original article

Simple and reliable eco-extraction of bioactive compounds from dark chocolate by Deep Eutectic Solvents. A sustainable study

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Summary The green solvents and eco-extraction methods are gaining increasing interest in chemical analysis for bioactive compounds in food matrices. Deep Eutectic Solvents (DES) developed as a greener and more sustainable alternative to organic solvents, owing to their non-toxic, highly stable, and biodegradation-friendly nature. DES application for polyphenols and antioxidant compounds extraction in dark chocolate samples has been evaluated in an integrated study for sustainability assessment, based on multivariate analysis and Life Cycle Assessment (LCA) methodology. A green extraction method based on DES was proposed testing different HBA:HBD pairs (ChCl:Fru, ChCl:Teg, Bet:Fru, and Bet:Teg). DES Bet:Fru resulted in the highest extraction yield in terms of both total polyphenols (0.34–3.37 g GAE/100 g) and flavonoids (1.13–8.32 g RUT/100 g), $P < 0.05$. Furthermore, the environmental performances of green and conventional solvents (MeOH:H_2O, H_2O, and MeOH) were evaluated by applying a comparative LCA (c-LCA). The c-LCA study highlighted that conventional extraction for polyphenols in dark chocolate was 60% more impactful than DES. DES pairs analysed quantitatively lowest impacted than conventional methods, considering the macro-categories Human Health ($9.99 \times 10^{-8} \div 1.54 \times 10^{-7}$ DALYs), Ecosystem ($2.29 \times 10–10 \div 3.57 \times 10^{-10}$ species.yr), and Resources ($6.57 \times 10^{-3} \div 8.96 \times 10^{-3}$ USD2013).

Keywords bioactive compounds, dark chocolate, Deep Eutectic Solvents, life cycle assessment, sustainability assessment.

Introduction

The increasing awareness of sustainability and environmental issues has promoted the need to replace conventional solvents with eco-compatible, harmless, and non-toxic extraction methods for bioactive compounds (BCs) in foodstuffs. For the chemical analyses of food matrices, conventional organic solvents (e.g., methanol, ethyl acetate, hexane, etc.) are widespread as an efficient extraction solvent for BCs; nevertheless, its significant impact on the environment, operator safety, and high toxicity is not overlooked. Therefore, in recent years, conventional methods have been joined or replaced by innovative green methods, focusing on both analytical parameters, in terms of extraction yield, purification, and sustainability concerns. Indeed, one of the objectives of Green Chemistry is to develop new analytical methods that reduce or completely replace the use of hazardous substances, thus reducing the risks to the environment (Ruesgas-Ramón *et al.*, 2017). In this regard, solvents such as supercritical fluids, Ionic Liquids (ILs), Deep Eutectic Solvent (DESs), and supramolecular liquids are used to replace conventional organic solvents (Paiva *et al.*, 2014; Benvenutti *et al.*, 2019; Manuela *et al.*, 2020). According to the principles of Green Chemistry, a process can be considered green or sustainable, when the procedure involves: (i) reduced use of hazardous solvents/reagents; (ii) energy-efficient design for minimised environmental and economic impact; (iii) biodegradation-friendly design for proper disposal of the waste produced (Socas-Rodríguez *et al.*, 2021).

One of the promising green solvents for efficient extraction of polyphenol and antioxidant compounds from food matrices are Deep Eutectic Solvents (DESs). DESs are mixtures of two substances consisting of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) which, when mixed in a specific molar ratio, have a lower melting point than the individual substances, allowing the formation of a clear solution,

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which is used as an extracting solvent (Alañón *et al*., 2020). The ability of the solvent to form hydrogen bonds with the bioactive compounds allows the analytes to be highly soluble. In addition, DES has a high selectivity; this allows for a decrease in the steps of the extraction procedure, as well as the amount of the solvent to be used and the analysis time (Ijardar *et al*., 2022). Due to their chemical properties and non-toxicity, DES could be suitable for numerous applications, from cosmetics to pharmaceuticals and food ingredients (Panzella *et al*., 2020).

This study was aimed at applying different Deep Eutectic Solvents as eco-extraction methods for simple and reliable polyphenol determination in dark chocolate, with an integrated approach for sustainability assessment.

Cocoa and cocoa-based products (i.e., chocolate) have unique sensory characteristics, whose remarkable nutritional properties have been widely exploited by dieticians and food technologists (Indiarto *et al*., 2021). It is worth noting that cocoa is constituted of about 600 different bioactive compounds, among which polyphenols and alkaloids represent the two main classes, responsible for health and quality-promoting effects on the human organism (Godočiková *et al*., 2020; Soares *et al*., 2022). However, these BCs not only determine the quality of the cocoa derivatives, but also are responsible for the psychoactive properties of food products; focusing on polyphenols, these arouse interest for their antioxidant, anti-inflammatory, and antitumor activity, as well as for their ability to inhibit pathological processes (Martini *et al*., 2018). The main groups of polyphenols in cocoa and its derivatives (i.e., cocoa powder, chocolate bars, etc.) are proanthocyanidins (58% of total dry weight), catechins (37%), anthocyanins (4%), whose content and type may vary depending both on the cocoa cultivar or origin as well as processing of cocoa beans and chocolate manufacturing processes (i.e., fermentation, roasting, conching, alkalinisation, etc.) (Giacometti *et al*., 2015). In particular, it was well established that alkalinisation might cause a progressive reduction of polyphenols, as well as their antioxidant activity (Miller *et al*., 2008). The greatest losses have been observed for epicatechins and catechins content, thus highlighting a reduction of 98% and 80% respectively. The changes in content might be ascribed to the oxidation of phenolic components under basic pH conditions (Giacometti *et al*., 2015; Sioriki *et al*., 2022).

To the best of our knowledge, there are no references in the literature, focusing on the comparison of eco-extraction methods applied for polyphenols in dark chocolate, by considering the green procedure in terms of both extraction efficiency and sustainability performances. In the present study, DES composed of different HBA (Choline Chloride, ChCl; Betaine, Bet), and HBD (Fructose, Fru; and Triethylene glycol, Teg) at specific molar ratios were tested, ChCl:Teg (1:2), ChCl:Fru (1:1), Bet:Teg (1:2), and Bet:Fru (1:1), thus evaluating different operating conditions in terms of time (50, 40, 30 min), temperature (60 °C, 70 °C, and 80 °C), and water content (10%, 20%, and 30%). DES extraction was also compared with conventional extractants (e.g., methanol/water, water, methanol, etc.), to assess the best extraction solvent for polyphenols in dark chocolate, both from a chemical and sustainable perspective. In addition, to evaluate the sustainability assessment as regards DES extraction considered, a comparative Life Cycle Assessment (c-LCA) was carried out to assess the environmental performances of different polyphenols extraction methods, thus evaluating the most sustainable extraction procedure in terms of impacts on Human Health, Ecosystem, and Resources (Vauchel *et al*., 2018).

Materials and methods

Bioactive compounds determination in dark chocolate samples

Reagents

Methanol (≥99.9%), distilled water, 2-Hydroxyethyl) trimethylammonium chloride (Choline chloride - ChCl), carboxymethyl)trimethylammonium hydrochloride (Betaine - Bet; ≥99%), D-(+)- Fructose (Fru; ≥99.5%) and 1,2-Bis(2-methoxyethoxy)ethane (Triethylene glycol dimethyl ether – Teg; ≥98%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocâlteu reagent, sodium nitrite ($NaNO_2$; ≥99.9%), sodium ≥98% carbonate (Na_2CO_3; ≥99.5%), Aluminium chloride ($AlCl_3$; ≥99%), Sodium hydroxide (NaOH; ≥98%), 3,4,5-Trihydroxybenzoic acid (gallic acid - GAE; ≥99%) and Quercetin-3-rutinoside trihydrate (Rutin - Rut; ≥99%) standard were purchased from Sigma-Aldrich (Milan, Italy).

Sample

Dark chocolate samples with 70% of cocoa powder were purchased from local supermarkets. The samples had a total fatty acid content of 40 g/100 g of product, carbohydrates: 31 g/100 g of product, simple sugars: 27 g/100 g of product, protein: 8.6 g/100 g of roduct, and fiber: 0.03 g/100 g of product. The samples were previously frozen at T = −18 °C and then grounded. The resulting powder was separated first with a 2 mm diameter filter and then with 0.7 mm. Chocolate powder with a diameter of less than 0.7 mm was used for sample analysis. The samples were then aliquoted and stored at temperatures below T=−18 °C, until the day of analysis.

Table 1 Composition of Deep Eutectic Solvents

HBA	HBD	Water Content (%)	HBA:HBD molar ratio
Choline chloride	Triethylene glycol	10	ChCl:Teg (1:2)
		20	
		30	
	Fructose	10	ChCl:Fru (1:1)
		20	
		30	
Betaine	Betaine	10	Bet:Teg (1:2)
		20	
		30	
	Fructose	10	Bet:Fru (1:1)
		20	
		30	

HBA, Hydrogen Bound Acceptor; HBD, Hydrogen Bound Donor; ChCl, Choline Chloride; Bet, Betaine; Fru, Fructose; Teg, Triethylene-glycol.

Deep Eutectic Solvents preparation

In this study, two different HBAs, Betaine (Bet) and Choline Chloride (ChCl), and two different HBDs, Triethylene glycol (Teg) and Fructose (Fru), were considered in specific molar ratios and different water content (Table 1). Before the ChCl-based DES synthesis, ChCl was dried under vacuum for 6 h at 80 °C (Manuela *et al*., 2020).

The DES was prepared according to the heating-stirring method (Ruesgas-Ramón *et al*., 2017): the HBD and HBA were placed in a capped round-bottomed flask and heated at T = 80 °C for 15 min under constant stirring (500 rpm). After the DES formation, no purification step was needed, and the mixtures were kept at room temperature in sealed flasks until their use for analysis.

Ultrasound-assisted solid–liquid polyphenol extraction (UAE)

For the Ultrasound-Assisted solid–liquid Extraction by Deep Eutectic Solvents, polyphenols were extracted according to the method previously described by Manuela *et al*., 2020 with some modifications. Briefly, 0.1 g of dark chocolate sample and 10 mL of DES solvent were added to a centrifuge tube, and the extraction was conducted by placing the sample in an ultrasonic and thermostatic bath (400 Hz). After, the samples were centrifuged at 3000 rpm for 10 min at room temperature; the supernatant was collected and filled up to volume in a 10-mL flask. After that, the extracts were analysed for spectrophotometric analyses.

For the optimization of the extraction procedure, the samples were treated at different operating conditions, considering solvents, temperature–time, and % water content. Acronyms used for their descriptions were named as follows: CONV refers to Conventional extraction, and DES to green extraction; solvent used for the extraction: MeOH:H_2O, H_2O, and MeOH (_a), Bet:Teg (_b), Bet:Fru (_c), ChCl:Teg (_d), ChCl:Fru (_e); temperature–time conditions were: T 60 °C for 50 min (_1), T = 70 °C for 40 min (_2), and T = 80 °C for 30 min (_3). Percentage (%) of water content was indicated as follows: 0% (_0), 10% (_10), 20% (_20), 30% (_30), 40% (_40), 100% (_100) (% water) (Table S1).

The DES extracts were compared with three different conventional extraction methods, considering the following solvents: (i) MeOH:H_2O (60:40 *v*/*v*); (ii) MeOH (100%); (iii) H_2O (100%). The conventional extraction procedures were conducted as follows: 0.1 g of sample was extracted with 5 mL of conventional solvent, homogenized in an ultrasonic bath (400 MHz), and centrifuged at 2900 ***g*** for 10 min. The supernatant was collected in a 10 mL flask, and the extraction procedure was repeated twice. The final volume was adjusted to 10 mL with conventional solvents.

The polyphenol extraction was carried out considering the above-mentioned operating conditions for DES extraction.

Total phenolic content by Folin Ciocâlteu assay

Total phenolic content (TPC) was conducted for all the DES and conventional extracts according to Ciano *et al*., 2022. Briefly, 0.5 mL of polyphenolic extract was mixed with 0.25 mL of Folin Ciocâlteu reagent in a 10 mL volumetric flask. After 3 min, 0.5 mL of an aqueous solution of sodium carbonate (7.5% *w*/*v*) was added and the flask was kept in darkness for 30 min. Then, it was filled up to volume with distilled water. The absorbance of the samples was read at λ = 750 nm in a UV–visible spectrophotometer (Jenway, Stone, UK). The results were expressed as grams of gallic acid equivalents per 100 g of dark chocolate extract (g GAE/100 g), and the results were obtained through a calibration curve ranging from 10 to 100 mg/L (R^2 = 0.9997).

Total flavonoid content by aluminum chloride assay

In all the DES and conventional extracts Total Flavonoid Content (TFC) was evaluated according to the Aluminium Chloride method described by Ozbek & Ozmen (2022). Briefly, 0.5 mL of the extract, 2 mL of distilled water, and 150 µL $NaNO_2$ (5%, *w*/*v*) were added to a volumetric flask. The solution was mixed and incubated in the dark for 5 min; then 150 µL of $AlCl_3$ (10%, *w*/*v*) was added and the solution was kept in the dark for 5 min. After that, 2 mL of 1 M NaOH was added to the solution and incubated for an additional 15 min, and then filled to a final volume of 5 mL. The absorbance of the extracts was recorded at 510 nm. The TFC results were expressed as grams of

Rutin equivalents per 100 g of dark chocolate extract (g Rut/100 g), by linear regression, ranging between 50 and 1000 mg/L ($R^2 = 0.9995$).

Radical scavenging activity by ABTS and DPPH assays
To assess the radical scavenging activity of the conventional and DES extracts were conducted by DPPH and ABTS assay following the methods described by Ciano *et al*., 2022. The DPPH radical scavenging assay was conducted as follows: 1.5 mL of DPPH reagent was added to 1 mL of polyphenol extract and kept in darkness for 30 min. The absorbance was read at 517 nm in a UV–visible spectrophotometer. Indeed, the ABTS radical scavenging was determined as followed: 3.6 mL of ABTS reagent was added to 0.4 mL of polyphenol extract and kept in darkness for 15 min. The absorbance was recorded at 734 nm.

The DPPH and ABTS scavenging capacity were expressed as a percentage of inhibition (I %) as in eq.1:

$$I\% = \frac{A_0 - A_f}{A_0} \times 100. \quad (1)$$

where A_0 is the ABTS or DPPH radical cation's initial absorbance, and A_f is the absorbance after the addition of sample extract.

Statistical analysis
The conventional and DES extractions were performed in triplicate as the spectrophotometric analysis. The results were expressed as mean value ± standard deviation ($n = 3$). Pearson's correlation coefficient was used to analyze the linear correlation within the obtained results. As result, the One-Way ANOVA test was applied, followed by the Mann–Whitney parawais posthoc test was possible to identify the significant difference ($P < 0.05$) between variables. Furthermore, multivariate analysis (Principal Component Analysis; PCA, and Cluster Analysis; CA) was performed on the results obtained for the different extraction conditions. Acronyms used for PCA and CA have been previously described (Section 2.1.4.) and were shown in Table S1. The statistical analyses were carried out with XLSTAT software (Addinsoft, 2022).

Sustainability evaluation of polyphenol extraction procedures using life cycle assessment methodology

Materials
The sustainability of conventional and green extraction methods for the evaluation of phenolic content in dark chocolate was assessed by applying the Life Cycle Assessment (LCA) methodology (2015ISO 14040:2006; I2006SO 14044:2006, 2006). The sustainability assessment was performed by using the software SimaPro 9.2.2. (Prè Sustainability B.V.).

Methods
Life Cycle Assessment was considered a standard tool for environmental impact assessment, and it should involve four phases: (i) Goal and scope definition; (ii) Life Cycle Inventory (LCI); (iii) Life Cycle Impact Assessment (LCIA), and (iv) Interpretation of results.

Goal and scope definition. The aim was to assess the environmental, health, and economic implications of the different polyphenol extraction methods used. In the study, the conventional solvent (MeOH:H_2O, 60:40 *v*/*v*) was compared with 4 DES pairs (Bet:Teg, Bet:Fru, ChCl:Teg, and ChCl:Fru) at 30% of water content, to identify possible hot spots of the polyphenol extraction process, as well as assessing the potential mitigation of environmental, health and economics impacts by applying green solvents. The functional unit (FU) considered was one extraction procedure for both polyphenols' extraction methods. The procedures took into consideration a *gate-to-gate* approach (Fig. 1).

Life Cycle Inventory (LCI). The main inputs and outputs for both polyphenols' extraction methods, conventional and DES, are shown in Table 2. LCI calculations were performed using data from EcoInvent v3.8, and ELCD databases, to model inputs for equipment, solvents, and electricity. Electricity consumption varied depending on operating conditions (i.e., ultrasonic bath, centrifugation, etc.), as detailed in Table 2. In the present work, the same quantity of dark chocolate samples (0.1 g) was used for the five extraction methods; therefore, as a comparative LCA was performed, dark chocolate was not included in the system boundaries.

Life Cycle Impact Assessment (LCIA). The ReCiPe 2016 Endpoint (H) V1.05 method was used for the impact calculations, thus considering the following impact categories: Global warming, Human health (GW, HH); Stratospheric ozone depletion (SOP); Ionising radiation (IR); Ozone formation, Human health (OF, HH); Fine particulate matter formation (FPMF); Human carcinogenic toxicity (HCT); Human non-carcinogenic toxicity (HCNT); Water consumption, Human health (WC, HH); Global warming, Terrestrial ecosystems (GW, TE); Global warming, Freshwater ecosystems (GW, FE); Ozone formation, Terrestrial ecosystems (OF, TE); Terrestrial acidification (TA); Freshwater eutrophication (FE); Marine eutrophication (ME); Terrestrial ecotoxicity (TE); Freshwater ecotoxicity (FET); Marine ecotoxicity (MET); Water consumption, Terrestrial ecosystem (WC, TE); Water consumption, Aquatic ecosystems (WC, AE); Land use (LU); Mineral resource scarcity (MRS); Fossil resource scarcity (FRS). These categories are

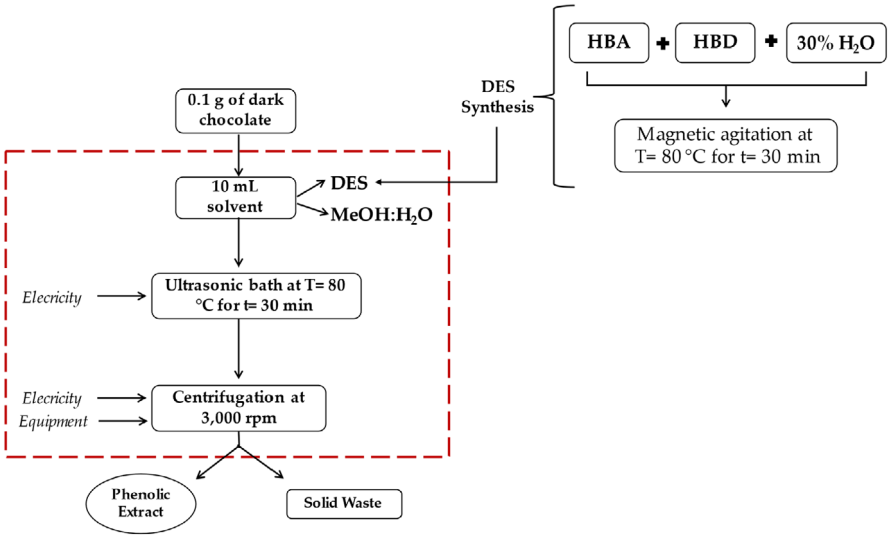

Figure 1 *Gate-to-gate* system boundaries considered for the polyphenols extraction methods in dark chocolate.

Table 2 LCI of conventional and DES extraction methods (Inputs referred to the FU: No. 1 extraction procedure)

Extraction conditions		Unit	MeOH:H_2O (60:40)	Bet/Fru (1:1)	Bet/Teg (1:2)	ChCl/Fru (1:1)	ChCl/Teg (1:2)
Inputs							
Solvents	H_2O	kg	0.0040	0.0030	0.0030	0.0030	0.0030
	MeOH	kg	0.0047	/	/	/	/
	Fru	kg	/	0.0035	/	0.0035	/
	Bet	kg	/	0.0023	0.0020	/	/
	Teg	kg	/	/	0.0049	/	0.0047
	ChCl (k)	kg	/	/	/	0.0027	0.0022
Equipment	Glass Pasteur kg pipettes		0.00873 (n. 3)	0.0029 (n. 1)	0.0029 (n. 1)	0.0029 (n. 1)	0.0029 (n. 1)
	Ultrasonic bath	kWh	0.00664	0.04	0.04	0.04	0.04
	Centrifugation	kWh	0.4995	0.04995	0.04995	0.04995	0.04995
	Homogenisation	kWh	0.0664	/	/	/	/
Total electricity		kWh	0.57254	0.08995	0.08995	0.08995	0.08995
Outputs							
Phenolic extract (mL)	10			10	10	10	10

H_2O, distilled water; MeOH, Methanol; Fru, Fructose; Bet, Betaine; Teg, Triethylene-glycole; ChCl, Choline Chloride.

considered endpoint indicators showing the environmental impacts on three macro-categories: Human Health (HH), Ecosystems (Es), and Resources (Rs). Results for the macro-category HH are expressed in Disability-adjusted life years (DALYs); for the macro-category Es results are expressed in species lost in a year (species.yr); for the macro-category Rs, results are in US Dollar 2013 (USD2013).

Result and discussion

Aiming to develop an eco-extraction method for dark chocolate polyphenols, different types of DES were tested as an alternative to organic solvent, commonly applied in the extraction of polyphenols from food matrices (Luo *et al.*, 2020). It is worth noting that the selection of DES varies depending on the chemical and physicochemical characteristics of the DES itself and the characteristics of the analytes to be extracted. Therefore, in this process, the highest DES extraction efficiency resulted from harmonisation of several important factors, such as HBD and HBA pairs choice, the molar ratio of DES mixture, temperature, and water content. In particular, this latter is strictly linked to viscosity, pH, polarity, and surface tension of DES mixture used (Zainal-Abidin *et al.*, 2017).

The extraction yield of the proposed solvents was evaluated by spectrophotometric assays (TPC, TFC,

Table 3 Quantitative results for total phenolic content for conventional and DES extraction methods

Solvent			Extraction conditions		
			60 °C × 50 min	70 °C × 40 min	80 °C × 30 min
Conventional	MeOH:H_2O		0.66 ± 0.07	0.87 ± 0.06	0.92 ± 0.18
	H_2O		0.85 ± 0.12	0.98 ± 0.14	0.99 ± 0.26
	MeOH		0.56 ± 0.09	0.65 ± 0.03	0.74 ± 0.18
Bet/Teg		10%	1.06 ± 0.06	1.25 ± 0.18	0.93 ± 0.16
		20%	1.16 ± 0.08	1.19 ± 0.09	1.06 ± 0.05
		30%	0.99 ± 0.13	1.21 ± 0.11	0.95 ± 0.14
Bet/Fru		10%	0.34 ± 0.02	nd	nd
		20%	2.36 ± 0.15	2.39 ± 0.21	1.08 ± 0.08
Green		30%	1.69 ± 0.11	2.59 ± 0.23	1.76 ± 0.05
ChCl/Teg		10%	nd	nd	nd
		20%	nd	nd	nd
		30%	0.06 ± 0.01	0.20 ± 0.06	0.07 ± 0.01
ChCl/Fru		10%	1.42 ± 0.17	1.09 ± 0.04	0.07 ± 0.02
		20%	0.86 ± 0.13	0.50 ± 0.10	0.15 ± 0.03
		30%	0.15 ± 0.02	2.35 ± 0.21	0.03 ± 0.01

Results are expressed as g GAE/100 g ± standard deviation (n = 3).
nd = not detectable.

ABTS, and DPPH assays), which revealed as simple and reliable methods for the analysis of bioactive compounds in food matrices (Yang *et al*., 2020).

Total polyphenols content in dark chocolate

Table 3 shows the TPC (g GAE/100 g of dark chocolate) obtained for conventional and different DES extracts. In the case of total polyphenols content, the results showed that the DES pairs examined resulted in three times higher extraction efficiency than conventional solvents (MeOH, H_2O, and, MeOH:H_2O). The amount of extracted polyphenols (0.07–2.59 g/100 g of dark chocolate) differs considerably in green solvents, thus highlighting that the selection of DES pairs is crucial to obtain a higher extraction efficiency in comparison to conventional solvents (Manuela *et al*., 2020). Among DES, the highest extraction yield was obtained for Bet/Fru at 30% hydration (1.76–2.59 g/100 g of dark chocolate), followed by ChCl/Fru at 10% (1.09–1.42 g/100 g), and Bet/Teg at 10% hydration (1.06–1.25 g/100 g dark chocolate). DES pair composed of ChCl/Teg at both 10% and 20% of water content revealed not applicable for polyphenols extraction in dark chocolate. It is important to note that the composition of the DES can seriously affect the FC assay. Indeed, when ChCl-based DES was used, the formation of a precipitate was observed, which caused interferences with the vertical spectroscopy lecture in the microplate reader. This phenomenon could be ascribed to ion exchanges between ChCl and potassium carbonate, leading to the formation of insoluble salts. Hence, the TPC quantification of plant biomass extracts by the FC method can lead to misinterpretation (mostly overestimation), so caution must be taken, especially when working with ChCl-based DES as extracting solvent. This could be probably attributable to the fact that DES pairs formed by Choline Chloride and Triethylene glycol gave interference with the Folin-Ciocâlteu assay. Different studies reported that the HBA-HBD pairs could interfere with the binding reaction between the reagent and the extractant (Mahesar *et al*., 2016; Zheng *et al*., 2021). When a ChCl-based DES was used, the formation of a precipitate was observed, which caused interference with the spectrophotometric analysis. This phenomenon could be attributed to ion exchanges between ChCl and potassium carbonate, which lead to the formation of insoluble salts (Ruesgas-Ramón *et al*., 2020).

Several extraction methods have been optimised for phenolic compounds, using DES as extraction solvent. The choice of DES type strictly depends on the phenolic class under consideration (Della Posta *et al*., 2022). In particular, considering the study of Manuela *et al*. (2020), who analysed cocoa by-products, Betaine-based DES resulted in the case of total polyphenols, with the highest extraction efficiency (15.33–22.82 mg/g dw); as well as it also resulted in the highest extraction efficiency of total procyanidins (0.3–1.41 mg/g dw). While considering ChCl-based DES mixture, Ruesgas-Ramón *et al*. (2020) optimised a procedure for the extraction of chlorogenic acids from coffee and cocoa co-products, thus showing ChCl-based DES as the most effective for the extraction of phenolic acids (i.e., chlorogenic acid, ferulic or

caffeic acids). These results were also confirmed by Khezeli *et al.* (2016), highlighting ChCl:Ethylene glycol (1:2), as the highest extracting solvent of phenolic acids from vegetable oils.

Moreover, temperature is reported to be the parameter that has the greatest influence on extraction yield. According to the literature, as the extraction time increases, the extraction yield tends to increase (Della Posta *et al.*, 2022). Nevertheless, high temperatures can degrade the analytes of interest causing the opposite effect. This factor can be controlled by operating on the time–temperature interaction. Based on studies in the literature, working at 60 °C for 1 h for polyphenols extraction from cocoa products (Ruesgas-Ramón *et al.*, 2017; Manuela *et al.*, 2020), we observed a particular extraction efficiency, increasing the temperature to 70 °C and reducing the extraction time to 40 min. This is probably because high temperatures affect the viscosity and surface tension of DES by enhancing the interaction between the green solvent and the target molecules (Chu *et al.*, 2022).

It was also found that the polyphenols extraction yield from 70% dark chocolate varied according to water content, which might affect the viscosity of the DES mixture and its polarity, thus playing a major role in the extraction of these molecules from food matrices (Ruesgas-Ramón *et al.*, 2017). The moisture content and the rheology of the solvents are considered, in fact, crucial factors in determining their extraction efficiency. In terms of physical–chemical properties, it is desirable that the solvent does not present high viscosity, since the lower the viscosity, the greater its diffusivity and therefore the stronger the efficiency of solubilization and extraction of the analyte of interest in the matrix (Cunha & Fernandes, 2018). There are several studies investigating the relationship between solvent rheology and extraction efficiency. Santana *et al.* (2019) characterized, by thermogravimetric analysis (TGA), infrared spectroscopy, and rheometry measurements, natural Deep Eutectic Solvents (NADES) prepared according to three different methods: controlled heating and stirring, ultrasound-assisted synthesis (UAS), and microwave-assisted synthesis (MAS). The results indicated that the solvent synthesised by ultrasound-assisted technique provided a lower viscosity value than other samples. Then, UAS was recognizedas fast and efficient synthesis technique, showing promise as new methods for the synthesis of NADES. Dai *et al.* (2015) investigated the effect of water content on the structure and characteristics of NADES. The authors found that the physicochemical properties can be tailored in a controllable way when diluted with water. The addition of water weakened the hydrogen bonding interactions between the solvent's components, lowering the viscosity and increasing the extraction ability. The "best" results were detected for 5% (v/v) of water dilution where NADES reached the highest solubilization capacity. Besides, the water content represents also the main factor for the best selectivity of extracting compounds (Ruesgas-Ramón *et al.*, 2017). According to Zainal-Abidin *et al.* (2017), DES containing high water content (about 25%) have a significant tendency to extract highly-polar compounds; on the contrary, DES with low percentage of water (<10%) showed high selectivity for less-polar compounds. Other researchers ascertained that the application of external stresses, including heating (Savi *et al.*, 2019) or physical forces (stirring or microwave radiations) (Dai *et al.*, 2015) improved the extraction efficiency. Specifically, higher temperature increases the entropy of the solvent system (increasing molecular motion) and consequently reduces the viscosity because of the weakening of the intermolecular bonds in the solvent. The above-reviewed literature survey investigating the effect of the physicochemical properties of solvents concerning their extraction efficiency will be considered as a basis on which to build future experimentation on our solvents. In this regard, thermal (e.g., TGA) and rheological analysis will be performed to study how synthesis parameters (e.g., water content) as well as process variables can maximize the extraction capacity of our solvents.

Total flavonoids content

To assess the different interactions of DES solvents with phenolic compounds, the total flavonoids (TFC) assay was performed using the Aluminum Chloride method and subsequent detection at $\lambda = 510$ nm. Table 4 shows the results of the TFC for the different DES pairs and conventional solvents under different extraction conditions.

The study showed that the DES examined were able to extract flavonoids from the 70% dark chocolate sample in different yields, thus highlighting a different interaction of DES pairs with molecules belonging to the flavonoid class (Zheng *et al.*, 2021). It was found that the HBA-HBD pair composed of Betaine and Triethylene glycol at 10% and 20% hydration, respectively had the best extraction yield of flavonoids for all three extraction conditions, which was about 3.5 times higher than the conventional extraction with MeOH: H2O at 60:40 *v*/*v*. Furthermore, it was seen that the extraction yield differs based on the hydration content of the DES, this being likely because the water content influences the viscosity and hydrogen bond formation between HBA and HBD by interacting differently with the target molecules (Panzella *et al.*, 2020). Concerning the three extraction conditions adopted in the study (60 °C × 50 min; 70 °C × 40 min and 80 °C × 30 min), they resulted in different extraction

Table 4 Total flavonoid content for conventional and DES extraction methods

			Extraction conditions		
	Solvent	**Water content (%)**	**60 °C × 50 min**	**70 °C × 40 min**	**80 °C × 30 min**
Conventional	MeOH:H_2O		3.73 ± 0.43	4.07 ± 0.22	4.35 ± 0.26
	H_2O		5.83 ± 0.62	6.08 ± 0.62	6.29 ± 0.44
	MeOH		2.47 ± 0.35	3.05 ± 0.41	3.57 ± 0.12
Green	Bet/Teg	10%	12.83 ± 1.09	12.40 ± 1.21	12.05 ± 0.98
		20%	13.12 ± 1.25	13.64 ± 1.23	10.43 ± 1.14
		30%	7.73 ± 0.45	6.82 ± 0.97	5.43 ± 0.34
	Bet/Fru	10%	2.63 ± 0.32	4.63 ± 0.65	1.13 ± 0.22
		20%	8.35 ± 0.74	5.79 ± 0.52	13.18 ± 1.11
		30%	4.14 ± 0.23	6.88 ± 0.67	5.26 ± 0.36
	ChCl/Teg	10%	4.88 ± 0.39	10.03 ± 1.03	4.92 ± 0.13
		20%	6.18 ± 0.84	4.14 ± 0.56	8.92 ± 0.47
		30%	7.87 ± 0.93	6.37 ± 0.63	7.57 ± 0.68
	ChCl/Fru	10%	1.90 ± 0.23	4.70 ± 0.21	2.35 ± 0.17
		20%	9.50 ± 1.05	5.05 ± 0.33	4.25 ± 0.21
		30%	3.17 ± 0.37	4.55 ± 0.25	5.81 ± 0.13

Results are expressed as g Rut/100 g ± standard deviation ($n = 3$).
H_2O, distilled water; MeOH, Methanol; Fru, Fructose; Bet, Betaine; Teg, Triethylene-glycole; ChCl, Choline Chloride.

yields among the DES pairs considered, and the increase in flavonoid extraction yield from dark chocolate to 70% did not follow the increase in extraction temperature or extraction time. The highest yield was obtained for extraction at 80 °C × 30 min with the DES consisting of Betaine and Triethylene glycol hydrated at 20% (13.64 ± 1.34 g RUT/100 g).

This result highlighted the importance of selecting the right HBA-HBD pair to extract the target compounds from food matrices; in fact, the literature showed that using Triethylene glycol as the HBD and varying the HBA between Betaine and Choline Chloride resulted in a different interaction between polar and non-polar phenolic compounds, affecting the extractive yield of TFC (Ruesgas-Ramón *et al.*, 2017). The composition of the DES greatly influences its polarity and thus its interaction with the target analytes. As was also found in the case of phenolic compounds, polar DES (i.e., DES based on organic acids) extract polar analytes better, while DES with medium or low polarity (i.e., DES based on polyalcohols) extract less polar analytes more efficiently (Della Posta *et al.*, 2022).

Different studies in literature highlighted the great efficiency of DES composed of organic acids as HBD (i.e., triethylene glycol) for flavonoids extraction from food matrices (Zhao *et al.*, 2015; Mansur *et al.*, 2019). Zhao *et al.* (2015) optimised a green extraction of Rutin from Sophora japonica flower buds, and based on 20 different DES, consisting of organic acids, sugars, polyalcohols and amines such as HBD, good results were obtained in terms of extraction efficiency using ChCl-triethylene glycol-based DES as extraction solvent. The worst results were obtained with sugar-based DES, probably due to their high viscosity. Mansur *et al.* (2019) selected ChCl-triethylene glycol-based DES as the most promising solvent for the extraction of several flavonoid classes in common buckwheat sprouts, thus revealing DES composed of organic acids such as HBD, were found to be unsuitable for flavonoid extraction. This could be explained by the low polarity of the target analytes, which are more to DES based on polyalcohols than those based on organic acids.

Radical scavenging activity by ABTS and DPPH assays

In addition to the determination of the total polyphenol and flavonoid content, the extraction capacity of the different DES on antioxidant compounds was studied by means of ABTS and DPPH assays.

For the ABTS assay, the results showed a different extraction yield of antioxidant compounds between the different DES (Table 5). Particularly, the highest anti-radical scavenging was determined in the 70% dark chocolate extracts obtained with the ChCl:Teg (91.33%–97.40% ABTS radical inhibition %, respectively). For this DES, no remarkable inhibition percentage changed between the different hydration rates of DES or the three conditions under which the extraction was conducted were revealed. Furthermore, the Bet/Teg pair also showed good anti-radical capacities, between 90.42% and 94.66% inhibition of the ABTS radical, for the 30% hydration, showing similar extraction capacities with the conventional solvent MeOH:H2O. The data also show that the formation

Table 5 ABTS assay result as inhibition percentage (I%) of ABTS radical ± standard deviation ($n = 3$)

			Extraction Conditions		
Solvents	**MeOH:H_2O**		**60 °C * 50 min**	**70 °C * 40 min**	**80 °C * 30 min**
			99.76 ± 0.12	**98.76 ± 0.41**	**98.54 ± 0.36**
Conventional	H2O		81.16 ± 0.34	83.16 ± 0.33	86.16 ± 0.14
	MeOH		40.77 ± 0.34	43.77 ± 0.11	45.77 ± 0.09
		10%	70.80 ± 0.31	76.13 ± 0.2 7	76.56 ± 0.28
	Bet/Teg	20%	75.45 ± 0.46	75.55 ± 0.39	76.21 ± 0.11
		30%	90.42 ± 0.32	94.66 ± 0.33	94.25 ± 0.51
		10%	3.48 ± 0.31	nd	nd
	Bet/Fru	20%	58.95 ± 0.21	64.08 ± 0.12	65.54 ± 0.36
Green		30%	79.75 ± 0.33	85.52 ± 0.24	65.96 ± 0.33
		10%	96.49 ± 0.32	95.79 ± 0.14	95.20 ± 0.25
	ChCl/Teg	20%	93.98 ± 0.41	92.17 ± 0.033	91.33 ± 0.36
		30%	97.40 ± 0.38	95.79 ± 0.21	96.28 ± 0.23
		10%	nd	10.05 ± 0.09	63.23 ± 0.11
	ChCl/Fru	20%	17.64 ± 0.12	54.14 ± 0.36	84.23 ± 0.07
		30%	75.68 ± 0.23	83.96 ± 0.07	86.36 ± 0.40

nd, not detectable.

of Deep Eutectic Solvents affects the extraction capabilities of antioxidant compounds (Hassani *et al*., 2019; Trombino *et al*., 2022).

As regards the scavenging activity of the extracts by DPPH radical assay, differences were found between extractions with conventional solvents and DES (Table 6). However, extracts obtained with DES showed a lower anti-radical capacity than conventional solvents MeOH:H_2O, and MeOH. Among the DES, ChCl/Fru at 10% (67.78%) and ChCl/Fru at 30% (61.74%) ChCl/Teg at 30% (51.00%), and Bet/Teg at 30% (42.00%) showed a higher percentage of DPPH radical inhibition. The extraction condition, which led to a better result in the anti-radical capacity for DES extracts, is 60 °C for 50 min.

In addition, for some DES pairs at different hydration rates, it was not possible to determine the antioxidant capacity because the components of the DES pair (HBA-HBD) may in turn interact with the assay reagent leading to the formation of the precipitate that

Table 6 DPPH assay result in inhibition percentage (I%) of DPPH radical ± standard deviation ($n = 3$)

			Extraction Conditions		
Solvents			**60 °C * 50 min**	**70 °C * 40 min**	**80 °C * 30 min**
	MeOH:H2O		73.99 ± 0.18	83.99 ± 0.34	93.99 ± 0.58
Conventional	H_2O		11.53 ± 0.07	15.53 ± 0.08	31.53 ± 0.27
	MeOH		67.13 ± 0.23	77.13 ± 0.53	87.13 ± 0.38
		10%	nd	nd	nd
	Bet/Teg	20%	nd	nd	nd
		30%	42.02 ± 0.16	37.80 ± 0.09	35.40 ± 0.08
		10%	nd	nd	nd
	Bet/Fru	20%	nd	nd	nd
		30%	nd	nd	nd
Green					
		10%	21.40 ± 0.08	21.00 ± 0.08	23.01 ± 0.07
	ChCl/Teg	20%	25.50 ± 0.05	31.80 ± 0.22	26.30 ± 0.14
		30%	51.00 ± 0.12	24.10 ± 0.22	19.40 ± 0.09
		10%	67.78 ± 0.35	60.72 ± 0.24	46.16 ± 0.11
	ChCl/Fru	20%	nd	nd	nd
		30%	61.74 ± 0.38	45.81 ± 0.12	nd

n.d., not detectable.

alters the data reading (Chen *et al*., 2021; Zheng *et al*., 2021). Indeed, the different results between the ABTS and DPPH assays are partly due to analytes to which the reagents bind, respectively ABTS with hydrophilic and lipophilic antioxidants while DPPH with only lipophilic. Furthermore, the DPPH radical does not react with phenolic acids and therefore the anti-radical capacity between ABTS and DPPH is partially different (Godočiková *et al*., 2020). In addition, the differences in anti-radical capacity in the two assays could be attributed not only to the extraction capacity of the target molecules by DES, but also to the interference between the reagents and other molecules present in the chocolate samples that are co-extracted from the test matrix, such as methylxanthines and pigments (Pierre *et al*., 2015).

Statistical analysis

The Pearson correlation coefficient (p) between the TPC, TFC, ABTS, and DPPH assays methods was calculated from the results obtained for the three-extraction condition considered. Table S2 showed the results of the Pearson correlation for all the extraction conditions. Considering the three different extraction conditions, the results of TPC, TFC, ABTS, and DPPH were positively correlated ($P < 0.05$). Specifically, for the extraction at T = 60 °C * 50 min, TPC was closely correlated to the ABTS ($r = 0.902$, $P < 0.05$) and DPPH ($r = 0.856$, $P < 0.05$) assays, as well as for the extraction at T = 70 °C * 40 min. In contrast, TFC correlated strongly with ABTS assay ($r = 0.991$, $P < 0.05$) for extraction conditions at T = 80 °C * 30 min. Furthermore, the results were subjected to the ANOVA One-way test, which showed that there were significant differences between the data averages for all the extraction conditions T = 60 °C * 50 min, ($P < 0.001$); T = 70 °C * 40 min ($P < 0.001$), and T = 80 °C * 30 min ($P < 0.001$). Therefore, the Mann–Whitney parawais *post hoc* test was subsequently performed to assess the significance of the different variables. The significant differences ($P < 0.05$) between the several variables are shown in Table S3. For the extraction at T = 60 °C * 50 min, the significant difference results for DPPH-ABTS ($P = 0.0298$, $P < 0.05$), TFC-ABTS ($P = 0.0051$, $P < 0.05$) and TFC-TPC ($P = 0.0098$, $P < 0.05$). Instead, for extraction at all differences between variables were found to be statistically significant ($P < 0.05$). Instead, for extraction at T = 80 °C * 30 min, the statistically significant variables ($P < 0.05$) are DPPH-ABTS ($P = 0.0154$), TFC-ABTS ($P = 0.0051$), TFC-DPPH ($P = 0.0001$) and TFC-TPC ($P = 0.0157$). To better understand the results obtained and to investigate the correlation between the spectrophotometric assays and the extraction under different operating conditions of the bioactive compounds with conventional extraction procedures and DES, PCA was applied. The analysis was performed on the matrix of observations for all extractions using Pearson's correlation. Furthermore, as some extractions present missing data, these were estimated by the software algorithm based on the arithmetic mean. Figure 2 shows the results of the PCA.

Comparing both graphically and *via* Kaiser's rule (*eigenvalue* > 1) the principal components resulting from the analysis, the cumulative variability obtained for the following model is 76.4%, which is explained by the first three components PC1, PC2, and PC3. The results showed that the samples were evenly distributed along the principal components. Despite this, three groupings can be identified. However, the grouping of variables in the third quadrant is negatively correlated with both PC1 and PC2. In the first quadrant, grouping is positively correlated with both PC1 and PC2. Finally, the grouping in the fourth quadrant is positively correlated with PC1 and negatively correlated with PC2.

Subsequently, a clustering analysis (CA) was performed on the results obtained from the PCA, based on the agglomeration method (weighted pair-group average) while the proximity type is based on similarity using Pearson's correlation coefficient. The results of the CA are shown in Fig. 3. The CA confirmed the PCA data by showing three different clusters. The dendrogram was divided based on the similarity principle, around values of 0.7118. Within Cluster 1 (C1), the element of similarity is represented by lower extraction temperature (60 °C) for a long time (50 min). In contrast, Cluster 3 (C3) is grouped according to high extraction temperature (80 °C) for short time (30 min). Within Cluster 2 (C2), dissimilar statistical units result, and therefore the variable points cluster to the closest cluster for low similarity value.

Sustainability analysis

In recent years, the application of DES solvents has been widely studied for the extraction of bioactive compounds from food matrices as they are regarded as "green" solvents with numerous advantages in their application (Ang *et al*., 2021). These compounds are classified as green solvents because they are non-toxic and biodegradable, but to date, there has been no comprehensive analysis of their real impact (Vauchel *et al*., 2018). Table 7 reported the impact results for all the eighteen damage impact categories studied for conventional and green polyphenols extraction.

For the LCIA, the ReCiPe 2016 Endpoint, with the 100-year perspective (H) V1.05 method was used for the impact calculations. The results showed that

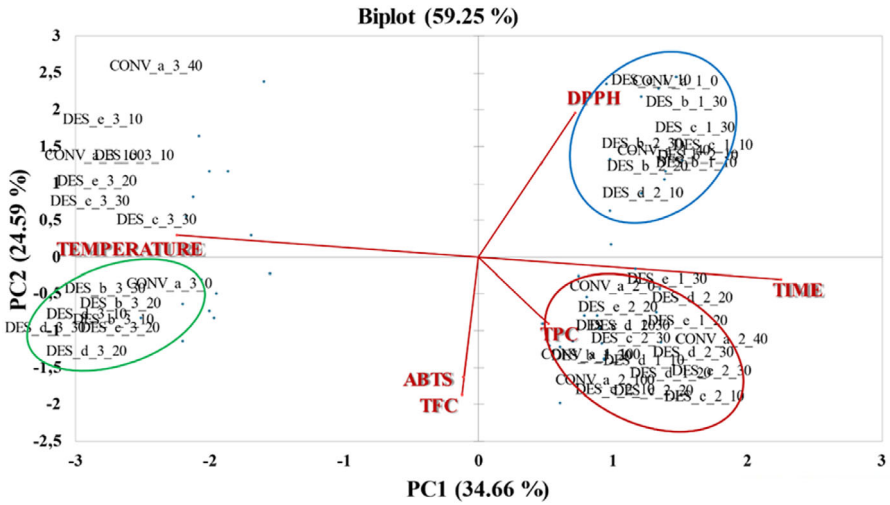

Figure 2 Biplot of Principal Component Analysis: Group in quadrant 1 (blue trace); Group in quadrant 3 (red trace); Group in quadrant 4 (green trace).

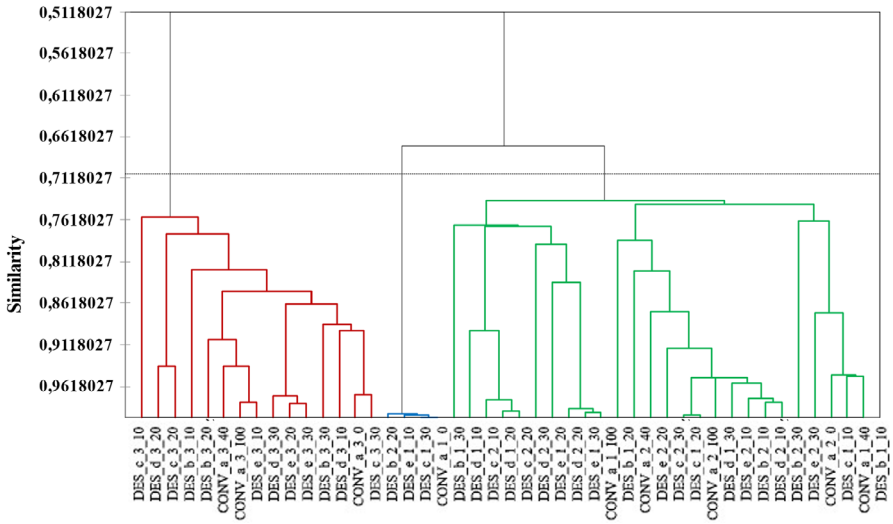

Figure 3 Dendrogram of Cluster analysis: C1 (red trace); C2 (blue trace); C3 (green trace).

conventional extraction (MeOH:H2O) had the highest impact for all eighteen categories considered, except for "Marine Eutrophication" category where the extraction method with the highest impact is attributed to the HBA-HBD composed by Bet/Fru. This is probably since both Betaine and Fructose are compounds of plant origin and therefore their production involves all those inputs typical of crops (such as fertilisers, insecticides, herbicides, etc.) that affect marine eutrophication, due to the accumulation in the soil and subsequent release into the sea from rivers and coastal settlements of nitrogen compounds, phosphorus and other phytostimulants (Kralisch *et al.*, 2015; Murugan *et al.*, 2021).

These results showed that DES extraction has a lower damage impact than conventional solvent, however, to better understand the different impacts on the "Human Health", "Ecosystem" and "Resources", the eighteen damage impact categories were grouped into three macro-categories to identify DES with a lower impact (Fig. 4). The results showed that the green extraction methods have a lower impact, ranging between 65% and 80%, than conventional extraction, however, among DES the HBA-HBD pair composed

Table 7 Impact of DES and conventional extraction for all the 18 damage impact categories

Damages impact category	Unit	MeOH:H^2O	Bet/Fru	Bet/Teg	ChCl/Fru	ChCl/Teg
Human health						
Global warming, Human health		2.34 E-07	7.25 E-08	6.13 E-08	5.17 E-08	5.14 E-08
Water consumption, Human health		5.8 E-09	2.32 E-09	1.81 E-09	1.33 E-09	1.29 E-09
Human carcinogenic toxicity		1.28 E-08	5.3 E-09	4.37 E-09	3.51 E-09	3.53 E-09
Human non-carcinogenic toxicity	DALY	2.11 E-08	6.74 E-09	6.31 E-09	4.08 E-09	5.01 E-09
Ionising radiation		2.24 E-10	5.18 E-11	5.13 E-11	4 E-11	4.47 E-11
Ozone formation, Human health		4.34 E-10	1.59 E-10	1.3 E-10	1.08 E-10	1.05 E-10
Fine particulate matter formation		1.68 E-07	6.68 E-08	5.1 E-08	4.21 E-08	3.85 E-08
Ecosystem						
Ozone formation, Terrestrial ecosystems		6.28 E-11	2.34 E-11	1.91 E-11	1.58 E-11	1.54 E-11
Terrestrial acidification		1.66 E-10	5.82 E-11	4.42 E-11	4.19 E-11	3.63 E-11
Freshwater eutrophication		3.63 E-11	1.44 E-11	1.16 E-11	9.33 E-12	9.23 E-12
Marine eutrophication		6.87 E-15	1.48 E-14	5.04 E-15	1.2 E-14	4.29 E-15
Terrestrial ecotoxicity		1.57 E-12	8.37 E-13	5.69 E-13	4.83 E-13	3.85 E-13
Freshwater ecotoxicity	species.yr	1.36 E-12	5.43 E-13	4.36 E-13	3.73 E-13	3.55 E-13
Marine ecotoxicity		2.88 E-13	1.13 E-13	9.21 E-14	7.63 E-14	7.47 E-14
Global warming, Terrestrial ecosystems		7.06 E-10	2.19 E-10	1.85 E-10	1.56 E-10	1.55 E-10
Global warming, Freshwater ecosystems		1.93 E-14	5.98 E-15	5.05 E-15	4.26 E-15	4.24 E-15
Land use		3.74 E-11	2.95 E-11	8.19 E-12	2.74 E-11	7.12 E-12
Water consumption, Terrestrial ecosystem		1.51 E-11	1.12 E-11	7.89 E-12	5.04 E-12	4.69 E-12
Water consumption, Aquatic ecosystems		9.78 E-16	7.47 E-16	5.02 E-16	3.47 E-16	2.95 E-16
Resources						
Mineral resource scarcity	USD2013	1.69 E-05	8.03 E-06	6.13 E-06	6.74 E-06	5.78 E-06
Fossil resource scarcity		2.51 E-02	8.95 E-03	8.00 E-03	5.79 E-03	6.57 E-03

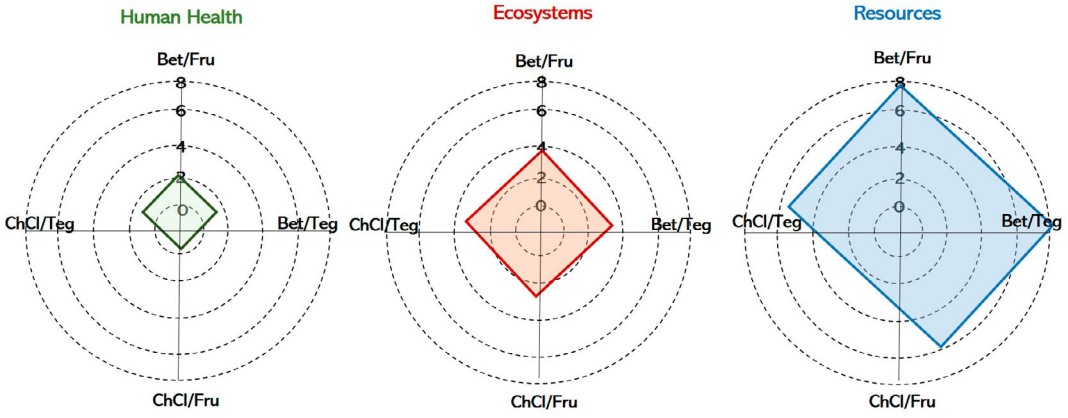

Figure 4 Macro-categories results for all the DES extraction methods.

of Bet/Fru is the most impactful for all the three macro-categories. For the "Human Health" macro-categories, Bet/Fru has about 35% higher impact than the DES composed of ChCl as HBA and 19% higher than Bet/Teg. Indeed, the lower impact for the "Ecosystem" macro-categories was obtained with ChCl/Teg, which has 36% lower impact than Bet/Fru. For the macro-categories "Resources" a lower impact than Bet/Fru was obtained for the DES composed of ChCl/Fru (−35%). This is probably due to the process's synthesis of the molecules of which DES is composed (Kralisch *et al.*, 2015). These could be attributed to the different origins of these molecules; furthermore, chemical LCA studies have some limitations attributable to the absence of some chemical inputs (Parvatker & Eckelman, 2018). In fact, to consider all HBA-HBD pairs in the LCA study, the inputs, following the reaction stoichiometry, for the synthesis of Chlorine Chloride and Betaine were reconstructed, as they are absent within the software

databases. However, the creation of these new chemical 'processes' may result in estimated missing data in the life cycle inventory phase, leading to greater variability in the output data (Kralisch *et al*., 2015; Parvatker & Eckelman, 2018).

Conclusions

In this work, the application of DES revealed as a sustainable and greener alternative for the extraction of bioactive compounds from dark chocolate samples. The study highlighted DES dual advantages in terms of both extraction yield and environmental assessment. Based on the results obtained, both DES pairs consisting of betaine and choline chloride as HBA exhibited, on average, a 35% higher extraction yield than conventional solvents. The impact of different operating conditions in terms of time, temperature, and water content significantly influenced the extraction performance of DESs, as multivariate analyses confirmed. Herein, the sustainability assessment of solvents for polyphenols eco-extraction from chocolate was carried out for the first time. The comparative-LCA study highlighted the conventional solvent as 60% quantitatively more impactful than DES on the eighteen damage impact categories considered, especially in terms of mineral and fossil resources availability. Furthermore, results revealed DES pairs of natural origin (ChCl:Fru, and Bet:Fru) as the best extractive solvents both in terms of extraction efficiency and environmental performance. Due to their plant-based synthetic nature, these green compounds could be directly used in the formulation of foods, and additives as well as in cosmetics and pharmaceutical preparations. Therefore, this work presents a valuable step towards the application of simple and reliable eco-extraction methods for bioactive compound determination in food matrices, thus promoting their direct adoption in the agro-industrial application and ready-to-use extracts for functional food.

Future experiments will be aimed at optimising the efficiency of the synthesised solvents, analytically studying the influence of viscosity (and therefore water content as well as process parameters) on their extraction performance.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Giuliana Vinci: Conceptualization (lead); data curation (lead); supervision (lead); validation (lead); writing – original draft (lead); writing – review and editing (lead). **Lucia Maddaloni:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); software (equal); writing – original draft (equal). **Sabrina Antonia Prencipe:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); software (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal). **Eleonora Orlandini:** Formal analysis (equal); investigation (equal); methodology (equal). **Matteo Sambucci:** Conceptualization (equal); data curation (equal); writing – review and editing (equal).

Ethical approval

Ethical approval was not applicable to the present study.

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.16315.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Acronyms used for Principal Component and Cluster Analyses.

Table S2. Person correlation ($P < 0.05$) for all the extraction conditions.

Table S3. Significance difference ($P < 0.05$) between variables of all the extraction condition.

3.3 Research article no. 3

Title: A Multimethodological Approach for the Valorization of "Senatore Cappelli" Wheat Milling By-Products as a Source of Bioactive Compounds and Nutraceutical Activity

Year of publication: 2023

Journal: International Journal of Environmental Research and Public Health, MDPI

Authors: Vinci, G., **Prencipe, S. A.**, Armeli, F., & Businaro, R.

Research macro-area: **Sustainability (S), Food Quality (FQ), Food Safety (FS)**

Graphical abstract:

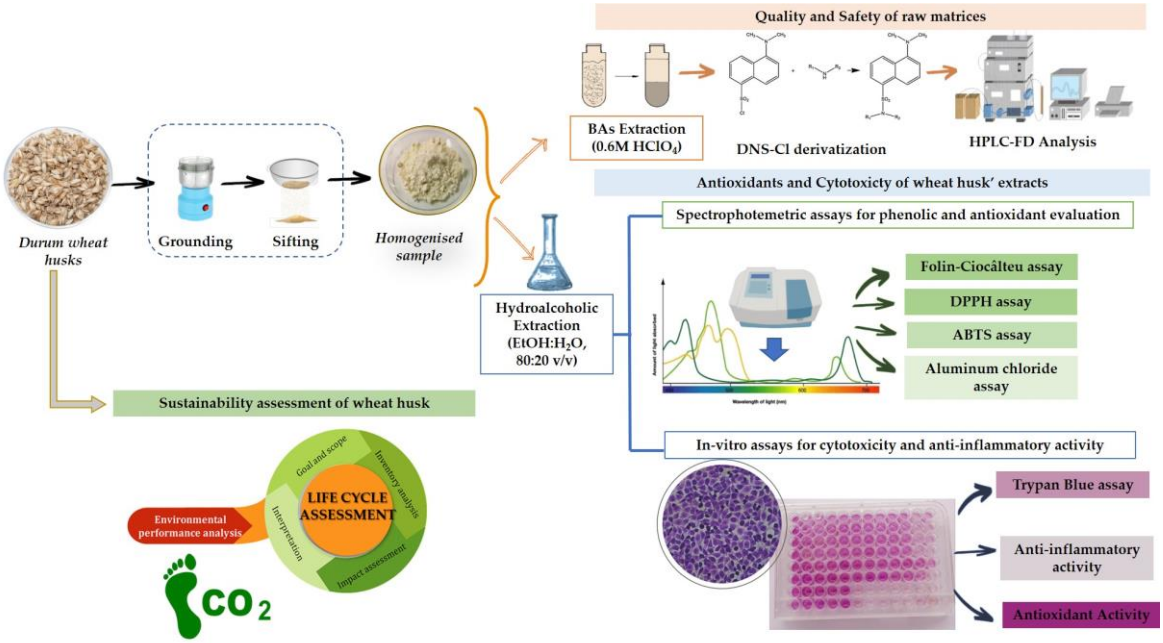

Figure 3.3. Graphical abstract of experimental study no. 3. Source: (Vinci G, Prencipe SA, Armeli F, Businaro R., 2023)

International Journal of
Environmental Research and Public Health

MDPI

Article

A Multimethodological Approach for the Valorization of "Senatore Cappelli" Wheat Milling By-Products as a Source of Bioactive Compounds and Nutraceutical Activity

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Abstract: Wheat is the third most cultivated cereal in the world and represents the major contributor to human nutrition. Milling wheat by-products such as husks (17–20% of the total processing output weight), even if still containing high-value-added bioactive compounds, are often left untreated or unused, thus resulting in environmental and human health burdens. In these regards, the present study is aimed at evaluating in a multimethodological approach the nutraceutical properties of durum wheat husks belonging to the ancient cultivar "Senatore Cappelli", thus assessing their potential as bioactive compound sources in terms of phytochemical, cytotoxic, and nutraceutical properties. By means of HPLC-FD analyses, wheat husk samples analyzed revealed a higher content of serotonin, amounting to 35% of the total BAs, and were confirmed to occur at biogenic amines quality index (BAQI) values <10 mg/100 g. In addition, spectrophotometric assays showed a significant variable content in the phenolic (189.71–351.14 mg GAE/100 g) and antioxidant compounds (31.23–37.84 mg TE/100 g) within the wheat husk samples analyzed, according to the different cultivar areas of origin. Considering wheat husk extracts' anti-inflammatory and antioxidant activity, in vitro analyses were performed on BV-2 murine microglia cells cultured in the presence or absence of LPS, thus evaluating their ability to promote microglia polarization towards an anti-inflammatory phenotype. Cytotoxicity assays showed that wheat extracts do not affect microglia viability. Wheat husks activity on microglial polarization was assessed by analyzing the expression of M1 and M2 markers' mRNA by RT-PCR. Wheat husk antioxidant activity was assessed by analysis of NRF2 and SOD1 mRNA expression. Moreover, the sustainability assessment for the recovery of bioactive components from wheat by-products was carried out by applying the life cycle assessment (LCA) methodology using SimaPro v9.2.2. software.

Keywords: wheat milling by-products; antioxidant activity; biogenic amines; microglia polarization; cytokines; anti-inflammatory activity; environmental sustainability; carbon footprint

Citation: Vinci, G.; Prencipe, S.A.; Armeli, F.; Businaro, R. A Multimethodological Approach for the Valorization of "Senatore Cappelli" Wheat Milling By-Products as a Source of Bioactive Compounds and Nutraceutical Activity. *Int. J. Environ. Res. Public Health* **2023**, *20*, 5057. https://doi.org/10.3390/ijerph20065057

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1. Introduction

Cereals and cereal-based products represent one of the major components of human nutrition, thus representing the base of the food pyramid and accounting for more than 55% of the total consumption in the Mediterranean diet. Among cereals, durum wheat (*Triticum turgidum* L. subsp. *durum*) has a core role in the Italian diet and in the national economy, thus resulting in a production ranging between 3850 and 3900 million tons in 2021, with an increase of around +1.5% over 2020 [1].

Numerous studies have confirmed that cereals exert a protective action on human health, and they are a rich source of bioactive components, thus providing an excellent amount of dietary fiber, proteins, and antioxidants that can have health-promoting effects (i.e., cholesterol-lowering properties, anti-inflammatory effects, chronic diseases prevention, etc.) [2]. In particular, the reasons for the protective effects of cereals on human health

could be mainly ascribed to the physio-chemical properties and structure of the grain (quantity, grain size, type of fiber, amount, and quality of phytochemical compounds as well as amylopectin and amylose content) [3]. Recent studies concerning the health benefits of wheat-based functional products have been increasingly focusing on the importance of introducing phytochemical compounds through the use of different wheat cultivars. Consequently, there is a renewed interest in the ancient varieties, particularly with regard to their potential nutraceutical quality [4].

During the milling process, most cereals, including wheat, undergo a series of treatments aimed at separating the outer fractions of the seed from the endosperm, intended for processing and transformation into cereal-based products (i.e., flour, pasta, bread, etc.). Nevertheless, increasing mechanization and industrialization have provided both food technologists and researchers with challenging problems arising from the production of processing by-products [5]. In particular, wheat husk (WH) is the major by-product of wheat milling. It is the outer layer of the grain, also called pericarp, that surrounds the endosperm and germ of the wheat grain. In whole wheat kernels, the WH is a multilayered tissue that accounts for 15–20% of the total processing weight, representing nowadays about 30 million tons of wheat milling by-products produced in the European Union [6]. Considering its physicochemical and organoleptic properties, WH consists of raw lignocellulosic material with a compact structure made up of cellulose (36–39%), hemicelluloses (18–21%), and lignin (16%) [7], which still contains a high content of bioactive compounds, particularly antioxidants such as phenolic compounds, carotenoids, etc. [7,8]. In these regards, different studies in the literature focus on the re-use of agricultural waste including wheat husks for renewable energy production [5,7], biofuel production, and biogas generation [9] as well as for the production of functional and value-added food products, cosmetics, feed for livestock use, natural bio-fertilizers, etc. [10].

Nevertheless, these residues or agro-industrial wastes are often left untreated or unused, so disposal is through dumping on land, incineration, or landfilling, thus resulting in the deposition of contaminants in the ecosystem and human health [11]. Therefore, the valorization of agricultural by-products through the extraction and recovery of molecules with high nutritional value (e.g., polyphenols, antioxidants, serotonin, etc.) as a new resource to be reused in other production processes could represent an alternative to incineration or composting.

In these regards, the present study is aimed at evaluating in a multimethodological approach the nutraceutical properties of two ancient Italian durum wheat husks belonging to the ancient cultivar "Senatore Cappelli" from two different cultivation areas (Puglia and Tuscany), thus assessing their potential as bioactive compound sources in terms of phytochemical, antioxidant, and anti-inflammatory properties. To this purpose, the content of total phenolic compounds (TPC) and total flavonoid (TFC) and antioxidant activity were carried out by ABTS and DPPH assays.

Neurological disorders such as AD and PD are characterized by the accumulation of misfolded proteins that contribute to chronic microglial hyperactivation. The release of pro-inflammatory mediators that trigger neuroinflammation, exacerbated by oxidative stress, leads to neuronal death [12]. Much attention is now focused on bioactive molecules present in functional foods and in industrial processing waste for their anti-inflammatory and antioxidant properties to counteract neuroinflammation [13]. Considering that wheat husk extracts anti-inflammatory and antioxidant activity, in vitro analyses were performed on BV-2 murine microglia cells cultured in the presence/or absence of lipopolysaccharide (LPS), thus evaluating their ability to promote microglia polarization towards an anti-inflammatory phenotype. Neuroinflammation is the main driver of several chronic neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and major depression. Microglial cells possess the mechanisms to worsen the inflammation or on the contrary to lead to the repair of the damage depending on the stimuli they receive from the microenvironment [14]. When microglia cells are activated by an inflammatory stimulus, they take on a pro-inflammatory M1 phenotype associated with the expression

of markers such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) that mediate inflammatory signaling through Toll-like receptor 4 (TLR4) [15]. The M1 phenotype is also associated with the release of pro-inflammatory cytokines, interleukins, and chemokine ligands such as CCL2 [16].

When the injury resolves, the microglia polarizes toward an anti-inflammatory M2 phenotype and, with the release of anti-inflammatory cytokines associated with Arginase-1 (Arg-1) and expressed by macrophages, plays a key role in immune response regulation, primarily through the competition between intracellular iNOS and Arg-1 for arginine. M2-activated microglia upregulate the expression of another anti-inflammatory mediator, namely CD206, a mannose receptor pattern-recognition receptor [17]. An additional anti-inflammatory marker is chitinase-like 3 (Chil3), which encodes for the protein Ym1 [18]. To evaluate M1 or M2 states in microglial cells untreated and extract-treated in the absence or presence of LPS, we analyzed the mRNA levels of M1 and M2 markers. In addition, we investigated the antioxidant effect of wheat-husk-derived extracts by analyzing the mRNA expression of key genes involved in the cellular antioxidant system.

In addition, to evaluate the quality and safety of raw matrices, the detection of eight biogenic amines, namely 2-phenylethylamine (B-Pea), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermine (Spm), spermidine (Spd), and serotonin (Ser), was investigated by means of high-performance liquid chromatography coupled with fluorometric detection (HPLC-FD).

Moreover, the sustainability assessment for the recovery of bioactive compounds from wheat by-products was carried out through the application of the life cycle assessment (LCA) methodology by using SimaPro v9.2.2. software.

2. Materials and Methods

2.1. Chemicals

2-phenylethylamine (B-Pea), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermine (Spm), spermidine (Spm), serotonin (Ser), Dansyl-Chloride (DSN-Cl), sodium bicarbonate ($NaHCO_3$), ammonium hydroxide (NH_4OH), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), Folin–Ciocalteu reagent ($H_3[P(W_3O_{10})_4]/H_3[P(Mo_3O_{10})_4]$, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (ABTS), sodium nitrite ($NaNO_2$), and aluminum chloride ($AlCl_3$) were purchased from Sigma-Aldrich (Milan, Italy). The following solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA): acetone (C_3H_6O), perchloric acid ($HClO_4$), acetonitrile, ACN (CH_3CN), methanol (CH_3;OH), and distilled water (d-H_2O), all of which were HPLC-grade. Further used materials include Dulbecco's Modified Eagle's Medium High Glucose (DMEM, Sigma Aldrich, St. Louis, MO, USA); Fetal Bovine Serum (FBS, Sigma Aldrich, St. Louis, MO, USA); L-glutamine of penicillin-streptomycin, non-essential amino acids, and sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA); 1× Tripsin-EDTA (Aurogene, Rome, Italy); Trypan Blue solution (1:1) (Corning, Glendale, AZ, USA); Lipopolysaccharide (LPS Sigma Aldrich, St. Louis, MO, USA); Qiazol Lysis Reagent (Qiagen, Hilden, Germany); and Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). RNA was extracted miRNeasy Micro kit (Qiagen, Hilden, Germany). The cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

2.2. Instruments

The instruments used for the analyses were the following: NEYA 10R refrigerated centrifuge (Exacta Optech, Modena, Italy), IKA T18 digital Ultra–Turrax (IKA-group, Saufen, Germany), Bandelin Sonorex RK100H water and ultrasonic thermostatic bath, Whatman 0.45 µm 100 (PTFE) syringe filters (Sigma Aldrich, Milan, Italy), and UV–vis spectrophotometer (Jenway, Stone, UK). The chromatographic analysis of biogenic amines was performed using an ATVP LC-10 HPV binary pump with an RF-10° XL fluorometric (FD) detector (Shimadzu, Kyoto, Japan) working at λ emission = 320 nm and λ excitation = 523 nm. A Supelcosil LC-18 column (250 mm × 4.6 mm, 5 µm) with a Supelguard LC-18 (Supelco,

Bellefonte, PA, USA) pre-column was used for the analysis of biogenic amines as well as a Steri-Cycle CO_2 Incubator (Thermo Electron Corporation, Waltham, MA, USA). Cell culture 6-well and 48-well plates (Sarstedt, Nümbrecht, Germany) were used; RNA was quantified with NanoDrop One/OneC (Thermo Fisher Scientific, Waltham, MA, USA), and quantitative real-time PCR (qPCR) was performed on an Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystem, Cheshire, UK) using the SDS2.1.1 program (Applied Biosystem, Foster City, CA, USA),

2.3. Sampling

The study analyzed two wheat husk (WH) samples of the ancient "Senatore Cappelli" (SC) cultivar from two different wheat production chains. The first SC cultivar was located in the hilly territory of Val d'Orcia (WH1) in the Tuscany region, while the WH2 samples were cultivated on the karstic Murge upland, which is located in the Puglia region, as shown in Figure 1. In particular, owing to the peculiar pedo-climatic characteristics of these areas (mild climate, distributed rainfall throughout the year), the soil is characterized by a high percentage of loam and clay and a lower percentage of sand; it is also flat and has good drainage. Both samples were cultivated from October 2021 to June 2022. Approximately 200 g of each SC durum wheat husk was previously ground finely using a blender and then sieved using a sieve with 0.7 to 2.0 mm diameter holes. The obtained particle size fractions were collected and stored at refrigerated temperature, namely T = −18 °C, until the day of analysis. Raw matrices WH1 and WH2 had a moisture content of 6% when analyzed.

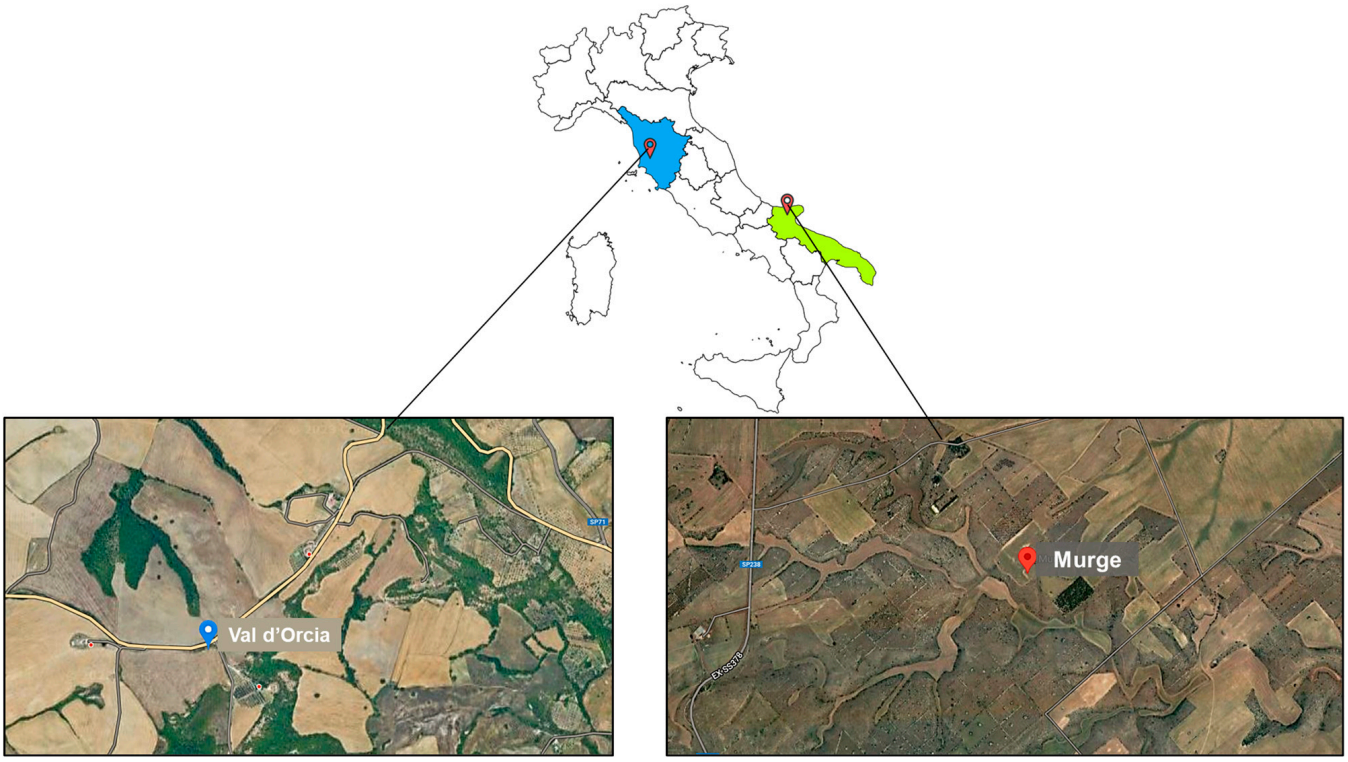

Figure 1. Geographical location and areas of origin for the wheat husk samples analyzed.

Both raw matrices and hydroalcoholic wheat husk extracts were characterized by HPLC chemical, spectrophotometric, anti-inflammatory, and cytotoxicity analysis, as shown in Figure 2.

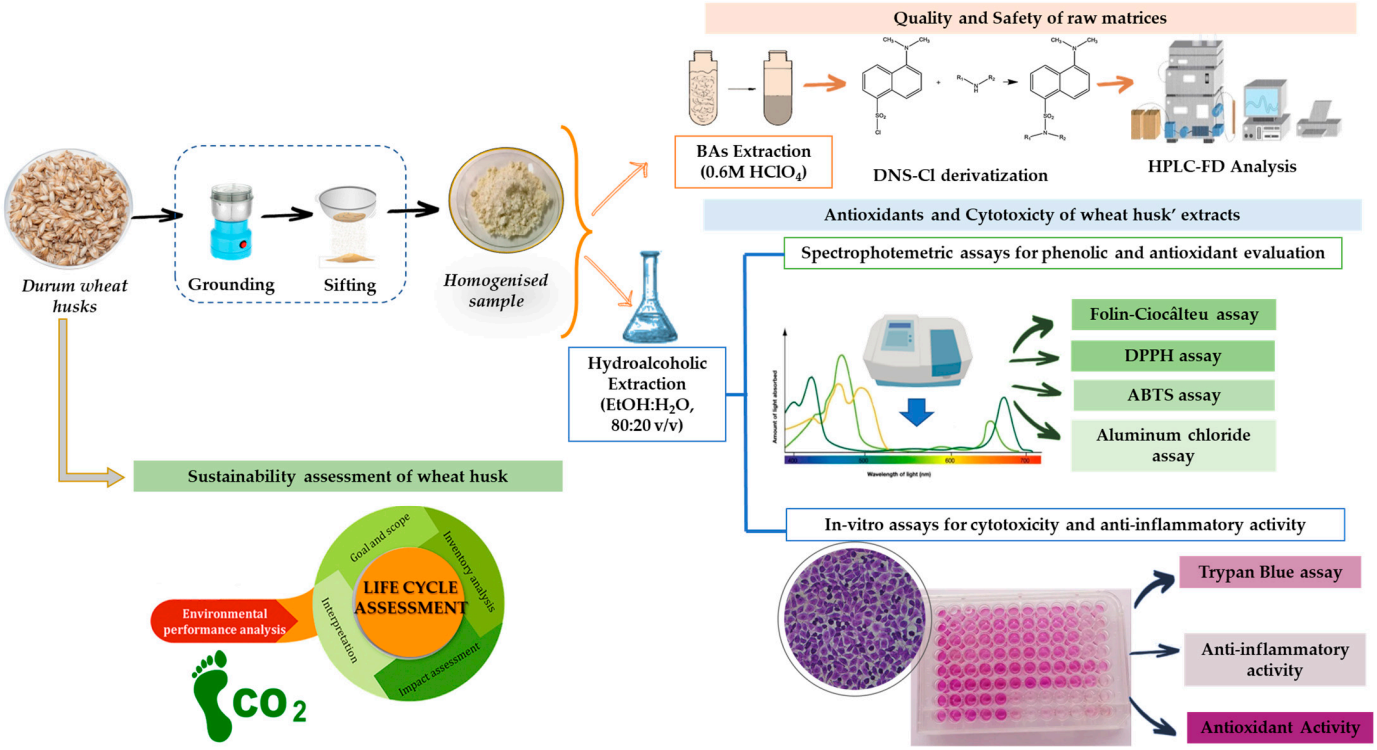

Figure 2. Multimethodological approach for the recovery of bioactive compounds from the wheat husk.

2.4. Quality and Safety of Raw Matrices

Biogenic Amines Extraction from Wheat Husk

The biogenic amines (BAs) determination was carried out according to a previously published method [19] with some modifications. Briefly, 1 g (±0.01 g) of SC wheat husk was added with 12 mL of 0.6 M $HClO_4$. The samples were then homogenized and centrifuged at 2700 rpm for 10 min at T = 25 °C. The supernatant was collected in a flask. The BAs extraction procedure was repeated twice. Then, the second extract was added to the first one and filtered through a 0.45 µm membrane syringe filter. The final volume was adjusted to 25 mL with 0.6 M $HClO_4$. For the derivatization procedure, a 1 mL aliquot of the final extract was added to 200 µL of 2 M NaOH, 300 µL of saturated $NaHCO_3$ solution, and 2 mL of dansyl chloride solution (10 mg/mL in acetone). After stirring, the samples were left in the dark for 60 min at 45 °C. To stop the dansyl chloride reaction, ~100 µL of 25% NH_4OH was added. The final volume was made up to 5 mL by adding acetonitrile. The dansylated extract was filtered using a 0.45 µm filter and injected into the HPLC system. Analytes were eluted using Supelcosil LC-18 column (250 mm × 4.6 mm; 5 µm) in reverse phase with Supelguard LC-18 pre-column coupled with fluorometric detection. The flow rate was set at 1.2 mL/min, and the column temperature was set at T = 30 °C. The elution sequence started with 3 min of isocratic elution (50% ACN; 50% water), reaching 100% ACN after 18 min. Subsequently, the starting isocratic condition (50% ACN; 50% water) was restored. Method accuracy (recovery > 95%), and precision (RSD < 4.6%) were evaluated by analyzing the SC extracts at three different concentrations of BAs. The results obtained from the triplicate analysis were expressed through a calibration curve for each BA, ranging from 0.1 and 25 mg/L. The biogenic amines quality index (BAQI) was calculated based on BAs results to determine the SC samples' quality loss. For BAQI values <10, the product can be considered safe [20]. It was calculated as follows and expressed in µg/g:

$$\text{BAQI} = \frac{(\text{PUT} + \text{CAD} + \text{HIS})}{(1 + \text{SPM} + \text{SPD})}$$

2.5. Phytochemical, Cytotoxic, and Anti-Inflammatory Properties of Wheat Husk Extracts

2.5.1. Hydroalcoholic Extraction of SC Wheat Husk

Sample extraction was performed according to the method of Zhang et al. (2021) with slight modifications [21]. About 10 g (±0.01 g) of each representative husk sample was weighted and placed into 50 mL glass centrifuge tubes, and 25 mL of ethanol in aqueous solution (80:20, *v*/*v*) was added. The samples were homogenized in an ultrasonic and thermostatic bath (Bandelin Sonorex, RK100H) at 400 MHz and at room temperature for 5 min and then centrifuged at 2700 rpm for 10 min at T = 25 °C with a NEYA 10R refrigerated centrifuge (Exacta Optech, Modena, Italy). The supernatant was collected in a 50 mL flask. The residue was added with 25 mL of ethanol in aqueous solution (80:20, *v*/*v*), mixed, and again centrifuged for 10 min. Then, the second extract was added to the first one and filled with ethanol/water (80:20, *v*/*v*) to the mark. For targeted analysis of "Senatore Cappelli" durum wheat husk samples (polyphenols, antioxidants anti-inflammatory activity), the extracts were filtered using 0.45 µm filter (Whatman® Puradisc filters, Sigma Aldrich, Milan, Italy). Extractions were performed on the day of analysis, and extracts were stored at T = 4 ± 2 °C.

2.5.2. Total Phenolic Content (TPC)

The total phenolic content of wheat husk samples was determined according to the Folin–Ciocâlteu method, as Gobbi et al. [19] reported. The absorbance of the samples was read at 750 nm against blank solution (EtOH:H_2O, 80:20 *v*/*v*). The results were expressed as milligrams of gallic acid equivalents per g of wheat husk (mg GAE/g wheat husk). The results were obtained through a calibration curve ranging from 20 to 250 mg/L (R^2 = 0.9998). All the measurements were carried out in triplicate.

2.5.3. Total Flavonoids Content (TFC)

The TFC of wheat husk samples was evaluated according to the aluminum-chloride method described by Abdel-Naeem et al. (2021), with some modifications [22]. To 0.5 mL of the hydroalcoholic extract, 2 mL of d-H_2O and 150 µL of $NaNO_2$ (5% *w*/*v*) were added to a 5 mL volumetric flask. The solution was stirred and incubated in the dark for 5 min, then 150 µL of $AlCl_3$ (10% *w*/*v*) was added, and the solution was put back in the dark for 5 min. Next, 2 mL of NaOH (1 M) was added to the solution and left in the dark for a further 15 min. Subsequently, samples were made up to final volume of 5 mL by adding d-H_2O. The absorbance of the wheat husk samples was read at 510 nm against EtOH:H_2O, 80:20 *v*/*v*. TFC results were expressed as milligrams of rutin equivalent (RE) per g of the wheat husk (mg RE/g) by linear regression, ranging between 50 and 1000 mg/L (R^2 = 0.9995). The results were expressed as means ± standard deviation (SD) of three replicates.

2.5.4. Antioxidant Activity Determination by ABTS and DPPH Assays

The antioxidant activity of wheat husk samples was evaluated using two different spectrophotometric analyses: DPPH and Trolox-equivalent antioxidant capacity (TEAC) assays. The free radical scavenging activity of wheat husks was evaluated by the DPPH assay, according to a previously reported method [23]. The scavenging activity was measured at 517 nm. All experiments were assessed in triplicate, and values were reported as a mean of EC50 ± standard deviation (SD); the EC50 corresponded to the concentration of the wheat husk samples (mg/mL of extract) that provided 50% of the radical scavenging activity. Eight different concentrations of gallic acid diluted in methanol (100–1 mg/L) were prepared and used as a positive control.

The TEAC of the wheat husk samples was estimated by the ABTS radical scavenging assay, according to Pierre et al. (2015) with slight modifications [24]. Briefly, 0.4 mL of wheat husk extract was added with 3.6 mL of ABTS radical solution and left in the dark for 15 min. ABTS radical decolorization was evaluated by measuring the absorbance at 734 nm. The results were expressed as milligrams of Trolox equivalent (TE) per g of wheat husk (mg TE/g) by a calibration curve ranging from 0.5 to 200 mg/TE (R^2 = 0.9963).

2.5.5. Trypan Blue Assay

The mouse microglia cell line (BV2) was seeded in DMEM containing 5% FBS, 1% L-glutamine, 1% penicillin-streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate at 37 °C in a humidified atmosphere with 5% CO_2. BV2 cells were arrayed in 48 wells (30,000 cells/400 µL) [25].

Treatment with the extracts obtained from the wheat husk (10, 50, and 100 ng/mL) was added, and the cells were incubated for 24 h at T = 37 °C. After 24 h, the cells were detached with Trypsin-EDTA 1× and counted through Burker's chamber in Trypan Blue solution (1:1). Both live and dead cells were counted.

2.5.6. Real-Time Quantitative PCR Analysis

BV-2 cells were seeded onto 6-well plates at a density of 10^6/well in 1 mL of DMEM. After a 45 min pre-treatment with extracts obtained from husks at concentrations of 10 and 100 ng/mL, cells were added with LPS 1 ng/mL and incubated at 37 °C for 4 h. The inflammatory stimulus was added with LPS 1 ng/mL for 4 h. After 4 h, cells were detached in 700 µL of Qiazol Lysis Reagent and stored at −80 °C. RNA was extracted and quantified from BV-2 cells. The cDNA was originated using the High-Capacity cDNA Reverse Transcription Kit. Quantitative real-time PCR (qPCR) was performed for each sample in triplicate on an Applied Biosystems 7900HT fast real-time PCR using the Power SYBR® Green PCR Master Mix. Primers for real-time PCR amplification were designed with UCSC GENOME BROWSER (http://genome.cse.ucsc.edu/ accessed on 11 January 2023); University of California, Santa Cruz) (Table 1). The comparative threshold cycle (CT) method was used to analyze the real-time PCR data. The target quantity, normalized with respect to the endogenous β-actin primer reference (ΔCT) and relative to the untreated control calibrator (ΔΔCT), was calculated using equation $2^{-\Delta\Delta CT}$ [25].

Table 1. Primers for Real-time PCR.

GENE	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Accession Number
mARG1	ATGTGCCCTCTGTCTTTTAGGG	GGTCTCTCACGTCATACTCTGT	NM_007482.3
miNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC	AF427516.1
mACT-β	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	NM_007393.5
mCOX2	AGGACTCTGCTCACGAAGGA	TGACATGGATTGGAACAGCA	NM_011198
mCCL2	GGAATGGGTCCAGACATACATTA	CTACAGAAGTGCTTGAGGTGGTT	NM_031530
mChil3	AGACTTGCGTGACTATGAAGCATTG	GCAGGTCCAAACTTCCATCCTC	NM_009892
mSOD1	GCCCGCTAAGTGCTGAGTC	AGCCCCAGAAGGATAACGGA	NM_017050
mNRF2	TCTGAGCCAGGACTACGACG	GAGGTGGTGGTGTCTCTGC	NM_031789
mCD206	TTCAGCTATTGGACGCGAGG	GAATCTGACACCAGCGGAA	NM_008625

2.6. Sustainability Evaluation of Wheat By-Products by Life Cycle Assessment (LCA)

The study evaluated the sustainability assessment of wheat by-products, which account for 17–20% of the total production, through the application of the life cycle assessment methodology in accordance with ISO 14040, 2006 and ISO 14044, 2006 [1]. SimaPro v9.2.2., software was used for the evaluation of environmental impacts.

2.6.1. Goal and Scope Definition

The goal of the study is to assess the environmental impacts associated with durum wheat by-products. In particular, the functional unit (FU) was identified as the production of 180 g of processing by-products (including husks) resulting from the milling process of 1 kg of wheat, thus making the assumption that this by-product corresponds to about 18% of the total wheat production according to a cradle-to-gate approach, as shown in Figure 3. The wheat husk system production includes the upstream process activities (i.e., seedling, water for irrigation, fuels, fertilization, etc.) for the agricultural production

of 1 kg of wheat. Primary information was obtained through face-to-face interviews with farmers and mill owners based on the in-field activities for agricultural and milling phases. The milling phase includes the stages from cleaning to polishing, thus considering the use of water and electricity. In addition, secondary source data from the literature were used for the energy-production phase. In the first scenario, wheat husks are destined to landfill disposal as a unitary quantity of 180 g used for zootechnical application and soil conditioners. Otherwise, the wheat husks extraction scenario considered both the extraction and processing phase of wheat husks.

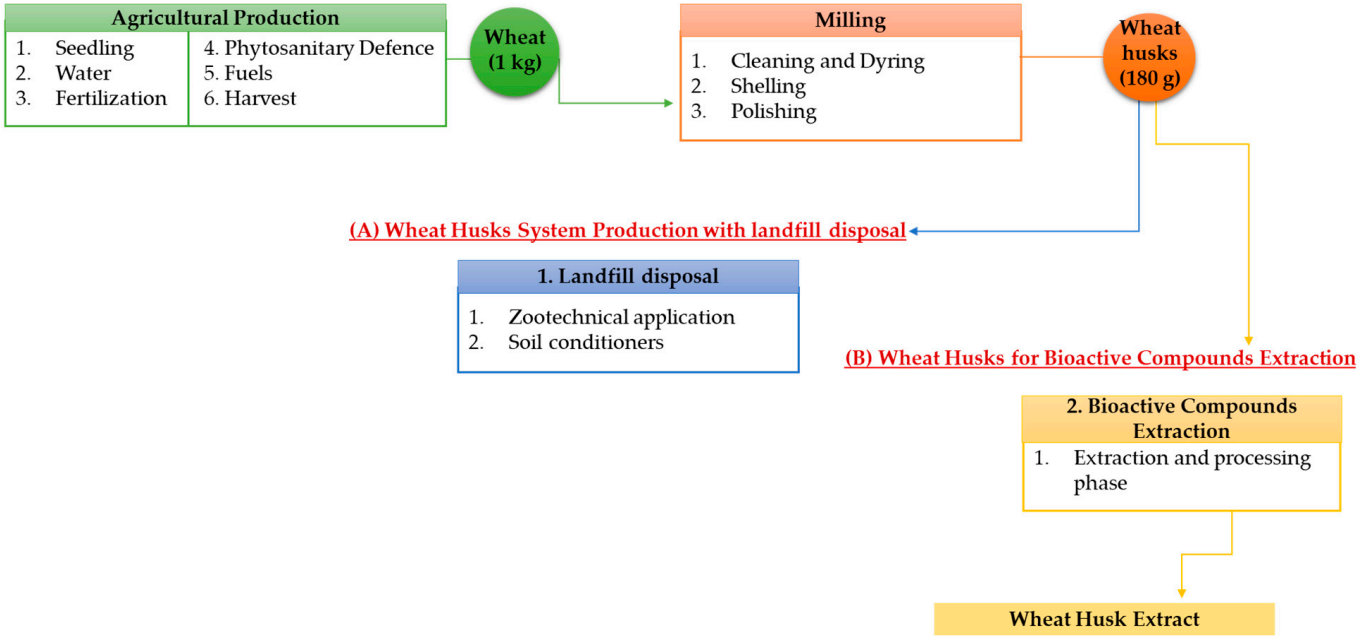

Figure 3. System Boundary of Durum Wheat Production considering (**A**) wheat husks for landfill disposal and (**B**) wheat husks for bioactive compounds extraction.

2.6.2. Life Cycle Inventory (LCI)

The unit processes of each phase were considered as well as the inputs from the agricultural phase to alternative scenarios for the extraction of bioactive compounds, thus considering the milling by-products generated. In particular, the inputs referred to a national average durum wheat production in the year 2020, thus focusing on the milling process of 1 kg of wheat; meanwhile, for the recovery of bioactive compounds from wheat milling by-products (husk), the inputs referred to the extraction process carried out on both samples, i.e., WH1 and WH2, analyzed in the study. Inputs for the wheat husk system production and recycling scenarios are shown in Table 2. All data referred to the same FU of 180 g of wheat husk resulting from the milling process of 1 kg of wheat, thus making the assumption that this by-product corresponds to about 18% of the total wheat production based on production estimations at national level [6]. LCI calculations were performed to model inputs for equipment, solvents, and electricity (i.e., ultrasonic bath, centrifugation, etc.) used during the extraction process. About 180 g of wheat husk was extracted for the recovery of bioactive compounds conventionally by using the hydroalcoholic solution of EtOH:H_2O (80:20, v/v) as extractant.

Table 2. Life cycle inventory for the wheat husk system production and recycling scenarios. Inputs referred to FU: 180 g of wheat husk.

Inputs	Unit	Value	Source
Up-stream Agricultural Process			
Agricultural fuel	g	1.58	
Water	g	85	*EcoInvent* v3.8
Mineral superphosphate (19% P_2O_5)	g	0.57	
Ammonium nitrate (26% N)	g	0.78	*WFLDB*
Urea (46% N)	g	0.92	*Agribalyse* v3.0.1
Seeds	g	0.03	*EcoInvent* v3.8
Herbicide	g	0.44	*WFLDB*
Insecticide	g	3.78	
Outputs			
Raw wheat grain	g	1000	
Wheat Milling Process			
Electricity	kWh	0.06	*EcoInvent* v3.8
Outputs			
Milled wheat grains	g	820	
Wheat by-products (husks)	g	180	
Bioactive Compounds Extraction			
Wheat husks	g	180	
Phase 1 (Hydroalcoholic Extraction)			
Chemicals (Ethanol)	g	896	
Ultrapure water	g	210	*EcoInvent* v3.8
Electricity for ultrasonic bath	kWh	0.12	
Phase 2 (Centrifugation)			
Electricity	kWh	0.45	*EcoInvent* v3.8
Outputs			
Wheat husk extract	mL	885	

EcoInvent version 3.8, *Agribalyse* v3.0.1, and *World Food LCA Database* (*WFLDB*) databases were used to calculate the environmental impacts of the extraction and processing phase of the wheat husks.

2.6.3. Scenario Analysis

To highlight the amount of CO_2 avoided as a result of the possible reuse of the by-product and their environmental compatibility, two scenarios were proposed: (S1) relating to disposal of the by-product in the landfill and (S2) concerning the valorization of wheat by-products through the recovery of bioactive compounds.

The scenarios were subsequently compared through carbon footprint (CF) calculation. CF was calculated based on the LCI and LCIA results. CF is a measure expressing the greenhouse gas emissions (GHGs) caused by a product, service, or process. In accordance with the Kyoto protocol, CF is expressed in kilograms of CO_2 equivalent (kg of CO_2 eq), and it was calculated according to Forster et al. (2007) [26] based on Equation (1):

$$\text{Carbon footprint} = \sum \text{G.G.}_i \times k_i \quad (1)$$

where G.G.$_i$ represents the amount of GHGs produced, and k_i corresponds to the CO_2-equivalent coefficient for that gas.

The CF was obtained employing the Green Gas Protocol V1.03/CO_2 eq (kg) method (GHGP 2020) by using SimaPro v.9.2.2. software.

2.7. Statistical Analysis

The data were obtained from the analysis of three replicates and were expressed as mean ± standard deviation (SD) from experiments. The significance of differences between the extracts was tested using a one-way analysis of variance (ANOVA) with $p < 0.05$. After ANOVA, multiple comparison tests were performed for statistically significant variables, using Dann's post hoc test (homogeneity of variance was assumed) at the level of $p < 0.05$. Statistical analyses were performed using unpaired Student's *t*-test (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Quality and Safety of Wheat Husks by Quantitative Determination of Biogenic Amines (BAs)

The content of eight BAs was evaluated in raw wheat husks by high-performance liquid chromatography with fluorescence detection (HPLC-FD). The analyzed wheat husk samples showed a variable content ($p < 0.05$) of total biogenic amines (Table 3).

Table 3. Biogenic amines content in "Senatore Cappelli" durum wheat husks. Values are expressed as means (mg/100 g) ± standard deviation (SD) of three replicates.

Biogenic Amines Concentration (mg/100 g)	WH1	WH2
B-PEA	9.57 ± 1.33 ^{b}	n.d.
PUT	1.81 ± 0.19 ^{b}	0.85 ± 0.11 ^{a}
CAD	0.58 ± 0.09 ^{a}	2.17 ± 0.11 ^{b}
HIS	n.d.	6.60 ± 0.79 ^{b}
SER	11.24 ± 1.79 ^{a}	17.26 ± 1.57 ^{b}
TYR	n.d.	1.12 ± 0.09 ^{a}
SPD	7.86 ± 0.81 ^{b}	0.82 ± 0.07 ^{a}
SPM	4.61 ± 0.47 ^{b}	0.74 ± 0.05 ^{a}
Total BAs	35.66 ^{a}	43.26 ^{b}
BAQI	0.42	5.46

β-PEA, β-phenylethylamine; SER, serotonin; TYR, tyramine; PUT, putrescine; CAD, cadaverine; HIS, histamine; SPD, spermidine; SPM, spermine; Total BAs, total amount of biogenic amines; B.A.Q.I., biogenic amines quality index; n.d., not detectable. The superscripts a and b in the same line denote significant ($p < 0.05$) differences.

In particular, WH2 samples showed a higher total BAs content (43.26 mg/100 g) than WH1 (35.66 mg/100 g) (p-value < 0.05). Thus, for both cases, SER is the most abundant biogenic amine, accounting for about 35% of the total content. This was in line with the literature results, which found an SER content ranging from 5.2 to 22 mg/100 g dw in wheat by-products [27]. Different authors reported that the occurrence of SER in plant-origin foods was affected by plant variety, degree of microbiologic contamination, and specific conditions to proliferate cells (pH, temperature, access to oxygen, etc.) [28]. Furthermore, it is well established that SER demonstrates positive effects on human health (psychoactive effects, vasoconstrictive properties, etc.); therefore, wheat husks relatively rich in SER could be of interest both to consumers and the food industry. It is relevant to underline BAs' higher amounts of PUT, SPD, and SPM, detected in the highest amount in WH1 (1.81 ± 0.19 mg/100 g dw; 7.86 ± 0.81 mg/100 g dw; and 4.61 ± 0.47 mg/100 g dw, respectively). According to different authors [29], polyamines putrescine as well as spermine

and spermidine are ubiquitous and endogenous in all plant-origin foods, and they have a relevant role in increasing food shelf life, thus representing a food-spoilage index [28,29]. In particular, the literature results highlighted PUT (0–8.6 mg/100 g), SPD (0–33 mg/100 g), SPM (0–4 mg/100 g), and SER (0–13 m/100 g) as the major BAs detected in durum wheat cultivars; their content in the outer layers of the grain (i.e., bran) is about 40–60% higher than in milling products such as whole and white flours [27]. Furthermore, the presence of exogenous monoamines CAD, HIS, and TYR detected in WH2 samples at a concentration of 2.1 ± 0.11 mg/100 g dw, 6.60 ± 0.79 mg/100 g dw, and 1.12 ± 0.09 mg/100 g dw, respectively, may be related to the storage and processing conditions of husks, thus representing a quality process marker. BAs such as HIS, CAD, and TYR are responsible for food-born illnesses such as Scombroid syndrome, cheese reaction, and food allergies, even if present at small concentrations [27]. The present results showed a variable content of these BAs among wheat milling by-products, but they were not as high as amounts reported for meat, fish, alcoholic beverages, cheese, and fermented vegetables (up to 1000–2000 mg/kg), thus representing the most involved foods in toxicological or allergic reactions. These trends are in line with the literature results reporting low BAs contents in cereals in comparison with the above-mentioned foods [27–29]. However, the biogenic amines quality index (BAQI) showed that the BAs amounts detected in wheat husk samples do not pose a health risk to the consumer since they presented values <10 mg/100 g [20].

3.2. Phenolic and Antioxidant Properties of "Senatore Cappelli" Durum Wheat Husks

During the wheat milling process, huge amounts of by-products are generated, accounting for 17–20% (*w*/*w*) of the total raw wheat. They generally consist of husks, outer germ layers, and bran, which still have biological and phytochemical properties such as polyphenols and antioxidants that could enhance health-promoting effects.

The phenolic and antioxidant profile of SC wheat husks was evaluated by means of spectrophotometric assays, as shown in Table 4.

Table 4. Phenolic and Antioxidant Properties of SC durum wheat husk.

	Cereal Milling Husks	
	WH1	**WH2**
TPC (mg GAE/100 g dw)	189.71 ± 3.97 [a]	351.14 ± 5.91 [b]
TFC (mg RE/100 g dw)	108.67 ± 3.44 [b]	156.90 ± 2.31 [a]
ABTS (mg TE/100 g dw)	31.23 ± 1.53 [a]	37.84 ± 4.69 [b]
DPPH (EC_{50} mg/mL)	1.45 ± 0.17 [a]	1.34 ± 0.12 [a]

Values are expressed as means (mg/100 g dry weight, dw) ± SD of three replicates. TPC, total polyphenols content; GAE, gallic acid equivalent; TFC, total flavonoids content; RE, rutin equivalent; TEAC, Trolox-equivalent antioxidant activity; TE, Trolox equivalent; EC_{50}, wheat husk samples' concentration providing 50% of radicals scavenging activity. The superscripts a and b in the same line denote significant ($p < 0.05$) differences.

Results highlighted a variable content of phenolic and antioxidant compounds among husk samples depending on the area of origin. In particular, WH2 resulted in the highest total phenolic content (351.14 ± 5.91 mg/100 g dw), total flavonoids (156.90 ± 2.31 mg RE/100 g dw), and antioxidant activity considering the ABTS assay (37.84 ± 4.69 mg TE/100 g dw). Meanwhile, wheat husk samples from the Val d'Orcia wheat chain (WH1) showed a 40% ($p < 0.05$) lower phenolic and antioxidant potential than WH from the Puglia chain. However, the results of the DPPH assay differed from those of the ABTS assay; this may be probably attributed to the different types of radical agents used. The DPPH reagent, in fact, is a stable nitrogen radical that interacts mainly with peroxide radicals involved in lipid peroxidation, whereas the ABTS reacts with both hydrophilic and lipophilic radicals. Therefore, the reactivity of DPPH is only limited to the lipophilic fraction [19]. In particular, the different content of phenolic and antioxidants among wheat husk samples analyzed may be attributable to the varying pedo-climatic characteristics for the wheat's area of origin despite belonging to the same cultivar ("Senatore Cappelli"). To this purpose, Dinelli et al. (2013)

affirmed that bioactive compound content may greatly vary depending on durum wheat genotype as well as differences in genetic and agricultural crop management [3]. In these regards, it is well established that the biosynthesis and accumulation of phenolic compounds during kernel development is greatly influenced by the wheat variety, environmental conditions, as well as abiotic and biotic stresses [30].

Considering TPC values, most literature data reported similar trends in durum wheat by-products in terms of free soluble fraction, which accounts for 50–75% of the total amount of phenolics, thus highlighting flavonoids and phenolic acids as the most abundant in wheat milling by-products [31]. In these regards, considering TPC, values ranging from 80.90–610.49 mg GAE/g were found in durum wheat by-products [32] as well as values that ranged within 10.84–26.73 mg TE/g and 3.61–1194.8 mg/mL for ABTS assay [33]. Nevertheless, antioxidant activity by DPPH assay showed different trends strictly depending on the chemical composition of cereals; in particular, this result may be due to the lipophilic fraction contained in the wheat by-product, which may have contributed to the extract's activity during the extraction process [33].

3.3. Cytotoxicity of "Senatore Cappelli" Durum Wheat Husks in BV2 Cells

Microglia play a key role in driving neuroinflammation, a mechanism underlying several neurodegenerative diseases; therefore, they represent a suitable model for investigating the anti-inflammatory and antioxidant activity of plant-derived bioactive molecules. Our results dealing with the cytotoxic activity of husk extracts on microglial cell cultures are depicted in Figure 4. The percentage of live cells in WH1 in the total number of cells at the concentration of 100 ng/mL is 92.6%; at the concentration of 50 ng/mL, it is 77.9% and at the concentration of 10 ng/mL is 93.2%. The percentage of live cells in the untreated cells (CTRL) out of the total corresponds to 78.64%. The percentage of live cells for WH2 is 90.2% at the concentration of 100 ng/mL; at 50 ng/mL, the percentage of live cells is 86.2% and at 10 ng/mL is 91.7%. The percentage of live cell control is 85.7%. Extracts obtained from husk, from the two different supply chains mentioned above, added to the in vitro cultures showed no toxicity at the concentrations of 100, 50, and 10 ng/mL. Indeed, BV2 cells treated with the husk extracts at 100 and 10 ng/mL showed a significantly higher proliferation compared to untreated cells (CTRL).

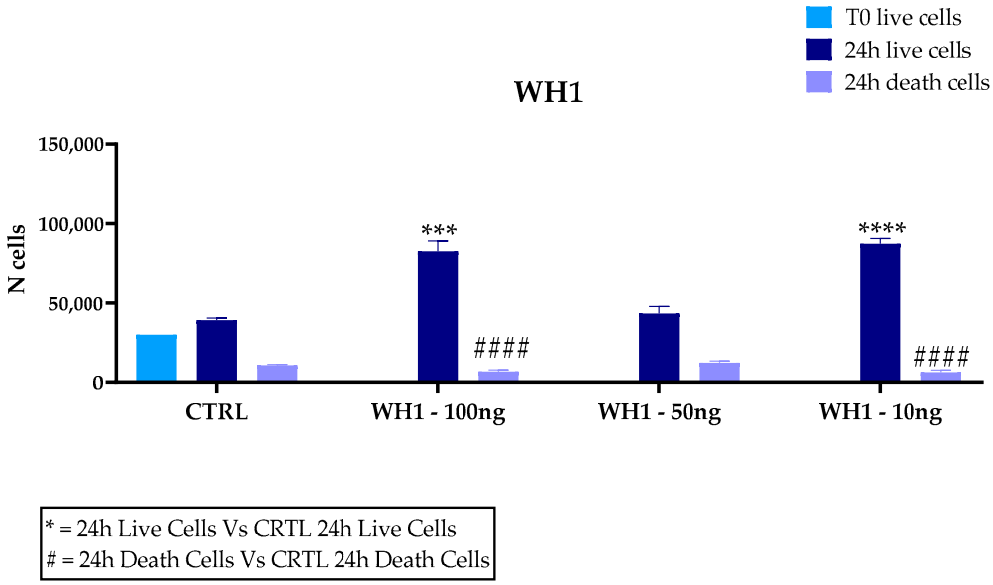

Figure 4. *Cont.*

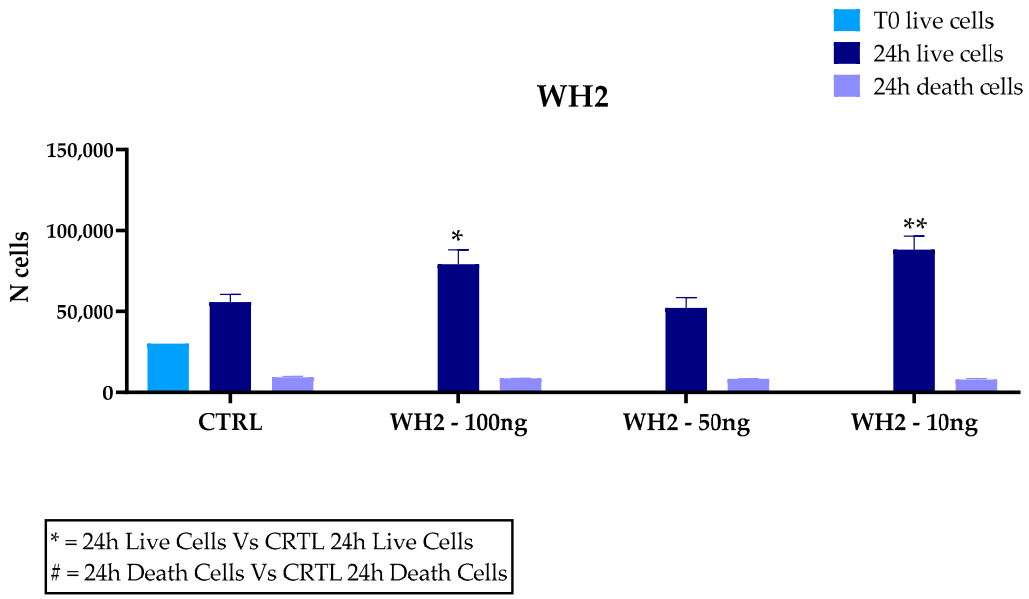

Figure 4. Trypan Blue assay for cytotoxicity of extracts on BV2 microglia cells. Cells were treated with 10, 50, or 100 ng/mL extracts of the husk (WH) of two supply chain. Data are reported as mean ± SD and normalized to the control of at least three independent experiments, and statistical analysis was reported using unpaired Student's *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; #### $p < 0.0001$.

An increase in dead cells was observed in WH1 compared to controls, although there was an increase in cell number. At 50 ng/mL concentration, no significant differences with untreated cells were recorded in both WH1 and WH2.

3.4. M1 mRNA Markers Expression

To evaluate the possible pro-inflammatory effect of the chaff-derived extracts of the two aforementioned dies, we analyzed the mRNAs of key M1 markers in BV2 cells. Results for mRNA expression of iNOS, COX2 and CCL2 mRNAs, and M1 markers highlighted a significant increase following LPS treatment compared with untreated cells (CTRL) (Figure 5).

Pretreatment with husk-derived extracts from both strands (WH1 and WH2) did not modulate iNOS, COX2, and CCL2 mRNAs expression. Husk extracts are devoid of any pro-inflammatory activity because they do not increase the expression of M1 markers.

Several randomized control trials have shown that the intake of whole grains compared to refined grains reduces the expression of pro-inflammatory cytokines such as IL-6 and TNF-alpha and a causes a reduction in serum levels of C-reactive protein in obese patients or patients with metabolic syndrome [34]. In a 2016 study, obese patients under 50 years of age experienced an improvement in diastolic blood pressure after a whole-grain diet [35]. Considering the high mortality risk associated with chronic diseases such as cardiovascular disease, low-calorie diets based on whole-grain foods may reduce this risk [36].

3.5. M2 mRNA Markers Expression

To evaluate the M2 status of BV-2 cells here, we performed RT-qPCR analysis and assessed mRNAs expression of Arginase-1 (Arg-1), which is associated with repair mechanisms [16]; and CD206 and Chil3 (Figure 6).

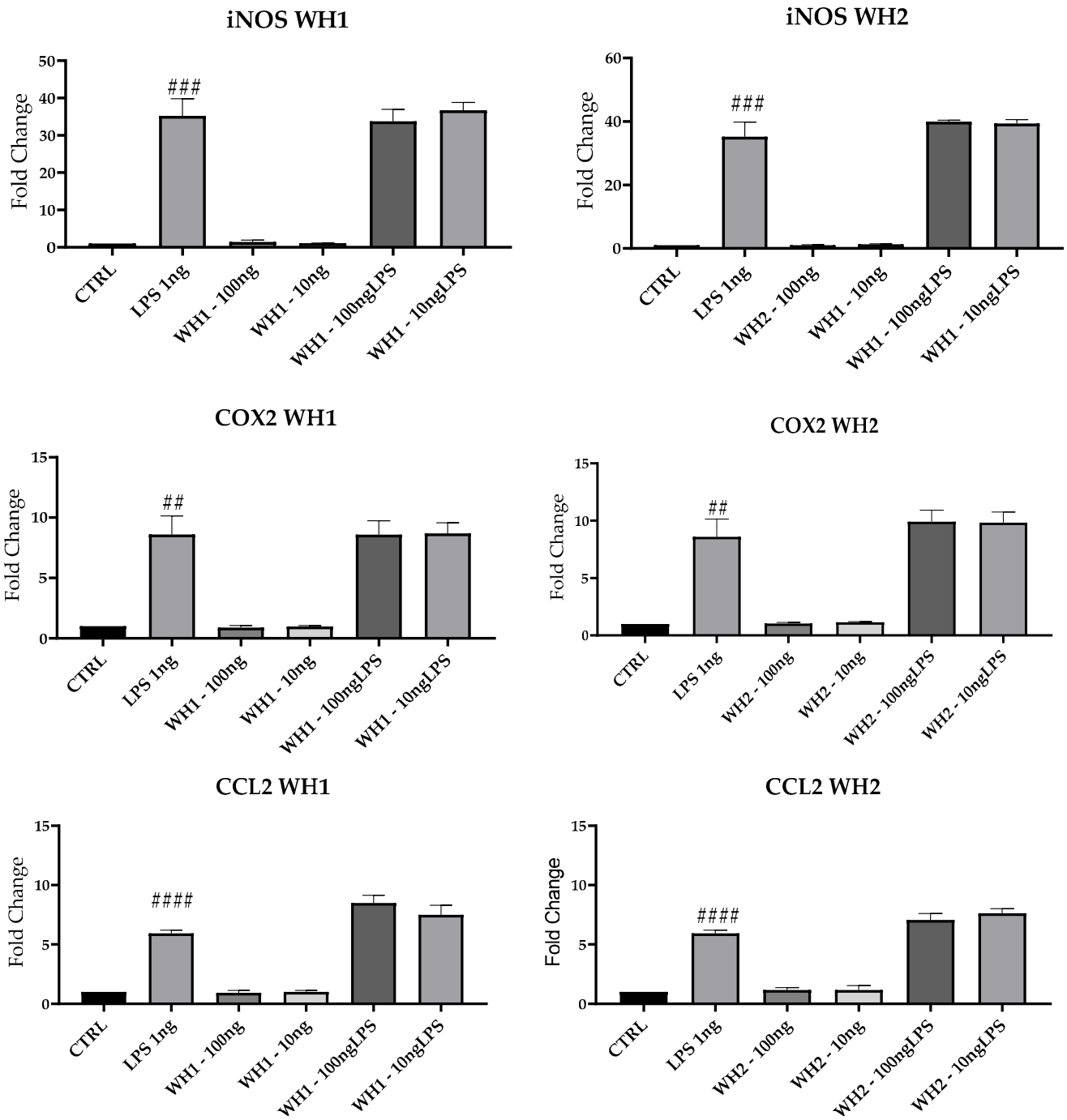

Figure 5. mRNA expression of iNOS, COX2, and CCL2 was evaluated by qRT-PCR. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the $2^{-\Delta\Delta CT}$ method. # VS CTRL; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

Expression of ARG-1 mRNA was induced by the addition of extracts obtained from WH1 and WH2 husk at both 100 and 10 ng/mL concentrations and compared with CTRL. Pretreatment with 10 ng/mL of WH2 husk extracts significantly increased ARG-1 mRNA expression also after the addition of LPS. The mRNA expression of CD206 increased significantly compared with CTRL after addition of WH1 100 and 10 ng/mL and WH2 10 ng/mL. In both chains, ARG-1 expression increased in cells pretreated with the extracts obtained from Pula in the presence of LPS.

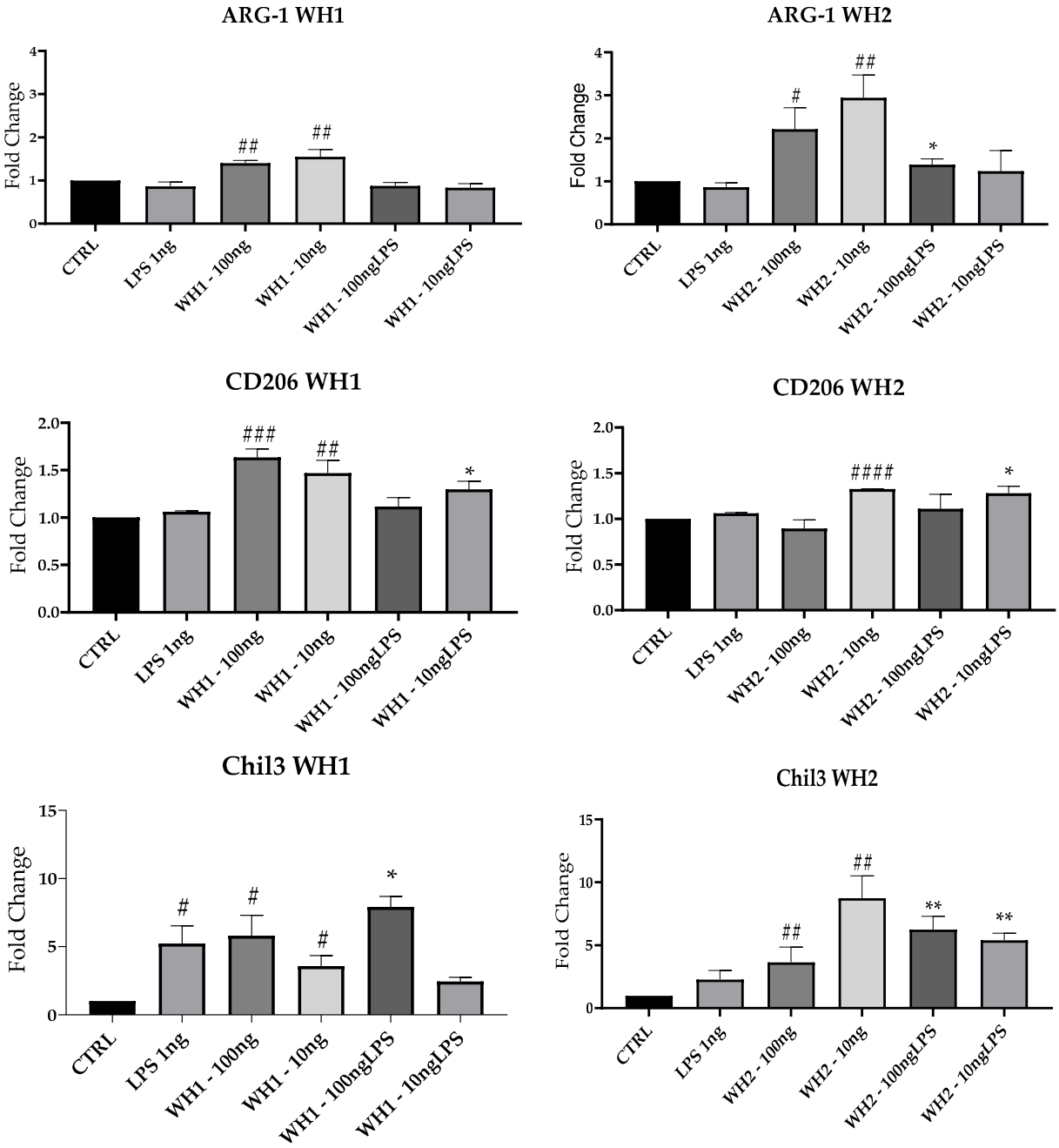

Figure 6. mRNA expression of ARG-1, CD206 and Chil3 evaluated by qRT-PCR. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the $2^{-\Delta\Delta CT}$ method. # VS CTRL; * VS LPS; * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

Chil3 mRNA expression was induced by the extracts alone in both chains. In the presence of LPS, the WH2 extracts at the concentrations of 100 and 10 ng/mL and WH1 extracts at the concentration of 100 ng/mL stimulated Chil3 mRNA expression.

Therefore, the extracts obtained from husk by themselves do not induce any inflammatory effect; instead, they stimulate mRNA synthesis of M2 markers, reverting microglia toward an anti-inflammatory phenotype. Neuroinflammation and oxidative stress are hallmarks of neurodegeneration, contributing to the etiopathogenesis of diseases such as AD, PD, and depression. There are still no approaches that resolve or prevent the onset of these diseases, so the identification of preventive therapeutic approaches seems urgent [37]. The ancient variety "Senatore Cappelli" has more polyphenolic components characterized

by nutraceutical properties than modern varieties [38]. Polyphenols introduced through the diet manage to cross the blood–brain barrier by modulating microglia cell-mediated inflammation in neurodegenerative diseases [39].

3.6. Antioxidant Activity of SC Durum Wheat Husk

Neuroinflammation underlies many neurodegenerative diseases that are incurable to date; in light of these observations, research is focusing on new therapeutic targets. The transcription factor Nrf2 regulates the expression of antioxidant genes that assist anti-inflammatory mechanisms [40]. Therefore, we evaluated by RT-PCR the mRNAs expression of NRF2 and SOD1 (superoxide dismutase1), which are enzymes protecting against oxidative stress [41]; they were significantly decreased in the presence of LPS compared with CTRL (Figure 7).

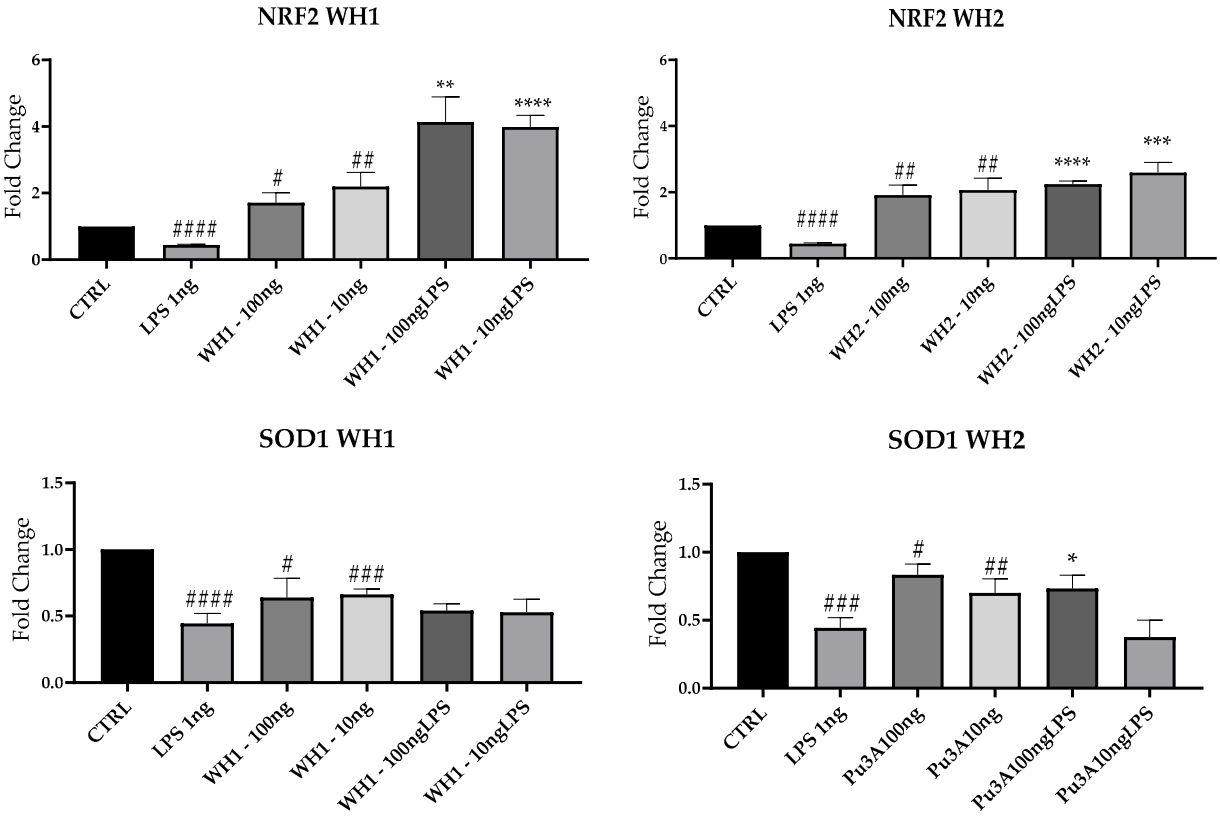

Figure 7. NRF2 and SOD1 mRNAs were evaluated by qRT-PCR. Data are shown as mean ± SD from three independent experiments performed in triplicate. Expression profiles were determined using the $2^{-\Delta\Delta CT}$ method. # VS CTRL; * VS LPS; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

WH1 and WH2 extracts stimulated NRF2 and SOD1 mRNA expression at both 100 and 10 ng/mL, both in the absence or in the presence of LPS.

NRF2 signaling pathways could become a promising therapeutic target. Extracts of "Senatore Cappelli" wheat derivatives restore NRF2 expression related to the upregulation of SOD1, an antioxidant gene. It is known that NRF2 pathways counteract ROS production and inflammation in neurodegenerative disorders, suggesting that stimulation of NRF2 factor could play a key role as a therapeutic approach [11]. These results are in accordance with a study from 2016, where different in vitro assays showed that wheat chaff with ultrasound or hydrothermal or alkali pretreatments for enzymatic conversion showed antioxidant activity correlated with the concentration of reducing sugars [42].

The increase in mortality due to the rising incidence of cardiovascular diseases has focused attention on identifying wheat species with antioxidant and anti-inflammatory properties. Ancient varieties exhibit these characteristics to a greater extent than modern wheat cultivars. Studies on rats fed a diet of ancient wheat showed lower concentrations of reactive oxygen metabolites in plasma compared to rats fed a diet of modern wheat [43].

3.7. Life Cycle Assessment of Wheat By-Products

Life cycle assessment represents a well-established tool to measure the basis of environmental sustainability of a product's or process's life cycle across its entire value chain from extraction of raw materials to its disposal or recycling [11].

The inventory results were analyzed in order to calculate the environmental impacts for each impact category, namely GW, global warming; LU, land use; TEC, terrestrial ecotoxicity; TA, terrestrial acidification; FR, fossil resource scarcity; and WC, water consumption, as indicated in Table 5. The ReCiPe 2016 Midpoint (H) V1.05 method was used for the impact calculations.

Table 5. LCIA results for wheat production process compared to wheat by-products.

Impact Categories	Unit	Wheat Production	Milling Process	Wheat By-Products
Global warming	kg CO_2 eq	2.39×10^{-1}	9.01×10^{-2}	1.05×10^{-1}
Terrestrial acidification	kg SO_2 eq	2.64×10^{-3}	0.6	8.83×10^{-4}
Terrestrial ecotoxicity	kg 1.4-DCB	1.79×10^{-1}	4.02×10^{-2}	1.14×10^{-1}
Land use	m^2a crop eq	1.20	2.40×10^{-4}	3.11×10^{-1}
Human non-carcinogenic toxicity	kg 1.4-DCB	4.59×10^{-1}	1.68×10^{-1}	1.93×10^{-1}
Water consumption	m^3	1.02×10^{-2}	4.50×10^{-3}	2.04×10^{-3}

In the wheat production system, the results were calculated for agricultural production and the milling process of 1 kg of wheat and relative impacts associated with milling wheat by-products, representing nearly 18% of the total production. The milling process has the least impact in all investigated environmental categories.

The results showed that wheat production greatly impacts the environment, showing high values for GW (2.39×10^{-1} kg CO_2 eq), LU (1.2 m^2a crop eq), TA (2.64×10^{-3} kg SO_2 eq), and WC (1.02×10^{-2} m^3). Out of these values, the wheat by-products account for 23–34% of the total impacts related to the entire production process, as highlighted in Figure 8, where the results were characterized and expressed as a relative impact, where the scenario with the highest value in the impact category is set as the reference value (100), and the other is calculated accordingly.

Principally, these impacts are mainly attributable to the emissions associated with using fertilizers from crop fields and fuel consumption for in-field operations, which contribute to 86% of the environmental impacts. According to the reviewed literature, most LCA studies on agro-food production mainly highlighted the energy-intensive production of fertilizers and the large amount applied to crop fields as the first factor responsible for climate-altering emissions [11,44–46], contributing most to the acidification, eutrophication, and respiratory effect categories [47].

In addition, it is worth noting that wheat production also greatly impacts human non-carcinogenic toxicity (HNCT), thus generating an amount of 4.59×10^{-1} kg 1.4-DCB, from which wheat husks contribute 24% of the total impacts related to human toxicity. In particular, the environmental problems causing human toxicity are mainly linked to the release of heavy metals (i.e., nickel and arsenic) and polycyclic aromatic hydrocarbons into the air [48].

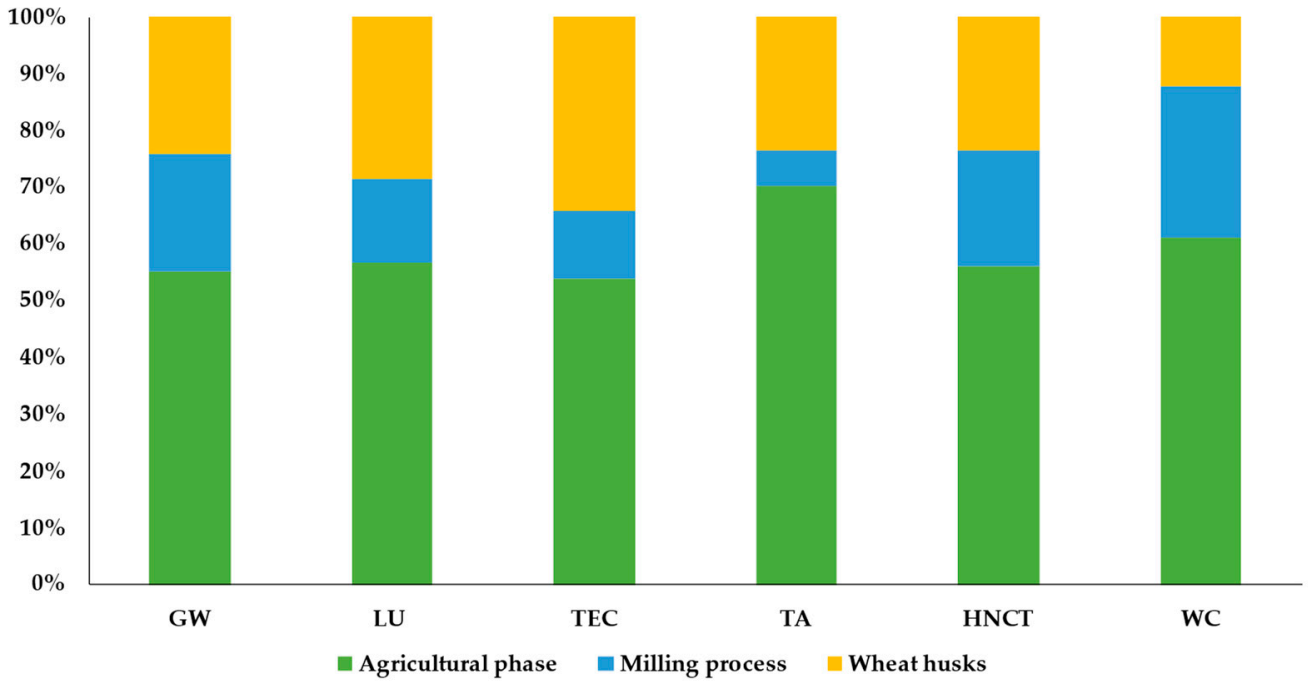

Figure 8. Percentage contribution (%) of each wheat process life cycle stage to environmental impact categories. GW, global warming; LU, land use; TEC, terrestrial ecotoxicity; TA, terrestrial acidification; HNCT, human non-carcinogenic toxicity; WC, water consumption.

Carbon Footprint of Alternative Scenarios for Wheat Husk System Production

Considering the environmental analysis of wheat production, it is worth noting that 25.6% of the environmental impacts in the GW category are associated with husk. As most studies highlighted, agricultural by-products are often discharged, thus creating the main disposal and environmental issue for the wheat-processing industry. In these regards, the possibility of evaluating potential scenarios for mitigating CO_2 emissions related to wheat by-products could represent a strategic option from an environmental perspective.

In these regards, evaluating the potential CO_2 emissions associated with the landfill disposal of 180 g of husk (obtained from the milling process of 1 kg of wheat), which is commonly used for animal breeding or soil conditioning purposes [10], it can be seen that it generates 0.073 kg CO_2 eq, corresponding to 19.8%, of the total emissions generated. In the case of recycling this by-product for the extraction of high-value-added bioactive compounds, about 0.054 kg CO_2 eq would be generated, accounting for about 14.5% of the total GHGs. Therefore, considering the possible valorization of the husk as an alternative to landfill disposal results in an avoided CO_2 amount of 0.0193 kg CO_2 eq, as shown in Figure 9. Different studies in the literature focused on applications possibilities for wheat milling by-products, thus focusing on their recycling processes for renewable energy production [7,44,49], biofuel and bio-gasification processes [9], as well as for the production of feedstock to produce various products including biosurfactants [50].

However, by comparing this value to the total production of durum wheat in Italy, which today amounts to about 3.5 million tons/year produced in 2022 [51], it is worth noting the possibility of avoiding nearly about 12,160 kg CO_2 eq per year.

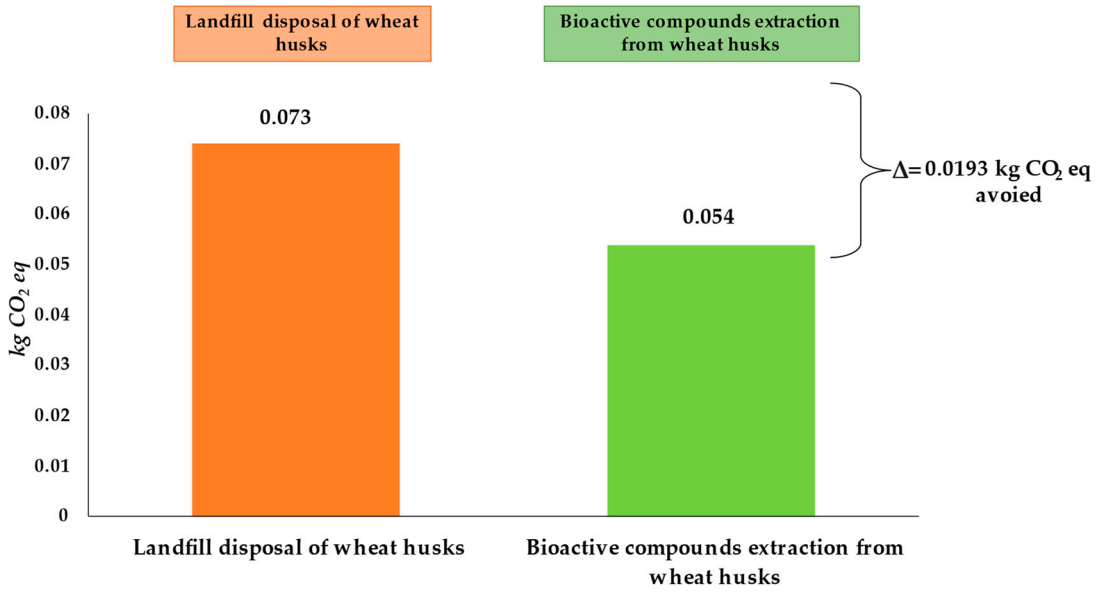

Figure 9. Carbon footprint of alternative scenarios for wheat husk system production.

4. Conclusions

The present work was aimed at valorizing two wheat milling husks of the ancient "Senatore Cappelli" cultivar by the recovery of bioactive compounds, thus evaluating their phenolic and antioxidant potentials and nutraceutical activity. To this purpose, a multi-methodological approach was carried out to assess both the quality and safety of raw matrices as well as the antioxidant and nutraceutical properties of wheat husk extracts.

By means of HPLC-FD analyses, wheat husk samples analyzed revealed a higher content of SER amounting to 35% of the total BAs and were confirmed to occur at BAQI values <10 mg/100 g, thus denoting no loss in the quality of analyzed samples. In addition, spectrophotometric assays showed a significant variable content in the phenolic (189.71–351.14 mg GAE/100 g) and antioxidant compounds (31.23–37.84 mg TE/100 g) within the wheat husk samples, according to the different cultivar areas of origin and the related pedoclimatic characteristics, which influence the different distribution in the content of bioactive compounds. Considering the growing interest in the renewability of food resources, this multi-methodological study builds on the potential neuroprotective role in terms of reduction of neuroinflammation and oxidative stress of waste products from sustainable agricultural supply chains.

Based on in vitro assays on BV2 cells, the two wheat husks of the ancient cultivar "Senatore Cappelli" are non-cytotoxic and increased mRNA expression of anti-inflammatory markers such as ARG-1, CD206, and Chil3. They also stimulated the expression of genes involved in the antioxidant system.

Moreover, through the application of LCA methodology, it was possible to highlight that the impacts associated with the disposal of wheat milling by-products account for approximately 9% of total wheat production. Nevertheless, the extraction of high-value-added compounds from the wheat husk can mitigate environmental and health impacts, thereby inducing 0.41% CO_2 savings per year (12,160 kg CO_2 eq) compared to the overall wheat-production chain. In this framework, it could be worth putting forth greater efforts in terms of energy efficiency and water productivity, thus ensuring a rationalized and sustainable production by a unit of input. This will result in long-term environmental benefits in terms of resource saving and reduced environmental pollution, which could also affect human health.

To improve the environmental performance of wheat by-products, we mainly focused on the recovery of bioactive compounds by conventional extraction methods using hydroalcoholic solvents. Potential mitigating approaches may include the use of green solvents

such as natural deep eutectic solvents (NADESs) that, due to their natural composition, can be used directly for food-fortification processes as well as for pharmaceutical and cosmetic purposes.

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3.4 Research article no. 4

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Journal: Beverages, MDPI

Authors: Gobbi L., Maddaloni L., **Prencipe S.A.**, Vinci G.

Research macro-area: **Sustainability (S), Food Quality (FQ), Food Safety (FS)**

Graphical abstract:

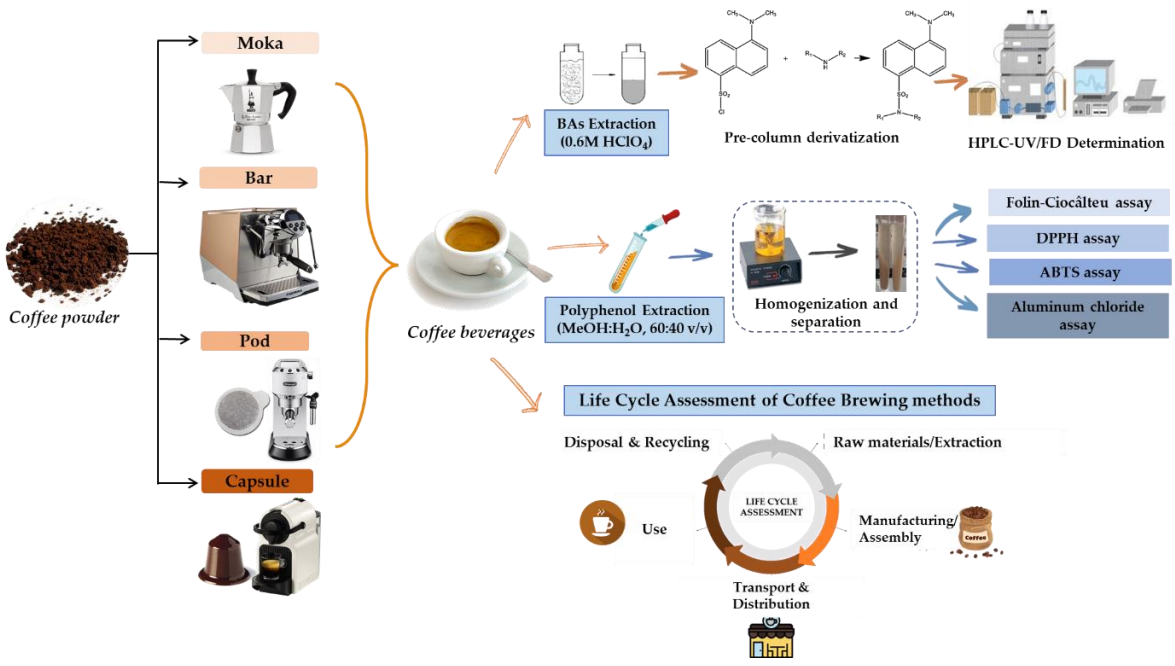

Figure 3.4. Graphical abstract of experimental study no. 4. Source: (Gobbi L, Maddaloni L, Prencipe SA, Vinci G., 2023)

beverages

MDPI

Article

Bioactive Compounds in Different Coffee Beverages for Quality and Sustainability Assessment

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Abstract: Coffee is one of the most widely consumed beverages worldwide, mainly due to its organoleptic, and psychoactive properties. Coffee brewing techniques involve the use of different extraction/infusion conditions (i.e., time, temperature, pressure, water/powder ratio, etc.), which can influence the quality of the final product. The study aimed to analyze the effect of four brewing coffee techniques (industrial espresso machine, Moka machine, pod machine, and capsule machine), which are the most used coffee brewing methods in Italy, on the quality and safety of the coffee brews, taking into account the profile of biogenic amines (BAs), total polyphenol content (TPC), total flavonoid content (TFC) and anti-radical activity (DPPH and ABTS assay). Eight coffee powders and brewed beverages from two different brands belonging to the 100% Arabica variety (country of origin Brazil) were analysed. The brewing techniques all resulted in a reduction of both BA content (27–30%), TPC (55–60%), TFC (50–55%), and anti-radical assays (45–50%) in coffee beverages compared to ground coffee samples. The study also showed that Moka is the method that yields the highest TPC (2.71–3.52 mg GAE/g coffee powder) and TFC (8.50–8.60 mg RUT/g coffee powder) content and highest anti-radical capacity in coffee beverages. The multivariate statistical analysis revealed a difference between coffee powder and infusions and coffee infusions obtained by different extraction techniques. Moreover, an analysis of the environmental impacts related to the different coffee preparation methods examined was conducted. This was performed by applying the Life Cycle Assessment (LCA) methodology through SimaPro v.9.2.2. software.

Keywords: coffee brewing methods; coffee powder; coffee beverage; biogenic amines; polyphenols; antioxidants; food quality; sustainability; life cycle assessment

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1. Introduction

Coffee, valued mainly for its organoleptic-nutritional characteristics, is one of the most consumed foods/drinks in the world. Moreover, coffee is one of the most traded commodities globally [1]. In 2020, coffee production reached 10.7 million tonnes with a value of approximately USD 102 million. Brazil (34.7%) is among the main producing countries, followed by Vietnam (16.5%), Colombia (7.8%), Indonesia (7.3%), Ethiopia (5.5%), and Peru (3.5%) [2]. According to the International Coffee Organisation (ICO), more than 169 million 60 kg bags of coffee were produced in 2020, with an increase of 0.3% compared to the previous year. As coffee production and consumption are expected to steadily grow in the coming years, the amount of coffee by-products produced by the coffee industry is also expected to increase [1,2].

Hot and cold coffee drinks can be produced from a different variety of beans (i.e., Arabica and Robusta). The two most important species in terms of economics are *Coffea arabica* L. (generally referred to as Arabica), which accounts for about 70% of production, and *Coffea canephora* (generally referred to as Robusta). Arabica and Robusta coffees differ in terms of ideal pedo-climatic conditions (soil composition, climate, temperature, etc.), physical aspects (size of the green coffee, etc.), chemical composition, and the characteristics of the brew obtained after roasting [1]. In particular, considering the chemical

composition, Robusta coffee contains higher amounts of antioxidant compounds, caffeine, and soluble solids, resulting in increased body and strong flavor and aroma. The caffeine content in seeds ranges from 0.3% to 2.7% and is twice as high in Coffee Robusta as in Coffee Arabica, which contains almost 1.5%. While Arabica coffee offers superior cup quality and aroma, different secondary metabolites (i.e., minor isomers of chlorogenic acids and diterpenes), are not present in *C. Arabica* [1–3].

There are many coffee preparation techniques, involving the use of different extraction/infusion conditions (i.e., time, temperature, pressure, water/powder ratio, etc.) [4,5]. Concerning the coffee brewing methods mostly used in Italy, 87% of the home-brewed coffee beverage is mainly characterized by Moka, and the semi-sweet brewing method [6]. However, nowadays, coffee preparation with traditional techniques is slowly giving way to faster brewing methods such as pods and capsules. These are small containers containing previously roasted and ground coffee beans that are used in special systems to brew them. The introduction of these new systems resulted in an increase in domestic coffee consumption [7,8].

The chemical composition of coffee beverages mainly depends on the processing techniques (i.e., pre-roasting and roasting) of green coffee beans. Moreover, processing methods at the harvesting stage and industrial processes of green coffee, as well as the methods used by consumers to prepare the coffee beverage, contribute to changes in the concentration of certain compounds within the finished product. The study of the chemical composition of coffee also referred to as "coffeeology", has highlighted more than 1000 different volatile and non-volatile compounds, exhibiting several functional properties, such as antioxidant, anti-inflammatory, antihypertensive and antimicrobial activities, that act both positively and negatively on the consumer's health [9,10].

Considering the physiochemical properties, coffee beans consist of (i) an outer skin (exocarp), which is rich in caffeine, chlorogenic acids, and tannins [9]; (ii) a middle pulp and a mucilaginous layer (mesocarp), which is a source of carbohydrates, such as glucose, and pectin; (iii) parchment, composed of cellulose, caffeine, and minerals; (iv) silver skin (integument), composed of polysaccharides, such as cellulose, and hemicellulose, as well as proteins and phenolic compounds [9], and (v) finally, the seeds (endocarp), containing significant concentrations of caffeine, polyphenols, flavonoids and triacylglycerols (TAGs), bioactive compounds with high antioxidant and antimicrobial activities [10]. The content of bioactive compounds (i.e., antioxidants, biogenic amines, etc.) depends on the coffee species, growing conditions, harvesting techniques, and the processing techniques the bean undergoes (i.e., roasting at high temperatures) [11–13]. Among its bioactive compounds, there are biogenic amines (BAs), consisting of basic low-molecular weight compounds derived from microbial and/or thermal decarboxylation of amino acids. These compounds, which can have both undesirable effects on health (e.g., histamine, cadaverine, tyramine, etc.) and positive effects (e.g., serotonin), are considered an indicator of food quality and safety. Studies have reported that the concentration of BA, especially polyamines (spermine, spermidine, and putrescine) changes considerably during the formation of the fruit and the processing phases, especially during the roasting phase of green coffee. Among the most abundant amine is putrescine, followed by spermine, spermidine, and serotonin [1,13]. Whereas the BA present in smaller quantities is cadaverine, histamine, and tyramine. Among other bioactive compounds, coffee contains significant amounts of polyphenols, i.e., molecules produced by the secondary metabolism of plants that can have positive effects on human health (i.e., anti-atherosclerotic activity, antioxidant activity, etc.). Among the polyphenols most commonly found in coffee are chlorogenic acid, hydroxycinnamic acid, and their derivatives (i.e., caffeic acid, ferulic acid, etc.), as they are molecules with a high scavenging activity towards free radicals [14]. The polyphenol content in coffee varies considerably between green and roasted coffee, indeed the roasting process of the beans leads to the degradation of approximately 70–75% of the polyphenols contained in green coffee [14–16].

Bioactive compounds in coffee beverages have been studied using different brewing preparation techniques (espresso machines, capsule machines, pod machines, and Moka) with different infusion conditions (i.e., time, temperature, pressure, etc.). Some studies have considered the possibility of evaluating multiple bioactive compounds in coffee powder and respective brewed coffees obtained by different techniques [13]. Therefore, this study aimed to evaluate the effect of four different coffee extraction methods (professional coffee machine, Moka machine, pod machine, and capsule machine), on the quality and safety of the final product, as shown in Figure 1. Therefore, the effect of these four coffee preparation methods was evaluated for the content of eight biogenic amines (serotonin, histamine, spermidine, putrescine, tyramine, cadaverine, and β-phenylethylamine), the total polyphenol (TPC) and flavonoid (TFC) content, and the anti-radical capacity (ABTS and DPPH assay). For both coffee powders and the infusions obtained from them by the different extraction techniques, the quantitative determination of BA was carried out by HPLC-FD, while for TPC, TFC, and the DPPH and ABTS assays were performed by means of UV-Vis spectrophotometric analysis. Indeed, univariate, and multivariate statistical analysis was performed on the spectrophotometric and chromatographic results of coffee powder and coffee beverages. Furthermore, concerning the influence that different brewing techniques have on the final coffee beverage and packaging, an analysis of the environmental impacts could be of relevance for an all-encompassing quality and sustainability assessment. Over the latest ten years, several studies have been carried out to assess the environmental performances of the coffee production process and packing stages. Brommer et al. (2011) estimated GHGs emissions associated with the preparation of 2000 cups of coffee based on a cradle-to-gate approach, thus highlighting coffee cultivation as the most impactful phase responsible for 55.4% of total GHGs emissions, followed by post-consumer phases (36%), and coffee packaging, and distribution (6.6%) [17]. Concerning the packaging of different brewing methods, Dubois et al. (2011) observed the environmental burdens associated with 40-mL Nespresso capsules based on the different types of packaging materials (i.e., polypropylene or PP, polyethylene or PE, polyethylene terephthalate or PET, Aluminium or Al, and PE-Al-PET multi-layer bags). They highlighted that the most impactful materials derived from the waste disposal of PE capsules in landfill are due to direct CH_4 emissions associated with the degradation of starch [18].

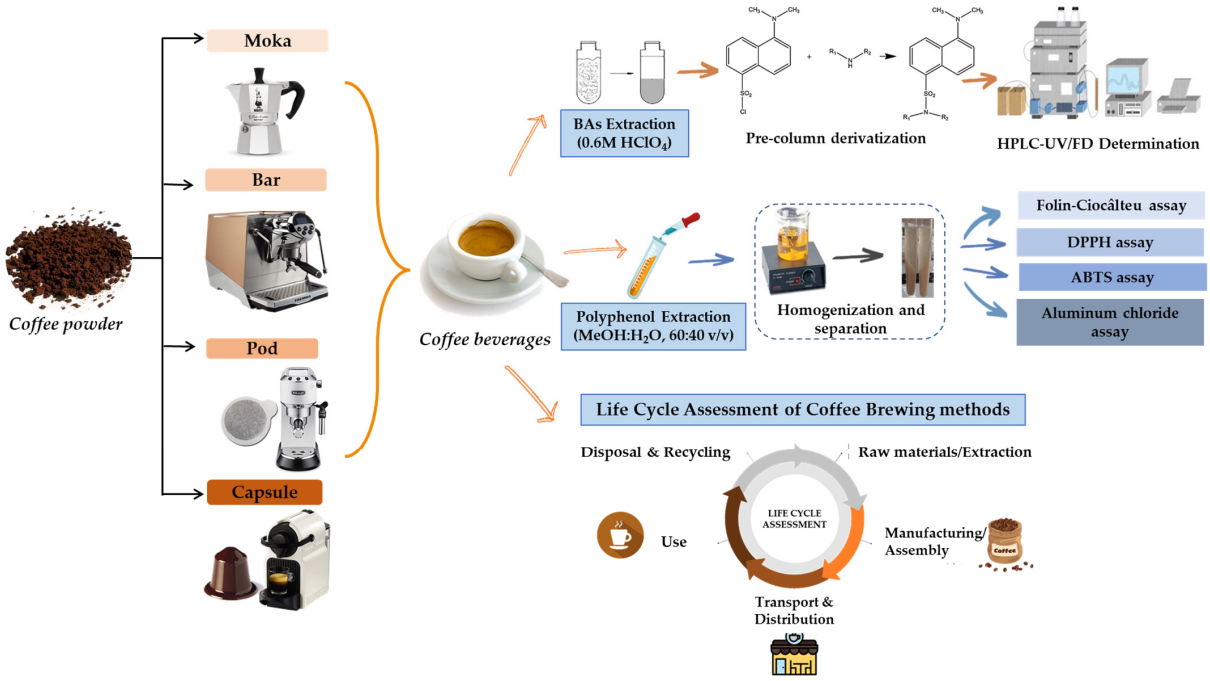

Figure 1. Scheme of quality and sustainability assessment of coffee brewing methods.

The results of the above-mentioned studies were only slightly comparable since they differ in several factors (such as coffee varieties, coffee beverage volume from 40 to 237 mL, etc). Besides, with coffee brewing methods, the method-energy efficiency significantly affects the overall environmental impacts associated with a single-use phase.

In this regard, the environmental sustainability of different coffee brewing methods analyzed in the study was evaluated through the application of Life Cycle Assessment methodology (ISO 14000:2006) using the software SimaPro v. 9.2.2. The LCA analyses focused on a gate-to-gate approach, thus allowing the comparison of coffee brewing methods based in detail on the preparation technique, its energy-demand, and packaging materials.

2. Materials and Methods

2.1. Chemicals

2-phenylethylamine (B-Pea), Putrescine (Put), Cadaverine (Cad), Histamine (His), Tyramine (Tyr), Spermine (Spm), Spermidine (Spd), and Serotonin (Ser) were purchased from Supelco (Bellefonte, PA, USA), as well as the derivatizing agent (Dansyl-Chloride, DSN-CL), sodium bicarbonate ($NaHCO_3$), ammonium hydroxide (NH_4OH) and sodium hydroxide (NaOH). Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (ABTS), sodium nitrite ($NaNO_2$) and aluminum chloride ($AlCl_3$). In addition, the following solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA): acetone (C_3H_6O), perchloric acid ($HClO_4$), acetonitrile; ACN (CH_3CN), methanol (CH_3OH) and double-distilled water (d-H_2O).

2.2. Instruments

The following instruments were used for the analysis: Bandelin Sonorex RK100H water and ultrasonic thermostatic bath, IKA T18 digital Ultra–Turrax (IKA-group, Saufen, Germany), and Whatman 0.45 µm 100 (PTFE) syringe filters (Sigma Aldrich, Milan, Italy), UV-Vis spectrophotometer (Jenway, Stone, UK), NEYA 10R refrigerate centrifuge (Exacta Optech, Modena, Italy). The chromatographic analysis of biogenic amines was performed using an ATVP LC-10 HPV binary pump with an RF-10° XL fluorimetric (FD) detector (Shimadzu, Kyoto, Japan) operating to λ emission = 320 nm, and λ excitation = 523 nm. A Supelcosil LC-18 column (250 mm × 4.6 mm, 5 µm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column was used for the determination of BAs.

2.3. Sampling

Eight coffee powders and brewed beverages from two different brands belonging to the 100% Arabica variety (country of origin Brazil) were analysed. For each brand, n = 2 coffee powders for domestic use (Moka), n = 2 coffee powders for professional use (Bar), n = 2 coffee powders for the capsule brewing method, and n = 2 coffee powders for the pod brewing method were considered, as shown in Figure 1. All types of coffee powder packaged for the above-mentioned coffee brewing methods refer to the same brand. The samples were purchased from local retailers in the city of Rome, Italy. The coffee packages were opened just before analysis to avoid and limit oxidative damage. Extracts (coffee beverages) were obtained in triplicate for each type of coffee powder and stored at T = +4 °C until the day of analysis. Different extraction methods were used for the analyses: Moka coffee machine (model Bialetti, Omegna, Italy), capsule machine (model Krups Nespresso INISSIA XN100, Naples, Italy), pod machine (model De'Longhi Dedica EC685.W, Treviso, Italy), and professional espresso machine (model Faema Hot Steam, Milan, Italy). For each type of extraction, the amount of coffee required for the specific machine was used with a known amount of demineralized water. The extraction conditions are shown in Table 1.

Table 1. Extraction conditions for different coffee brewing techniques.

Extraction Condition	Coffee Powder Weight (g)	Water Volume (mL)	Pressure (Pa)	Time (s)	Temperature (°C)
Moka	5	25	10×10^5	300	95
Espresso Professional	7	25	18×10^5	25	96
Espresso Pods	7.5	20	18×10^5	30	90
Espresso Capsules	5.5	20	15×10^5	30	90

For espresso coffee preparation by the traditional method, the professional coffee machine was used. After weighing 7 g of coffee powder for catering use, i.e., the quantity required for the filter holder of the machine, the coffee was pressed into the filter. While, the single-serving Moka was prepared by weighing 5 g of coffee powder for domestic use, without pressing it into the filter, and 25 mL of water, relative to the volume of the machine's boiler. The capsule coffee was extracted by placing the capsule, containing weighted amounts of coffee powders, and sealed in a protective atmosphere (5 ± 0.5 g), into the machine with a compatible capsule system. Each capsule was used only once and then disposed of. For coffee pods, another machine specifically for this product was used, the pods consisted of pre-packaged coffee (7 ± 0.5 g) and hermetically sealed between two sheets of filter paper. In both methods, the volume of water used was approximately 20 mL. The samples obtained by the different extraction methods were stored at refrigerated temperatures (T = 4 ± 2 °C) until analysis.

2.4. Determination of Biogenic Amines

Biogenic amine extraction in coffee powder samples was performed according to the method previously described by Vinci et al. (2021) [19] with some modifications. 1 g of coffee powder was weighed and placed in a centrifuge tube, then 10 mL of 0.6 M $HClO_4$ was added and homogenized in an ultrasonic bath for 10 min at 400 Hz at room temperature. Then, after centrifugation (3000 rpm for 10 min), the supernatant was stored in an amber vial. For the coffee beverage samples, the extraction was performed according to the method of Vinci et al., 2021 [19], with some modifications. Briefly, 1 mL of sample was placed in a 10 mL amber flask then acidified by adding 0.6 mL of 10.3 M $HClO_4$ to obtain a final concentration of 0.6 M and made up to volume with distilled H_2O. The extracts thus obtained for coffee powders and coffee infusions were stored at a temperature of 4 ± 2 °C.

Following BA extraction in coffee powders and coffee beverages, the samples were then derivatized by adding 200 µL NaOH 2 N and 300 µL saturated $NaHCO_3$ solution to 1 mL of acid extract. Subsequently, 2 mL of DNS-Cl at a concentration of 2 mg/mL in acetone is added. The sample was placed in the dark for one hour at 45 °C in the ultrasonic bath (Bandelin Sonorex RK100H). Subsequently, the solutions were made up to a volume of 5 mL with acetonitrile (ACN) and filtered through 0.45 µm FPTE syringe filters. Biogenic amines were detected by HPLC-FD following the standardized method defined by ISO 19343:2017, with some modifications [20]. Analytes were eluted using Supelcosil LC-10 column (250 × 4.6 mm; 5 µm) in reverse phase with Supelguard LC-18 pre-column (Supelco), coupled with fluorimetric detector (λ excitation = 320 nm; λ emission = 523 nm). The flow rate was set at 1.2 mL/min, while the column temperature was set at 30 °C. The elution program starts with 3 min of isocratic elution (50% ACN; 50% water) reaching 100% of ACN after 18 min to finish after 3 min of isocratic elution. After that, the start isocratic condition (50% ACN; 50% water) was restored. The results were obtained through a calibration curve ranging from 0.1 to 25 mg/L for each BA. Based on BA results, the Biogenic Amines Quality Index (BAQI) was calculated to assess the coffee samples' quality.

For BAQI values < 10, the product can be considered safe [21]. It was calculated as follows and expressed in μg/g of coffee powder:

$$BAQI = \frac{(PUT + CAD + HIS)}{(1 + SPM + SPD)}$$

2.5. Polyphenol, Flavonoid, and Antioxidant Activity Determination

A hydroalcoholic extraction was performed on the coffee powder samples and their infusions. The hydroalcoholic extraction procedure in the coffee powder samples was as follows: 0.1 g of coffee powder was weighed, to which 5 mL of MeOH:H_2O (60:40, *v*/*v*) was added [22]. The solutions were centrifuged at 3000 rpm for 10 min at room temperature and the supernatant was collected in a 10 mL volumetric flask. A second extraction was performed on the supernatant as described above. For the coffee infusions, the extraction was performed by placing 1 mL of sample in a 50 mL volumetric flask and making up to volume with MeOH:H_2O (60:40, *v*/*v*). Extractions were performed on the day the infusions were prepared and all extracts were stored at 4 ± 2 °C.

2.5.1. Total Polyphenols Content

The total polyphenols content (TPC) was assessed for both hydroalcoholic extracts of coffee powder and coffee infusions according to the method described by Vinci et al. (2022) [22] with some modifications. 0.5 mL of the hydroalcoholic extract was mixed with 0.25 mL of Folin Ciocâlteu reagent in a 10 mL amber volumetric flask. After 3 min, 0.5 mL of aqueous sodium carbonate solution (7.5% *w*/*v*) was added and the flask was kept in the dark for 30 min. It was then made up to volume with distilled water. The absorbance of the samples was read at 750 nm. The results were expressed as milligrams of gallic acid equivalents per gram of coffee powder (mg GAE/g coffee powder). The results were obtained through a calibration curve ranging from 10 to 100 mg/L (R^2 = 0.9998) and the blank was the solvent used for sample extraction.

2.5.2. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was evaluated in all powdered and infused coffee extracts. The TFC was determined according to the method described by Abdel-Naeem et al. (2021), with some modifications [23]. To 0.5 mL of extract, 2 mL of distilled water and 150 μL of $NaNO_2$ (5% *w*/*v*) were added to a 5 mL volumetric flask. The solution was stirred and incubated in the dark for 5 min, then 150 μL of $AlCl_3$ (10% *w*/*v*) was added and the solution was put back in the dark for 5 min. Next, 2 mL of NaOH (1 M) was added to the solution and left in the dark for a further 15 min. Subsequently, 5 mL was made up to a volume of 5 mL. The absorbance of the extracts was read at 510 nm. TFC results were expressed as milligrams of rutin equivalents (Rut) per gram of coffee powder (mg Rut/g coffee powder). As the TPC assay, the TFC blank corresponds to the solvent used for the extraction of polyphenols from coffee samples.

2.5.3. Determination of Antioxidant Activity

The antioxidant activity of coffee powders and infusion extracts was evaluated by using two different reagents: ABTS and DPPH [24,25]. The scavenging activity of the ABTS radical in the samples was evaluated by measuring the decrease in absorbance at 734 nm. A 7 mM solution of ABTS was prepared by dissolving 0.19 g of ABTS powder in 50 mL of distilled water, while the PBS solution was prepared by dissolving 0.38 g of PBS powder in 10 mL of d-H_2O. 25 mL of 7 mM ABTS and 0.4 mL of PBS (1.9 mg/mL) were placed in an amber flask. The solution was kept in the dark for 16 h at room temperature to activate the reagent. 3.6 mL of the reagent was added to 0.4 mL of hydroalcoholic extract, and the sample with the reagent was placed in the dark for 15 min and then read on the UV-Vis spectrophotometer. The scavenging activity of coffee samples was also assessed for both types of extracts by DPPH assay. A 2.5 ng/mL DPPH solution was prepared by

dissolving 125 mg of standard powder in 50 mL of methanol. To 1 mL of hydroalcoholic extract, 1.5 mL of DPPH solution (2.5 ng/mL) was added and kept in the dark for 30 min at room temperature. The absorbance (λ = 517 nm) was then measured against methanol using a UV-Vis spectrophotometer. The results were calculated using the inhibition rate (I%) of the radical cation for both assays, according to the following equation:

$$I\% = (A_0 - A_1)/A_0$$

where A_0 is the absorbance of the control (blank) and A_1 is the absorbance of the DPPH or ABTS radical in the extract.

2.6. Life Cycle Assessment (LCA)

Following standards ISO 14040:2006 and ISO 14044:2006, Life Cycle Assessment (LCA) is considered a standardized and valuable tool for environmental impact assessment, and it should involve four phases [26,27]: (1) Goal and scope definition, describing the objective of the study, the functional unit (FU) and the system boundary; (2) Life Cycle Inventory (LCI), collecting the input data for the environmental assessment of a product, process, or activity; (3) Life Cycle Impact Assessment (LCIA), which is aimed at evaluating sustainability in terms of impacts on the environment, human health, and resources; and (4) Interpretation of results, in which LCIA results are interpreted according to the objectives and scope definition. SimaPro 9.2.2., software was used for the evaluation of environmental impacts.

2.6.1. Goal and Scope Definition

The study was aimed at assessing the environmental performances of different brewing methods (Moka, espresso bar, espresso pods, and capsules) for coffee beverages, by taking into account operational conditions in terms of time, temperature, coffee powder, water consumption, and packaging materials. The functional unit (FU) is a 40-mL cup of espresso coffee with no additional ingredients (i.e., milk, sugar, etc.) and by the Italian Coffee Committee's disciplinaries [28]. The system boundaries are referred to as a gate-to-gate approach. In this regard, the life cycle included the use of the aforementioned coffee machines, considering their operational conditions (in terms of electricity usage), roasted and ground coffee, water use, and primary packaging materials used (i.e., paper filters, low-density polyethylene, polypropylene, etc.). Nevertheless, the system boundaries did note GHGs emissions arising from the capital goods production, such as coffee machines, as well as their maintenance and disposal due to: i. lack of data, ii. the exclusion of operating goods in previous LCA studies on coffee brewing methods [17,28,29] and, iii. the assumption that these inputs could be considered negligible because of their minor contribution to a single cup of espresso coffee. Therefore, the coffee production chain (cultivation, transportation of coffee beans, as well as coffee roasting and grounding) was excluded from the study, since it was assumed to be the same for the coffee beverages obtained from the different brewing systems analyzed [28,29]. Waste disposal of both spent coffee grounds, and all packaging materials used, were excluded from the study.

2.6.2. Life Cycle Inventory (LCI)

The input data concerning the preparation of a 40-mL cup of coffee beverage (FU) are shown in Table 2.

The primary data were provided by a coffee company, located in Rome (Lazio, RM, Italy), and referred to roasted and ground coffee, as well as primary packaging materials. The secondary data for electric coffee machines usage were extracted from the Ecoinvent v3.8 database, provided by SimaPro 9.2.2. software [30].

2.7. Statistic Analysis

The data were obtained from the analysis of three replicates and were expressed as mean ± standard deviation. The normality of the data distribution was checked using the Shapiro–Wilk test, the homogeneity of variances using Levene's test, and the significance of

differences between the extracts was tested using one-way analysis of variance (ANOVA) with $p < 0.05$. After ANOVA, multiple comparison tests were performed for statistically significant variables, using Dann's post hoc test (homogeneity of variance was assumed) at the level of $p < 0.05$. Following the characterization of the coffee samples with different brewing methods, a multivariate analysis was carried out to interpret the results using principal component analysis (PCA). The data were pre-treated (autoscaling) to exclude variance related to the different units of measurement of the analyses performed. Analyses were performed using CAT software.

Table 2. Inventory data for different coffee beverage brewing methods. All data referred to a 40-mL cup of coffee beverage (FU).

INPUTS		**Coffee Brewing Methods**			
	Unit	**Coffee Moka**	**Coffee Bar**	**Coffee Capsule**	**Coffee Pod**
Roasted and ground coffee	g	8.0	11.2	14.0	11.0
Water for preparation	g	100	100	54.08	40.3
Electricity	kW·h	0.266	0.014	0.022	0.021
Primary packaging					
Low-density polyethylene (LDPE)	g	0.352	0.493	-	-
Paper filters	g	-	-		2.47
Poly-laminated bag (PE-Al-PP)	g	-	-	2.01	
OUTPUTS					
1 cup of coffee beverage	mL	40	40	40	40

3. Results and Discussion

3.1. Biogenic Amiens Content in Coffee Samples

The BA content in coffee powder and beverage samples is shown in Table 3. The differences in BA concentrations in the ground and respective brewed samples could be associated with some factors involved in the extraction mechanisms (water/coffee ratio, temperature, pressure, brewing time, etc.), but also with the particle size of coffee powder [31,32]. During the coffee extraction phase, water can extract the soluble compounds from the coffee powder [1,11]. Furthermore, through the pressure generated by water on the sample, less soluble or physically bound compounds are extracted by physical mechanisms. Another aspect to be considered is the extraction temperature, which can lead to the degradation of thermolabile compounds and help the emulsification of fats in the final extract. Therefore, to make the data as comparable as possible, we eliminated the granulation effect by choosing coffee powders with the same degree of grinding. In addition, similar extraction parameters were selected, always bearing in mind the peculiarities of each infused coffee preparation technique [1].

The total BA amounts in the coffee powder samples ranged between 67.01 µg/g of coffee powder and 96.83 µg/g of coffee powder, thus in line with the results found in the literature [33,34]. These BA concentration differences in coffee powder samples could be related to the different agronomic techniques and the different transformation processes that coffee beans undergo along the supply chain. Furthermore, it has been shown that the presence of high BA concentrations in food may also be related to product storage and shelf-life [1]. Among all the BAs considered, only Cad and Put were not found in the samples. In coffee powder samples, the BAs present the highest concentrations are Ser (62.13–84.24 µg/g of coffee powder), followed by β-Pea (2.22–11.93 µg/g of coffee powder) and His (2.22–7.30 µg/g of coffee powder), with a large degree of variability depending on the sample considered.

Table 3. Biogenic amines amount (μg/g of coffee powder) ± standard deviation (SD) in ground coffee and brewed coffee samples.

Samples		Biogenic Amines (μg/g of Coffee Powder)								BAs Tot	BAQI
		Ser	B-Pea	Put	His	Cad	Tyr	Spd	Spm		
Coffee powder	Bar 1	71.77 ± 1.02 ^{c}	8.97 ± 0.23 ^{b}	0.33 ± 0.07 ^{a}	2.78 ± 0.23 ^{b}	nd	nd	0.72 ± 0.09 ^{b}	0.74 ± 0.12 ^{a}	84.59	1.26
	Bar 2	75.50 ± 1.21 ^{a}	11.93 ± 0.33 ^{a}	0.35 ± 0.04 ^{a}	2.32 ± 0.20 ^{a}	nd	nd	0.51 ± 0.07 ^{b}	0.67 ± 0.17 ^{b}	90.77	1.22
	Capsule 1	63.46 ± 0.76 ^{b}	3.26 ± 0.12 ^{a}	0.52 ± 0.13 ^{c}	3.62 ± 0.14 ^{c}	nd	nd	0.19 ± 0.03 ^{c}	0.78 ± 0.11 ^{c}	71.85	2.10
	Capsule 2	57.87 ± 0.84 ^{c}	3.65 ± 0.17 ^{a}	0.45 ± 0.11 ^{a}	4.19 ± 0.17 ^{a}	nd	nd	0.10 ± 0.01 ^{a}	0.74 ± 0.13 ^{a}	67.01	2.52
	Moka 1	82.61 ± 1.32 ^{a}	5.81 ± 0.36 ^{b}	0.43 ± 0.06 ^{a}	7.30 ± 0.51 ^{b}	nd	nd	0.29 ± 0.02 ^{a}	0.38 ± 0.03 ^{a}	96.83	4.60
	Moka 2	84.24 ± 0.91 ^{b}	5.35 ± 0.36 ^{b}	0.42 ± 0.01 ^{a}	5.78 ± 0.32 ^{a}	nd	nd	0.21 ± 0.05 ^{b}	0.39 ± 0.05 ^{b}	96.38	3.89
	Pod 1	62.13 ± 0.53 ^{c}	2.75 ± 0.25 ^{a}	0.40 ± 0.03 ^{b}	2.22 ± 0.13 ^{b}	nd	nd	0.34 ± 0.01 ^{b}	0.55 ± 0.07 ^{b}	67.88	1.89
	Pod 2	72.47 ± 1.32 ^{c}	2.22 ± 0.12 ^{a}	0.35 ± 0.02 ^{a}	3.20 ± 0.21 ^{a}	nd	nd	0.33 ± 0.02 ^{b}	0.57 ± 0.08 ^{a}	81.15	1.87
Coffee beverages	Bar 1	14.38 ± 0.56 ^{b}	0.52 ± 0.08 ^{b}	0.18 ± 0.02 ^{b}	8.29 ± 0.58 ^{a}	nd	nd	0.14 ± 0.02 ^{a}	0.24 ± 0.11 ^{c}	23.60	6.81
	Bar 2	12.75 ± 0.41 ^{c}	0.52 ± 0.05 ^{b}	0.16 ± 0.03 ^{a}	7.97 ± 0.74 ^{c}	nd	nd	0.12 ± 0.03 ^{b}	0.20 ± 0.09 ^{b}	21.61	6.76
	Capsule 1	14.42 ± 0.65 ^{a}	0.41 ± 0.31 ^{a}	nd	0.77 ± 0.21 ^{b}	nd	nd	0.11 ± 0.03 ^{a}	0.32 ± 0.08 ^{a}	16.02	0.54
	Capsule 2	19.95 ± 0.25 ^{c}	0.48 ± 0.11 ^{b}	nd	0.85 ± 0.26 ^{b}	nd	nd	0.06 ± 0.01 ^{c}	0.22 ± 0.14 ^{b}	21.75	0.57
	Moka 1	33.46 ± 0.84 ^{b}	0.51 ± 0.08 ^{b}	nd	14.66 ± 0.54 ^{b}	nd	nd	0.04 ± 0.02 ^{a}	0.25 ± 0.06 ^{a}	48.92	11.38
	Moka 2	31.82 ± 0.67 ^{c}	0.44 ± 0.05 ^{a}	nd	20.57 ± 0.86 ^{a}	nd	nd	0.07 ± 0.01 ^{b}	0.26 ± 0.09 ^{b}	53.15	15.50
	Pod 1	21.38 ± 0.35 ^{a}	0.85 ± 0.14 ^{a}	nd	0.37 ± 0.36 ^{b}	nd	nd	0.03 ± 0.01 ^{c}	0.20 ± 0.04 ^{a}	22.83	0.30
	Pod 2	18.46 ± 0.62 ^{b}	0.31 ± 0.04 ^{a}	nd	0.22 ± 0.05 ^{a}	nd	nd	0.14 ± 0.02 ^{b}	0.15 ± 0.06 ^{c}	19.28	0.17

Ser: Serotonin; B-Pea: B-Phenylethylamine; Put: Putrescine; His: Histamine; Cad: Cadaverine; Tyr: Tyramine; Spd: Spermidine; Spm: Spermine; nd = not detectable; BAs Tot: Total amount of biogenic amines; BAQI: Biogenic amine quality index. The superscripts a, b, and c denote significant ($p < 0.05$) differences.

The data showed that in the coffee beverages obtained by the different extraction methods, the profile of biogenic amines reflected the coffee powder samples, except for Put, which was only found in the sample obtained with the professional espresso machine (Bar 1 and 2). In all coffee beverages, there was a reduction in the biogenic amine content varying between 50 and 77%. This is probably because ABs at high temperatures tend to degrade; therefore, the extraction process may influence the concentration of BAs in the final extract. The total content of biogenic amines for the different beverage extraction techniques varies between 16.02 and 5.15 μg/g of coffee powder. In coffee beverages, the most represented amine is serotonin (12.75–33.46 μg/g of coffee powder), followed by histamine (0.22–20.57 μg/g of coffee powder). The concentration in the infusions obtained with a professional espresso machine and Moka coffee machine is higher than in ground coffee, probably due to the time/temperature and extraction pressure [1]. However, the Biogenic Amines Quality Index (BAQI) showed that AB concentrations do not pose a health risk to the consumer. Furthermore, the histamine content in coffee beverages was found to be below alert levels defined by Regulation (EU) 2073/2005 [35].

3.2. Total Polyphenol Content

The Folin-Ciocâlteu assay was used to determine the total content of polyphenols (TPC) in coffee and respective brews obtained from the different extraction processes (Moka, professional espresso, espresso pods, and espresso capsules). The TPC expressed as mg gallic acid equivalent (GAE) per g of coffee powder is shown in Table 4.

The highest TPC was found in capsule coffee preparations (28.46–29.61 mg GAE/g of coffee powder), this trend, however, was not maintained by the infusion processes. The coffee infusions that presented a higher concentration of total polyphenols in the final extract were the samples obtained with the Moka machine (2.71–3.52 mg GAE/g of coffee powder) and the professional bar machine (1.51–1.72 mg GAE/g of coffee powder). This is probably due to the longer contact time between water and ground coffee and the higher

extraction temperatures. The water volume and the coffee powder amount ratio used has been highlighted as limiting factor for the extraction of TPC from coffee for all types of extraction techniques; in addition, other factors that could influence the extraction of polyphenols from coffee in the different extraction methods are temperature, contact time between water and coffee powder, pressure and powder size [16,31–33].

Table 4. Total polyphenols content (mg GAE/g of coffee powder) ± SD in coffee powder and beverage samples. The superscripts a, b, and c denote significant ($p < 0.05$) differences.

Sample	TPC (mg GAE/g of Coffee Powder)	
	Coffee Powder	Coffee Beverages
Bar 1	25.37 ± 0.52 ^{b}	1.51 ± 0.26 ^{a}
Bar 2	23.33 ± 0.61 ^{c}	1.72 ± 0.21 ^{a}
Capsule 1	22.96 ± 0.38 ^{b}	1.46 ± 0.02 ^{b}
Capsule 2	23.87 ± 0.71 ^{b}	1.49 ± 0.05 ^{a}
Moka 1	23.03 ± 0.23 ^{c}	2.71 ± 0.25 ^{b}
Moka 2	24.06 ± 0.41 ^{b}	3.52 ± 0.08 ^{a}
Pod 1	29.61 ± 0.26 ^{b}	1.30 ± 0.06 ^{b}
Pod 2	28.46 ± 0.53 ^{a}	1.44 ± 0.07 ^{c}

3.3. Total Flavonoid Content

The results of the total flavonoid content (TFC) are shown in Table 5. In coffee powder samples, the TFC varied between 82.33 ± 0.84 and 113 ± 1.05 mg RUT/g of coffee powder. The samples with the highest TFC content were capsule coffee powder (99.40–113.69 mg RUT/g of coffee powder) and Moka coffee powder (93.64–97.67 mg RUT/g of coffee powder). After infusion, there was a reduction in the total flavonoid content in all infused coffee samples. However, the best method of preparation of the infused coffee was the use of the Moka technique (8.55–8.60 mg RUT/g of coffee powder) [31,32]. Furthermore, the variability in flavonoid concentration in coffee beverage samples may be related to time, extraction temperature, and the ratio of water to the coffee powder, as pointed out by Uslu (2021) [36].

Table 5. Total flavonoid content (mg RUT/g coffee powder) ± SD for ground coffee and respective beverages samples. The superscripts a, b, and c denote significant ($p < 0.05$) differences.

Sample	TFC	
	Coffee Powder	Coffee Beverages
Bar 1	86.78 ± 0.42 ^{c}	5.13 ± 0.21 ^{c}
Bar2	79.90 ± 0.56 ^{a}	4.77 ± 0.36 ^{b}
Capsule 1	82.33 ± 0.84 ^{b}	4.39 ± 0.25 ^{b}
Capsule 2	90.02 ± 0.72 ^{a}	5.01 ± 0.14 ^{b}
Moka 1	93.64 ± 0.63 ^{a}	8.55 ± 0.24 ^{a}
Moka 2	97.67 ± 0.82 ^{b}	8.60 ± 0.31 ^{c}
Pod 1	113.69 ± 1.05 ^{b}	4.41 ± 0.09 ^{a}
Pod 2	99.40 ± 0.94 ^{b}	3.94 ± 0.11 ^{b}

3.4. Determination of Antioxidant Activity

To assess the antioxidant activity of the coffee samples examined, two antiradical assays were performed: ABTS and DPPH. The ABTS assay showed that for the coffee powder samples and the extracts obtained from them, the extraction process did not affect the antiradical activity of coffee (Table 6).

Table 6. Inhibition percentage (I%) ± SD of coffee powder and beverage samples for ABTS and DPPH assays. The superscripts a, b, and c denote significant ($p < 0.05$) differences.

Sample	ABTS		DPPH	
	Coffee Powder	Coffee Beverages	Coffee Powder	Coffee Beverages
Bar 1	99.03 ± 0.21 ^{a}	97.63 ± 0.11 ^{a}	61.09 ± 0.23 ^{b}	27.03 ± 0.12 ^{a}
Bar 2	99.12 ± 0.23 ^{c}	97.71 ± 0.13 ^{a}	60.89 ± 0.11 ^{a}	27.42 ± 0.18 ^{a}
Capsule 1	97.59 ± 0.11 ^{b}	99.35. ± 0.21 ^{b}	48.76 ± 0.15 ^{a}	7.84 ± 0.06 ^{c}
Capsule 2	96.31 ± 0.22 ^{c}	99.42 ± 0.25 ^{b}	49.04 ± 0.13 ^{a}	9.06 ± 0.09 ^{a}
Moka 1	99.88 ± 0.25 ^{b}	99.62 ± 0.13 ^{b}	64.65 ± 018 ^{b}	16.01 ± 0.25 ^{c}
Moka 2	99.96 ± 0.27 ^{b}	99.73 ± 0.17 ^{b}	64.82 ± 0.16 ^{c}	14.78 ± 0.17 ^{a}
Pod 1	98.23 ± 0.19 ^{a}	97.68 ± 0.15 ^{a}	54.75 ± 0.09 ^{b}	24.23 ± 0.24 ^{a}
Pod 2	98.52 ± 0.17 ^{b}	97.76 ± 0.14 ^{a}	55.21 ± 0.05 ^{a}	22.04 ± 0.15 ^{b}

The ABTS assay was characterized by high antiradical activity for both ground coffee (96–99% of Inhibition) and coffee infusions (97–99% of Inhibition), in agreement with the literature studies [32,36]. Concerning the DPPH assay, the results obtained showed a difference in radical inhibition between the ground coffee samples and the respective infusions (Table 6). Among the coffee powder samples, the Moka samples were found to have a higher antioxidant capacity of about 65% DPPH radical inhibition for both samples, followed by the ground coffees for professional brewing (60.89–61.09% of Inhibition).

However, the extracts obtained with the different infusion methods showed a lower scavenging capacity towards the DPPH radical, probably due to the influence of the extraction factors on the antioxidant compounds [36,37]. Furthermore, the results obtained showed a difference in the antiradical activity within the ABTS and DPPH assays, and this is probably due to the different target molecules of the two reagents [38,39]. In particular, ABTS is mainly oxidized by peroxyl radicals and it is soluble in both aqueous and organic solvents, so can be used to determine both the hydrophilic and lipophilic antioxidant capacity (AOC) of extracts [40]. Therefore, it could be worth noting the strong scavenging abilities of both coffee powders and coffee brews against ABTS radicals.

3.5. Statistics Analysis

The data obtained from the biogenic amines analysis and the spectrophotometric assays for the determination of polyphenols, flavonoids, and scavenging activities were analyzed by univariate and multivariate analysis. The Pearson correlation between the eight biogenic amines analyzed and the spectrophotometric assays (TPC; TFC; ABTS and DPPH) was evaluated (Table 7).

Table 7. Pearson correlation ($p < 0.05$) between spectrophotometric assays (TPC, TFC, DPPH, and ABTS) and biogenic amines determined in coffee samples.

	ABTS	DPPH	TPC	TFC	Ser	B-Pea	Put	His	Spd
ABTS		0.163	0.187	0.195	0.414	0.448	0.034	0.087	0.465
DPPH			0.878	0.904	0.900	0.810	0.906	−0.272	0.607
TPC				0.990	0.925	0.678	0.912	−0.213	0.555
TFC					0.933	0.713	0.927	−0.228	0.542
Ser						0.809	0.847	−0.090	0.667
B-Pea							0.649	−0.232	0.879
Put								−0.195	0.444
His									−0.247

The results showed that there was a strong positive correlation ($r > 0.900$; $p > 0.05$) between the total polyphenol content, the total flavonoid content, and the DPPH antioxidant assay. Regarding BA concentration, the analysis showed that serotonin, β-phenylethylamine, and putrescine are positively correlated ($r > 0.678$; $p < 0.05$) with total polyphenol content, total flavonoid content, and scavenging activity towards the DPPH radical. Spermidine is positively correlated ($r > 0.607$; $p < 0.05$) to the DPPH antioxidant assay. Furthermore, β-Pea correlated positively with Ser ($r = 0.809$; $p > 0.05$), while Put and Spd positively correlated with both serotonin and β-Pea content ($r > 0.667$; $p > 0.05$).

In addition, an exploratory analysis using Principal Component Analysis (PCA) was performed on the data obtained from the analyses of the spectrophotometric assays and the biogenic amine content. Firstly, PCA was performed considering all the results obtained from the analyses (Figure 2).

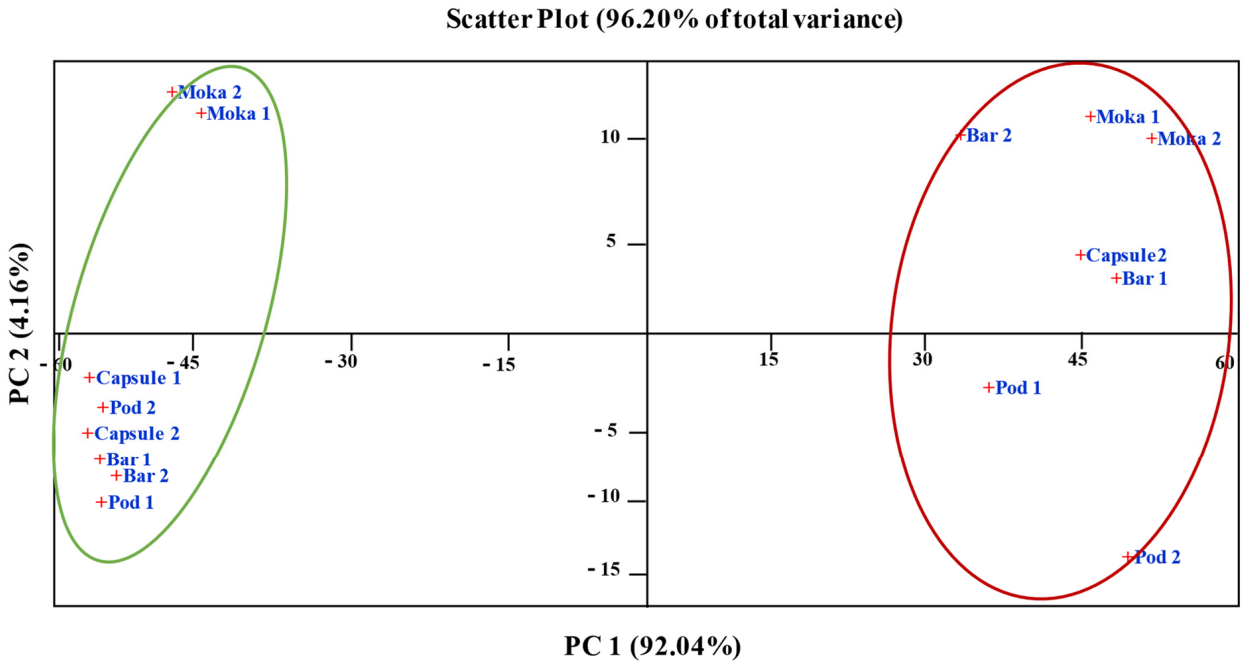

Figure 2. Scatter Plot of the main components (PC1 vs. PC2) of ground coffee (orange), and infused coffee (green) samples.

Considering this exploratory investigation, it was found that the first two principal components explain approximately 96% of the variance. Furthermore, from the Scatter Plot between Principal Component 1 (PC1) and Principal Component 2 (PC2), two groupings along the *x*-axis (PC1) were formed between the ground coffee and infused coffee samples. Loadings analysis showed that the variables that weigh positively on Principal Component 1 are total polyphenol content and total flavonoid content, followed by serotonin, whereas the variables that weigh positively on PC2 are serotonin concentration and histamine concentration followed by β-phenylethylamine. The variables that weigh negatively on PC2 are TFC and TPC. The Scatter plot also shows a separation along the PC2 component for the Moka-infused coffee samples; therefore, to better assess how the variables affected the Moka-infused coffee samples, the principal component analysis was performed for these samples only (Figure 3). The total variance explained by the two principal components in this second PCA analysis was around 90%. It was also shown that the coffee beverage samples were separated according to the extraction procedure. Therefore, it can be concluded that the multivariate principal component analysis showed a difference between the ground and infused coffee samples and that even with a small number of samples, clustering between the infused coffee samples is evident for the variables studied [41,42].

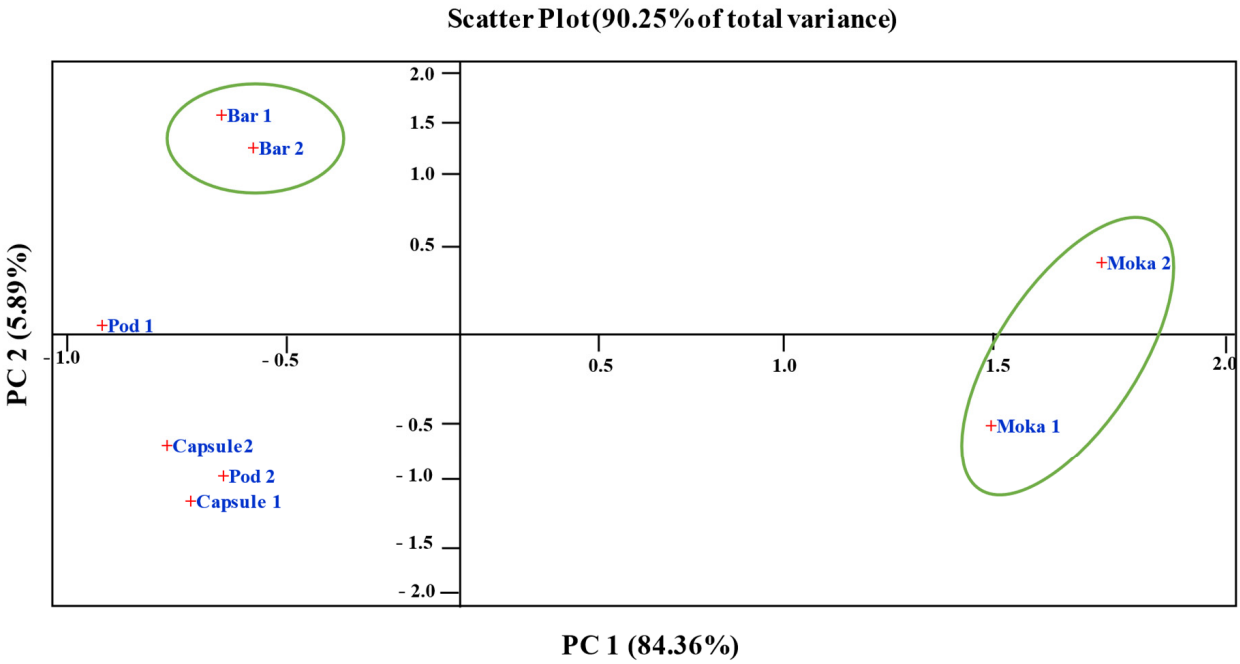

Figure 3. Scatter Plot of the main components (PC1 vs. PC2) of coffee infusion obtained with different brews techniques.

3.6. Life Cycle Assessment Impact Assessment of Coffee Brewing Methods

The LCA analysis was conducted to assess the environmental impact of the four different coffee preparation methods examined (Moka, industrial coffee machine, capsule machine, and pod machine). The analysis was carried out by applying the global ReCiPe Midpoint (H) method, which considers 18 different impact categories [43]. The data obtained showed that the method with the highest weighting on almost all 18 impact categories is the capsule method. Table 8 showed LCA results obtained for the coffee brewing methods analyzed in the study. The results of LCA comparison among the four coffee brewing methods (Moka, bar, capsule, and pod) analyzed in this study, revealed a variation depending on the preparation method used by the consumer. A remarkable difference among coffee brewing methods was recorded for five impact categories: Global Warming, Terrestrial Ecotoxicity, Human Non-Carcinogenic Toxicity, Land Use, and Fossil Resource Scarcity.

The capsule was the coffee brewing method that weighs the most on 14 out of 18 impact categories considered. It heavily impacts Terrestrial Ecotoxicity (4.21×10^{-1} kg 1.4-DCB), Human non-carcinogenic toxicity (1.12×10^{-1} kg 1.4-DCB), and Land Use (1.35×10^{-1} m^2a crop eq). This could probably be related to the release of heavy metals into soil, air, and water mainly related to the primary packaging of the brewing method; in particular, aluminum (Al) used for capsules had a key role in human and environmental ecotoxicity. As stated by different authors [44,45], the environment may be contaminated by Al mainly from anthropogenic sources and through the weathering of rocks and minerals. Several chemical compounds with Al (i.e., Al nitrate, Al phosphate, Al sulfate, etc.) are widely used in various products and processes associated with human activities, such as for the manufacturing of cooking utensils and foils, as well as layers for capsule production. However, for some impact categories, the capsule has less impact than the other brewing methods considered. It was found that Global Warming (1.38×10^{-1} kg CO_2 eq), it has a lower impact than Moka by about 9%, Ionizing radiation by 23%, Human carcinogenic toxicity by 16%, and Fossil resource scarcity by 18%.

Table 8. LCIA results for different coffee brewing methods.

Impact Category	Unit	Pod	Moka	Capsule	Bar
Global warming	kg CO_2 eq	1.14×10^{-1}	1.87×10^{-1}	1.38×10^{-1}	1.08×10^{-1}
Stratospheric ozone depletion	kg CFC_{11} eq	7.78×10^{-7}	6.48×10^{-7}	9.90×10^{-7}	7.89×10^{-7}
Ionizing radiation	kBq $Co-_{60}$ eq	6.05×10^{-4}	1.14×10^{-3}	5.33×10^{-4}	3.98×10^{-4}
Ozone formation, Human health	kg NOx eq	3.57×10^{-4}	4.49×10^{-4}	4.46×10^{-4}	3.50×10^{-4}
Fine particulate matter formation	kg $PM_{2.5}$ eq	2.75×10^{-4}	3.13×10^{-4}	3.45×10^{-4}	2.72×10^{-4}
Ozone formation, Terrestrial ecosystems	kg NOx eq	3.71×10^{-4}	4.62×10^{-4}	4.63×10^{-4}	3.64×10^{-4}
Terrestrial acidification	kg SO_2 eq	1.33×10^{-3}	1.29×10^{-3}	1.67×10^{-3}	1.33×10^{-3}
Freshwater eutrophication	kg P eq	1.57×10^{-5}	1.46×10^{-5}	2.00×10^{-5}	1.58×10^{-5}
Marine eutrophication	kg N eq	2.25×10^{-4}	1.65×10^{-4}	2.86×10^{-4}	2.29×10^{-4}
Terrestrial ecotoxicity	kg 1.4-DCB	3.28×10^{-1}	3.64×10^{-1}	4.21×10^{-1}	3.28×10^{-1}
Freshwater ecotoxicity	kg 1.4-DCB	4.05×10^{-3}	2.99×10^{-3}	5.16×10^{-3}	4.12×10^{-3}
Marine ecotoxicity	kg 1.4-DCB	9.02×10^{-4}	8.29×10^{-4}	1.15×10^{-3}	9.10×10^{-4}
Human carcinogenic toxicity	kg 1.4-DCB	1.18×10^{-3}	2.39×10^{-3}	1.40×10^{-3}	1.09×10^{-3}
Human non-carcinogenic toxicity	kg 1.4-DCB	8.78×10^{-2}	8.31×10^{-2}	1.12×10^{-1}	8.86×10^{-2}
Land use	m^2a crop eq	1.04×10^{-1}	9.95×10^{-2}	1.35×10^{-1}	1.05×10^{-1}
Mineral resource scarcity	kg Cu eq	5.17×10^{-4}	5.74×10^{-4}	6.52×10^{-4}	5.14×10^{-4}
Fossil resource scarcity	kg oil eq	2.38×10^{-2}	4.94×10^{-2}	2.72×10^{-2}	2.15×10^{-2}
Water consumption	m^3	7.96×10^{-3}	7.95×10^{-3}	1.01×10^{-2}	8.08×10^{-3}

Furthermore, the type of primary packaging used for storing the coffee also plays a decisive role in Moka coffee beverage, thus resulting in a higher impact for the categories Terrestrial and Freshwater Ecotoxicity (3.64×10^{-1} kg 1.4-DCB; 2.99×10^{-3} kg 1.4-DCB, respectively) [46].

Characterized results obtained with the ReCiPe Midpoint method for the coffee brewing methods impact assessment are shown in Figure 4. In addition, Moka coffee beverage resulted in the highest value for Fossil Resource Scarcity (4.94×10^{-2} kg oil eq), thus representing 40% of the total impact generated by all coffee brewing methods considered for this impact category. This could be attributable to the differences in energy consumption of the respective preparation techniques [17]. The Moka method requires higher electricity consumption, which is based on the use of fossil fuels, thus being responsible for the direct emission of greenhouse gases such as CO_2 and N_2O, which inevitably affect Global Warming, as well as Fine Particulate Matter Formation [46,47].

Meanwhile, the preparation method with the lowest environmental impact is the industrial machine and this is probably related to less time spent on coffee preparation and reduced packaging of the raw material, as it is packaged in 3–5 kg LDPE bags, which are then collected as organic waste [28,29]. The study found that the concentration of coffee powder used, as well as the ratio of packaging mass to the volume of the beverage prepared, has a significant effect on the environmental impact of coffee preparation methods.

Cibelli et al. (2021) highlighted a greater amount of GHGs emissions associated with a multi-layer packing bag (i.e., Al-PP-PE) as well as post-consumer wastes, for espresso coffee machines than for capsule or pod coffee machines [28].

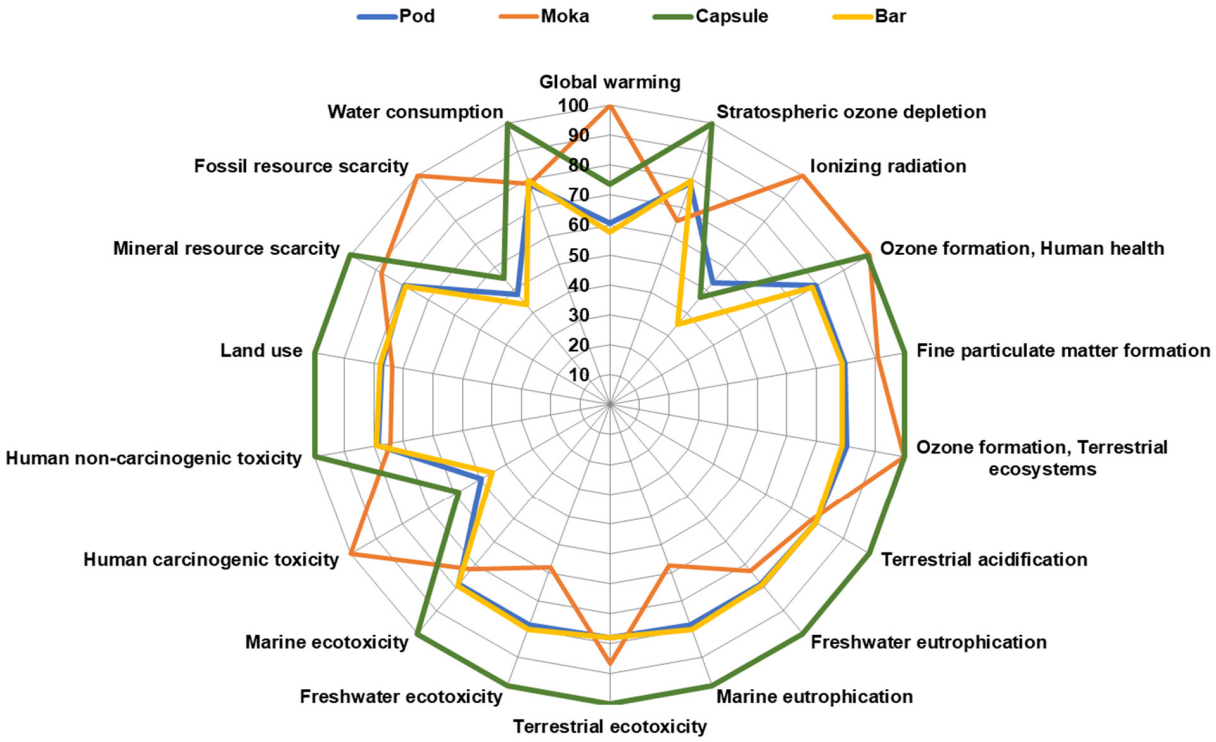

Figure 4. Characterized results for environmental impacts of coffee brewing methods.

4. Conclusions

In recent years, the market for nourishing foods and beverages has become increasingly diversified in response to structural changes in consumer demand, which calls for increased attention to the health-promoting effects, the preservation of the environment, and the socio-economic well-being of small producers. As the brewing method can be considered the main contributing factor for the coffee beverage chemical-nutritional composition, this study demonstrated that bioactive compound content (polyphenols, antioxidants, and BAs) greatly depends on the brew preparation technique adopted. Coffee powders used directly for professional espresso machines, and Moka, and coffee powders packaged for pods and capsules, and subsequently extracted by different brewing methods were considered. All four different coffee beverages obtained were then compared with the corresponding non-extracted coffee powders.

Analyses of coffee powders showed total BA concentration ranging from 67.01 μg/g to 96.83, thus highlighting a decrease of 39% in coffee beverages (16.02–53.92 μg/g). Among all BAs, Serotonin was the prevailing amine in both ground coffee samples (62.13–84.24 μg/g) and coffee beverage samples (12.75–33.46 μg/g). β-Pea, Put, His, Spd, and Spm were found in wide variations of concentration observed depending on the coffee brewing method. When considering coffee brews, phenolic compounds (polyphenols, flavonoids, antioxidants) are the class of bioactive compounds most abundant in coffee, which undergo significant variation during coffee beverage preparation. It was found that the total polyphenol content was higher in the starting ground coffee powders (22.96–29.61 mg GAE/g) and decreased significantly in coffee beverages, between 80% and 90%, depending on the different beverage preparation methods. The same trend was found for the TFC assay, thus observing in ground coffee samples a flavonoid content approximately 15 times higher than coffee beverages samples.

The overall reduction of bioactive compounds in coffee beverages could probably be due to the high brewing temperatures and pressures, which lead to the degradation of these compounds; in addition, the water/coffee contact surface and the particle size of the coffee powder may affect the extractant capacity of biogenic amines and phenolic compounds [1,5]. Furthermore, using multivariate analysis, it was possible to show that

the variables considered allowed the samples to be grouped into ground coffee and coffee beverages and that they were heavily influenced based on the brewing method adopted.

The application of LCA methodology allowed the sustainability assessment of coffee brewing methods, thus highlighting lower environmental impact for the industrial coffee machine compared to the capsule brewing method, which showed the highest environmental burden in 14 out of 18 impact categories analyzed. However, the LCA study presents limitations, since the coffee cultivation and production stages were not considered for the sustainability assessment, in a cradle-to-grave approach. For this reason, future studies will have to expand the boundaries of the system, also considering the disposal and reuse of processing by-products. In addition, different coffee preparation techniques (e.g., Turkish, French, American brewing techniques, etc.) can be compared to highlight the most efficient one in terms of both the quality and sustainability of the final coffee beverage.

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3.5 Research article no. 5

Title: Sustainability Assessment of Different Extra Virgin Olive Oil Extraction Methods through a Life Cycle Thinking Approach: Challenges and Opportunities in the Elaio-Technical Sector.

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Graphical abstract:

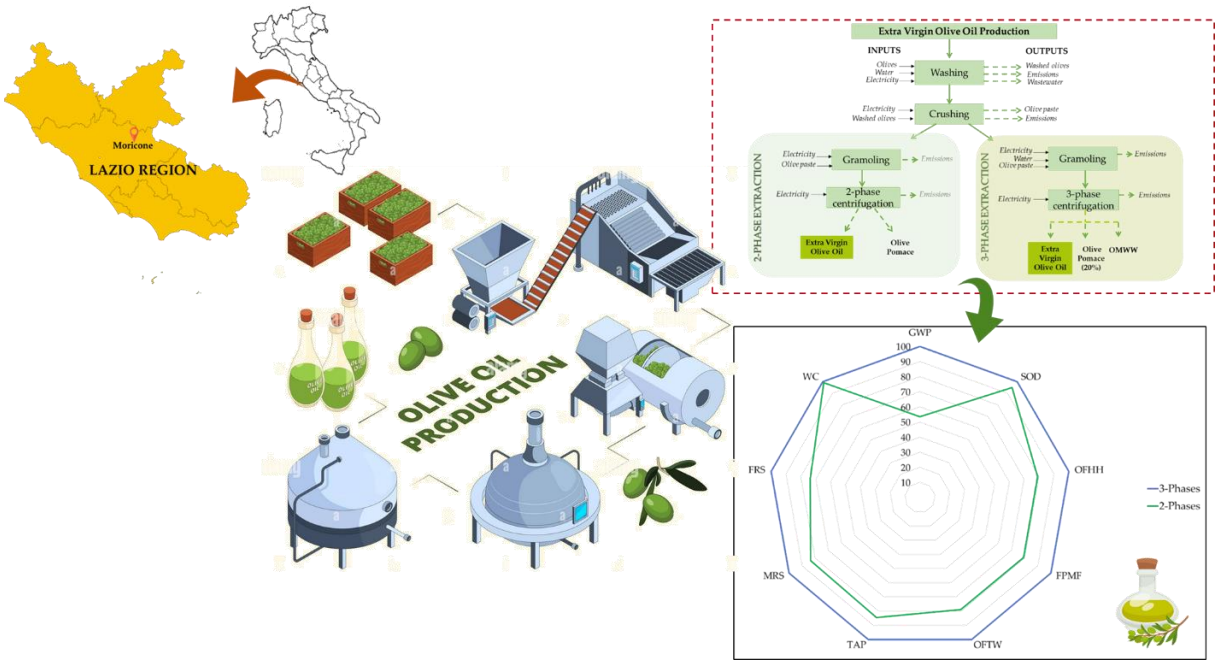

Figure 3.5. Graphical abstract of experimental study no. 5. Source: author's elaboration

sustainability

MDPI

Article

Sustainability Assessment of Different Extra Virgin Olive Oil Extraction Methods through a Life Cycle Thinking Approach: Challenges and Opportunities in the Elaio-Technical Sector

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Abstract: Owing to its important nutritional features, extra virgin olive oil (EVOO) is one of the world's highest-value products, mostly manufactured in Mediterranean countries. However, its production exerts several negative environmental effects, mainly related to the agricultural phase (and the use of fertilizers, pesticides, etc.) and waste management. Olive oil can be extracted from the olive paste using different extraction systems, including pressure, centrifugation, and percolation. In particular, EVOO by-product composition strictly depends on the extraction technologies, and two- or three-phase centrifugal extraction methods are usually employed. Therefore, due to olive oil's economic value, it might be useful to investigate its environmental impacts, to advise sustainable supply chain models. In this context, a valuable tool for assessing the product's environmental compatibility is the Life Cycle Assessment, which is part of a broader Life Cycle Thinking philosophy. This research focused on evaluating the EVOO environmental impact by comparing two- and three-phases extraction processes. Additionally, two scenarios, (i.e., composting and bio-gasification), were proposed to assess the best valorisation strategy for the produced pomace. The results showed that the two-step extraction process was more sustainable than the three-step one in nine out of nine considered impact categories. By milling 1000 kg of olives, the first technology approximately produces 212 kg CO_2 eq, the latter 396 kg CO_2 eq. Finally, pomace valorisation by bio-gasification was found as the best recovery process, able to confer greater environmental benefit than composting.

Keywords: extra virgin olive oil; two-phase and three-phase centrifugal extraction processes; life cycle assessment; biogas; composting

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1. Introduction

Globally, the olive oil sector experienced a positive consumption growth (+2.9%) over the last three crop years and should reach about 3,215,000 tons this year [1]. This trend is related to several established and emerging aspects. Among others, they include the Mediterranean diet acceptance, the recognition of extra virgin olive oil's health-promoting effects, the consumers' attitude after the pandemic to choose better quality products, and the widespread application of olive oil as an ingredient in cosmetic, pharmaceutical, and food formulations, as well as the support to single-origin premium olive oil and sustainable production practices [2,3]. At the temporary level, the annual olive oil production is further boosted by the scarcity on the market of sunflower oil, mainly produced and exported by Ukraine. As soon as the war will go on, production will suffer thus increasing the demand for other vegetable oils to be used in replacement. These issues fueled olive oil consumption, which rose faster than production, the latter expected to reach in the current year 3,100,000 tons [1]. In 2020/2021 the IOC members held 93% of the global olive oil production. Among producers, Spain dominated with about 47%, followed by Greece (9%) and Italy (9%) [1]. However, it should be underlined that, although Greece is expected to

increase olive production in 2023, threats were raised for Spain and Italy. Both countries suffered one of the most severe droughts of all time, leading to a decrease in production but also a lower quality of olive crops. Moreover, the high costs of energy, the rising demand, and the productive framework of both countries, further endanger the financial sustainability of the production process for many Spanish and Italian companies. Imports and exports are going to exceed 1 million tons (1,211,000 and 1,189,000 tons, respectively), with USA and Europe as the leading importers, followed by Brazil, Japan, Canada, and Russia. According to IOC outlooks, the European market will remain the more important producer, exporter, and consumer of olive oils with the Mediterranean countries (i.e., Spain, Italy, Greece, and Portugal) accounting for the largest shares worldwide. The same four countries in 2021 exported about 75% of the globally produced olive oil, led by Spain and Italy with about 46% and 20% export shares, respectively [1].

With 1.1 million hectares devoted to olive trees cultivation, 315,000 tons of olive oil produced in 2022, almost 50 Protected Designations of Origins (PDO) and protected geographical indication (PGI) certifications, and about 5000 olive oil mills spread all over the country, Italy is one of the key players in the olive oil market, as witnessed by the income produced by this sector that is proved to be one of the pillars of the Italian economy [4]. Nevertheless, in recent years, the Italian olive oil sector underwent a loss of competitiveness, due to the recent phytosanitary problems as well as to the scarce technological improvement and unproper process management in the mill plants [5]. Whereas the agronomic issues are going to be solved, the lack of technological innovation still needs to be addressed, being the high costs of machinery, the lack of infrastructures, the problems in waste disposal, and the small/medium industrial capacity are the most severe limiting factors [6].

Among the extra virgin olive oil (EVOO) production steps, the extraction phase shows a great deal of room for improvement to enhance yields, product quality, and the environmental load of the whole production cycle. For the abovementioned reasons, currently, in the Mediterranean countries, the three-phases centrifugal extraction system is still the most widespread, followed by the two-phases extraction method and by the traditional discontinuous pressing process, with strong differences depending on the zone [7]. Known since the 1970s, the three-phase decanter gained popularity as it reduced labor while increasing the processing capacity and the oil yield. However, the high-water demand during malaxation, the high energy consumption, and the large amounts of wastewater generated pushed towards the implementation of a two-phases decanter able to separate the liquid phase (oil) from a wet solid phase, without the addition of water. Typically, the two-phase obtained oil is richer in antioxidants and aroma compounds, also avoiding the generation of highly polluting liquid wastes. However, in the two-phase process, the energy requirements are generally higher, and a certain loss of oil is recorded as it remains absorbed into the pomace. This results in a wet solid by-product (55–60% water content), particularly difficult to handle from environmental and economic points of view. Moreover, optimal control of the waste characteristics (moisture and solids, in particular) cannot be achieved during the two-phase extraction, as the solid and liquid phases are delivered together. It follows that, depending on the method, different advantages or drawbacks can be underlined [5,8].

For this reason, at the industrial level, the proper management of the extraction process represents one of the parameters to take into consideration to improve the economic, qualitative, and environmental features of the whole process. In particular, the latter issue gained much attention during the last decades as olive oil production causes relevant environmental impacts, gathered in both space (Mediterranean basin) and time (September-December) [9]. Huge amounts of different wastes are generated during production, mostly pruning residues (leaves, woody fractions, and thin branches), stones, olive mill wastewaters, and pomace. Among them, leaves represent about 10% of the picked olives, the same value as the fruit stones. Olive mill wastewaters (OMWW) and olive pomace (OP) account for 35–45% of the processed drupes. The latter categories are the most problematic in terms

of quantities (30 million m^3 per year and 2 million tons per year, respectively) and polluting capacities (pH, chemical and biological oxygen demand, etc.).

Many attempts have been carried out to quantify the environmental loads of the olive oil supply chain, mostly by the application of the life cycle assessment (LCA) methodology [10–12] with or without carbon footprint (CF) and energy footprint (EF) estimates [7,13–16]. Sometimes also economic [17] and/or social evaluations [18] have been reported to further support the environmental assessment. Accordingly, it is generally recognized that farming practices exerted the most significant environmental burdens (chemicals, water, and energy consumption), although packaging/distribution (fuel consumption, emissions, and wastes) and transport (fuel consumption, air emissions) activities seemed to contribute as well [17]. As far as manufacturing operations are concerned, they were analysed for waste generation and to a lesser extent fuel and energy consumption. In this sense, the extraction method itself showed a limited impact (energy and water consumption, emissions), if compared to other supply chain phases [19,20]. Nevertheless, as the extraction method dramatically affects the characteristics of the by-products and wastes, its contribution should not be neglected, especially when a gate-to-gate approach is considered. In this regard, very few studies can be found in the literature, specifically focusing on the environmental assessment of the milling process [5,21,22]; more often this aspect is considered in a general approach concerning the whole supply chain where its effects are usually underrated in comparison to others, and/or it does not represent the main goal of the study. In a gate-to-gate approach, Cappelletti et al. evaluated different extraction methods (i.e., pressure, two-phase, three-phase, and de-stoning) finding similar performances for two- and three-phases methods (0.6 MJ L^{-1} of produced oil), although cultivation was also considered [23]. Cini et al. studied how the reuse of olive stones could limit the environmental load during EVOO production [24]. In this sense, energy evaluations were accomplished, including the extraction methods, but once again not specifically related to this aspect. Perone et al. deeply evaluated only the extraction protocols (two- or three-phase) in terms of energy performances [6]. The authors found about double values of both energy use efficiency and overall equipment effectiveness for the continuous process (two-phase), in comparison with the batch production (three-phase). The authors concluded that new strategies are necessary to meet the small producers' demands to improve energy management in batch processing, suggesting the rapid and non-destructive oil analysis during processing, the reduction of dead times, and pooling different batches to fit the malaxed capacity. Nevertheless, the research only focused on energy performances without a broader environmental analysis. It follows that a knowledge gap can be pointed out, as a deeper evaluation of the environmental loads of the olive oil extraction step could be of interest, mostly for producers, to optimize the overall environmental and economical performances of their mill plants. In this context, this work aims to evaluate the environmental compatibility of extra virgin olive oil production by comparing two extraction methods, one with two phases and another with three phases, through a Life Cycle Thinking approach, using LCA. Next, to verify the preferable disposal of the pomace produced by the two processes, a scenario analysis was carried out, integrating the evaluation of the two-phase and three-phases processes with two different valorisation routes, one for agricultural use, i.e., composting, and one for energy purpose, pomace bio-gasification. Both end-of-life options for spent pomace are considered sustainable solutions with high GHG-saving potential, playing a relevant role in the valorisation of agro-industrial by-products and contributing to the closing of organic matter cycles in a circular economy perspective, as also reported by literature studies. For example, Valenti et al. explore various management scenarios for spent pomace, showing how bio-gasification is the most sustainable solution [25]. Similarly, Batuecas et al. show how biogas production can be a feasible alternative to reduce the environmental burden of oil production, adding value to the supply chain [26]. Fernandez-Lobato et al. (2022) show how bio-gasification of olive pomace can be considered a key technology to reduce environmental impacts associated with the oil production process, generating 0.88 kWh of renewable electricity per kg of olive [27]. Panuccio et al. studied the application of

compost from OP as an agricultural soil conditioner showing how the chemical properties of soil treated with this by-product are positively affected [28]. Mamkagh et al. also used OP as a fertilizer, proving it to be of high quality, revealing how it could also be a viable alternative to synthetic pesticides for pest control [29]. Therefore, within this study, both pomace composting and its bio-gasification were compared from an environmental point of view. A farm in the province of Rome was chosen as the case study, and the evaluation was carried out using SimaPro 9.2.2. software.

2. Life Cycle Assessment

The Materials and Methods should be described with sufficient details to allow others. within this study, a Life Cycle Assessment was conducted by the following standards:

- ISO 14040:2006 [30]. Environmental management: Life Cycle Assessment—Principles and framework.
- ISO 14044:2006 [31]. Environmental management: Life Cycle Assessment—Requirements and guidelines.

The LCA is a valuable tool for comparing two or more options in terms of potential environmental impacts. It consists of four phases: (1) Goal and scope definition; (2) Life Cycle Inventory (LCI); (3) Life Cycle Impact Assessment (LCIA); (4) interpretation. The Simapro 9.2.2. software was used for the impact evaluation.

2.1. Goal and Scope Definition

The goal of the study was to analyse the environmental compatibility of the EVOO extraction process by centrifugation, comparing the two different extraction technologies, two-step and three-step decanters. As shown in Figure 1, the extraction phase follows several steps: washing (hulling carried out with mechanical equipment to remove stems and leaves and washing to clean dust and dirt on the drupe), crushing (breaking the cellular structure of the fruits to obtain the olive paste), and gramoling (mixing the olive paste).

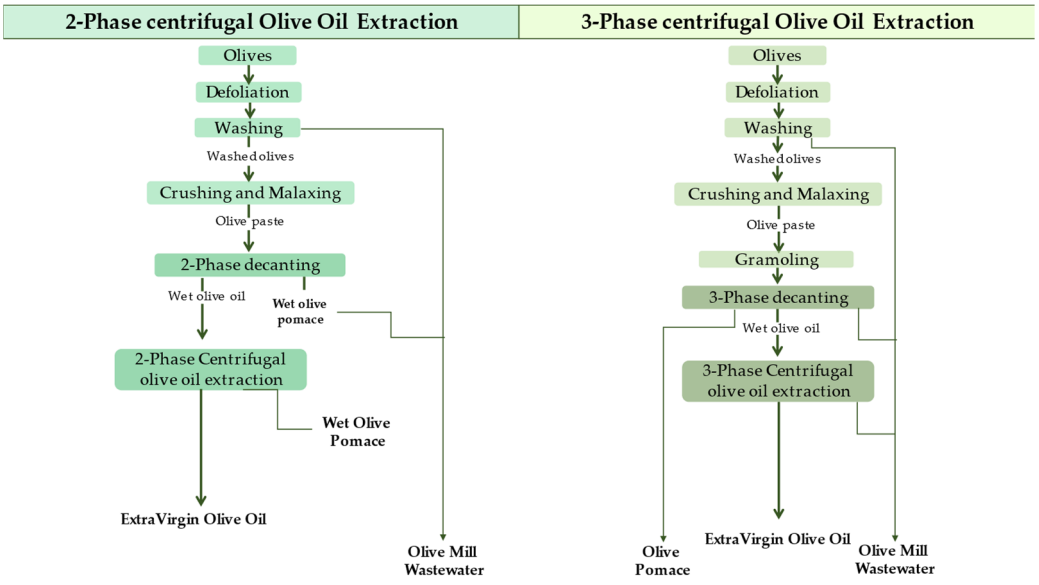

Figure 1. Olive oil extraction by 2- and 3-phase centrifugation.

Then, the extraction can take place. This process can be carried out by a two- or three-stage horizontal decanter. The latter involves the addition of water (usually about 50%) to the gramulated olive paste, generating a product consisting of three phases (oil–water–pomace), subsequently separated by centrifugation. On the other hand, two-phase extraction does not involve adding water to the gram paste. Thus, only two final products are obtained: oil and pomace, mixed with OMWW, thus obtaining oil in greater quantities,

with higher polyphenol content and a decrease in the aqueous by-product [32]. An oil mill located in Moricone, in the province of Rome (Lazio, Italy), was chosen as a case study.

Functional Unit (FU) and Systems Boundaries

The milling of 1000 kg of olives was chosen as the FU. Although among literature studies it is more common to use the liter as FU [14,33,34], in our case study the choice of FU was the milling of 1000 kg of olives, according to other literature data [10,35,36]. This choice well fits the analyses performed during olive oil production and is often referred to as the mass unit. Additionally, it should be considered that the oil yield is strictly related to the extraction process. Finally, performed LCA analysis is intended as a guidance to the company, to identify which of the two extraction processes can guarantee the best performance in terms of environmental impact. The two-phases decanter led to an 18.7% yield, and the three-phases one reached about 17.4% yield. So, considering these yields, approximately 187 kg (two phases) and 174 kg (three phases) of oil were obtained. "From gate to gate" (Figure 2) was chosen as the boundaries of the system, thus excluding the agricultural stage, and starting from the entrance of the olives to the mill not considering transportation, to focus only on the oil production process and pointing out the contribution of the different extraction systems.

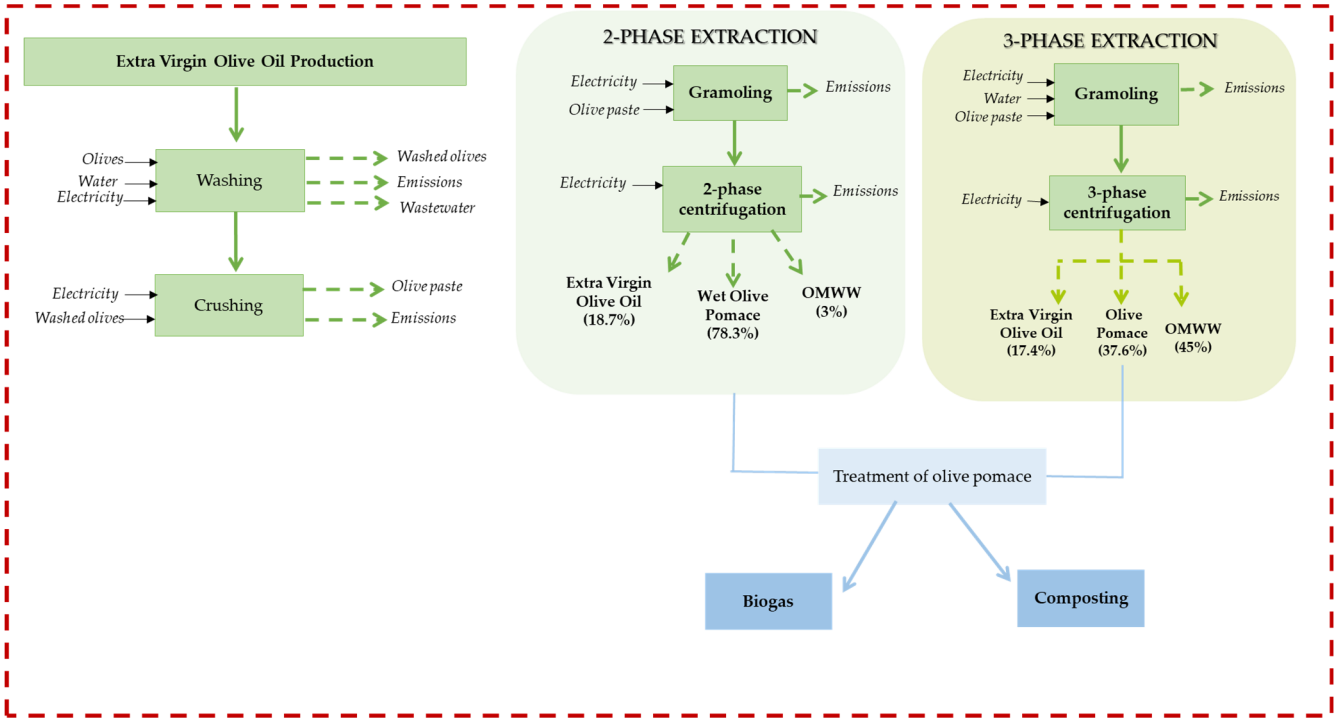

Figure 2. System boundaries of extra virgin olive oil production considered in the study (dotted arrows refer to the process outputs).

As regards the oil processing by-products, this study considers the possibility of reusing OP, obtained from the two centrifugal extraction processes, as input for composting and bio-gasification scenarios. In the evaluation of the environmental impacts associated with the considering recycling scenarios, it was assumed that 100% of the OP was reused for the scenarios.

While, OMWW management was not included in the system boundaries, because the oil mill analysed in the study, uses oil wastewater as soil conditioners, without prior treatment. For the same reason, bottling and distribution were also excluded, because each producer has a different approach (oil can be bottled in 0.75 L, 1 L, or 5 L bottles) [37]. In our study, the production of the machinery was not considered, due to the unavailability of

the data and the reduced environmental contribution of this factor, considering the long useful life and depreciation of the capital goods [37,38].

2.2. Life-Cycle Inventory (LCI)

Primary data were collected through the administration of the questionnaires to employees in the oil mill, in the period of October 2020 and January 2021. The oil mill is located in Moricone (Rome, Italy) (Figure 3).

Figure 3. The geographical position of Moricone in the Lazio Region (Italy).

The farm covers an area of 9 hectares (ha) in hilly surroundings, of which 7 hectares (ha) are planted with olive trees of the *Salviana* mono cultivar from Sabina, (42°14′48″84 N; 12°41′34″80 E), in the Lazio region. On average, the company produces a total of 5 tons of olives per year, and about 750 L of olive oil has been produced in the considered period. In particular, the oil produced presented an acidity of 0.22% (% oleic acid), thus resulting in the commodity classification of extra virgin olive oil [39]. The inventory data (Table 1) consisted of primary data for the year 2021, representing a single extraction. The latter data were subsequently combined with secondary data referred to the background production flows (i.e., olives production) obtained from databases Agribalyse v3.0.1, Ecoinvent v3.8, and World Food LCA Database (WFLDB), provided in SimaPro 9.2.2.

EC Regulation 1513/2001 defines virgin olive oil as all those *"oils obtained from the fruit of the olive tree only by mechanical or other physical processes, under conditions that do not cause alteration of the oil, and which have not undergone any treatment other than washing, decantation, centrifugation, and filtration, excluding oils obtained by solvent or with adjuvants having a chemical or biochemical action or by re-esterification processes and any mixture with oils of other kinds"* [40]. The following specification is added to extra virgin olive oil, defined as an oil "whose free acidity, expressed as oleic acid, is a maximum of 0.8 g per 100 g and having the other characteristics conforming to those laid down for this category". Therefore, the extra virgin olive oil production process, as stipulated in EC Regulation 1513/2001, involves the pressing of olives exclusively by mechanical means and methods and physical processes that do not cause alteration of the oil, not including inputs other than olives, water, and electricity. The outputs consist of EVOO, OP, OMWW, and pomace hazel (namely "nocciolino di sansa", basically the crushed olive kernel). In this study, the data refer to the milling of 1000 kg of olives and imply the use of 85 L of water for the two-phase process and 30 L for the three-phase, as well as ~55.4 kWh of electricity, were employed for both processes.

The obtained EVOO is approximately 187 kg for the two-stage process and 175 kg for the three-stage process, with a larger amount of pomace for the two-stage (784 kg) compared to the three-stage one (376 kg). In contrast, less OMWW is obtained in the two-stage process (60 L) in comparison to the three-stage counterpart (535 L). Data were modeled on the databases in Simapro 9.2.2, especially, Electricity and Water from Ecoinvent 3.4 [41], while Olive was from World Food LCA Database [39]. The Ecoinvent 3.4 database contains LCI data for energy production, transportation, and chemical production, while World Food LCA Database is a comprehensive LCI database, returning data for agricultural

and agri-food products. In the case of electricity, the Italian mix was considered for the year 2021–2022, which is composed as follows: natural gas (43.2%), renewable sources (41.7%), coal (7.9%), nuclear (3.5%), oil sources (0.5%), other (3.1%) [42]. The Italian electricity mix, in accordance with the Ecoinvent consequential system, is based on projections of future electricity market compositions (Ecoinvent v3.8, 2022) by national and international authorities, such as the European Commission (2016) and the International Energy Agency (2016). Therefore, the data for electricity, olives, and water have also been adjusted for Italian conditions based on Simapro 9.2.2 and the database (i.e., Ecoinvent v3.9) updates. So, the data used are based on the average process from the international databases mentioned above (since they are the only ones available in the LCA software package used in this study) and adapted to be as consistent as possible with the objective and scope of this study.

Table 1. Life Cycle Inventory for the olive milling process (Referred to as 1000 kg olives).

			2-Phases	**3-Phases**	**Ref**
INPUT		Olives	1000 kg	1000 kg	[42]
	Washing	Electricity	5.5 kWh	5.5 kWh	[43]
		Water	30 L	30 L	
	Crushing	Electricity	22 kWh	22 kWh	[43]
	Gramoling	Electricity	9.4 kWh	22 kWh	[43]
		Water	-	55 L	
	Centrifugal extraction	Electricity	18.5 kWh	28 kWh	[43]
Industrial yield (kg EVOO/kg olive, %)			**2-phases 18.7%**	**3-phases 17.4%**	
OUTPUT		Extra virgin Olive Oil	~187 kg	~174 kg	
		Pomace	~783 kg	~376 kg	
		Wastewater	~60 L	~535 L	
		Emissions to air (kg CO_2 eq)			
		CO_2	189.4	353.9	
		CH_4	17.4	32.5	
		NO_X	4.1	7.5	
		SF_6	1.1	2.1	
		Emissions to freshwater (kg P eq)			
		Phosphate	0.016	0.016	
		Phosphorus	0.001	0.001	
		Emissions to marine water (kg N eq)			
		Ammonium, ion	0.00730	0.00730	
		Nitrate	0.10141	0.10141	
		Nitrite	0.00002	0.00002	
		Nitrogen	0.00427	0.00427	

2.3. Life Cycle Impact Assessment (LCIA)

To have an assessment spectrum of the environmental performance of the two processes, the ReCiPe 2016 Midpoint (H) was used. This methodology was chosen and preferred over other calculation methods such as ILCD 2011, CML 2001, or TRACI, because having the availability of eighteen impact categories (compared to 16 of ILCD 2011 Midpoint, 15 of IMPACT 2002+, 11 of CML-IA Baseline, and 9 of TRACI) can provide more comprehensive, articulate, and specific results on the environmental impacts of olive milling than other methodologies with fewer impact categories. Therefore, Recipe 2016 Midpoint could give a broader picture with a greater degree of detail on the environmental impacts of production. It considers the following impact categories: Global Warming (GW); Stratospheric Ozone Depletion (SOD); Ionising Radiation (IR); Ozone Formation, Human Health (OFHH); Fine Particulate Matter Formation (FPMP); Ozone Formation, Terrestrial Ecosystems (OFTE); Terrestrial Acidification (TAP); Freshwater Eutrophication (FE); Marine Eutrophication (ME); Terrestrial Ecotoxicity (TEC); Freshwater Ecotoxicity (FEC); Marine

Ecotoxicity (MEC); Human Carcinogenic Toxicity (HCT); Human Non-Carcinogenic Toxicity (HNCT); Land Use (LU); Mineral Resource Scarcity (MRS); Fossil Resource Scarcity (FRS); Water Consumption (WC).

3. Results

The results of the LCA comparison between the two extraction systems are reported in Table 2.

Table 2. LCIA of the two extraction methodologies compared: three stages vs. two stages.

Impact Categories	Unit	Two-Phases	Three-Phases
Global warming	kg CO_2 eq	212	396
Stratospheric ozone depletion	kg CFC_{11} eq	0.000613	0.000646
Ionizing radiation	kBq Co-60 eq	0.362	0.362
Ozone formation, Human health	kg NO_x eq	0.294	0.373
Fine particulate matter formation	kg $PM_{2.5}$ eq	0.218	0.275
Ozone formation, Terrestrial ecosystems	kg NO_x eq	0.299	0.379
Terrestrial acidification	kg SO_2 eq	1.07	1.27
Freshwater Eutrophication	kg P eq	0.0173	0.0173
Marine Eutrophication	kg N eq	0.113	0.113
Terrestrial ecotoxicity	kg 1.4-DCB	114	114
Freshwater ecotoxicity	kg 1.4-DCB	0.724	0.724
Marine ecotoxicity	kg 1.4-DCB	0.438	0.438
Human carcinogenic toxicity	kg 1.4-DCB	1.05	1.05
Human non-carcinogenic toxicity	kg 1.4-DCB	140	140
Land use	m^2 a crop eq	183	183
Mineral resource scarcity	kg Cu eq	0.348	0.416
Fossil resource scarcity	kg oil eq	22.4	30.4
Water consumption	m^3	5.65	5.71

A remarkable difference was recorded in nine categories: Global Warming, Stratospheric Ozone Depletion, Ozone Formation—Human Health, Fine Particulate Matter Formation, Ozone Formation—Terrestrial Ecosystems, Terrestrial Acidification, Mineral Resource Scarcity, Fossil Resource Scarcity, and Water Consumption. Regarding the other impact categories, although the three-step process is more impactful than the two-step process, the differences are minimal. For example, regarding the Freshwater Eutrophication category, the two-phase, with 0.0173 kg P eq, has an impact of −0.01% compared to the three-phases process and a Marine Eutrophication of −0.0003%. Therefore, even evaluating the very low values of the other impact categories, and the considered perspective (Hierarchist, contemplating a 100-year time frame), the remaining categories were neglected. Then, the results concerning the impact categories with significant differences were expressed as relative impact (Figure 4).

In this case, through the software, the results of the study expressed in various units (kg CO_2 eq, kg SO_2, kg $PM_{2.5}$, etc.) are multiplied by the characterisation factors and expressed in terms of relative impact. In other words, the sum of the emissions of the various phases is set equal to 100%, and the various individual results are calculated accordingly as relative impact. This type of visualisation makes LCIA results more usable, especially when applying calculation methods, such as the ReCiPe 2016 Midpoint, which considers impact categories with different units of measurement. This permits to analyse the categories as reported to the same scale and underlining specific trends, simplifying the comparisons between the two processes. The recorded results clearly showed the three-step extraction process is more impactful than the two-step one in all the categories, making the latter more sustainable. GWP is the impact category where the difference between the two methods is more evident, with the three-phase extraction generating about 212 kg CO_2 eq, −46% compared to the other process (396 kg CO_2 eq). The second category showing a valuable difference is then FRS, where the three-stage system generated a depletion of 30.4 oil eq, +26% compared to the two-stage system, which returned FRS

value of 22.4 oil eq. In the range of 20–21% is the difference related to FPMP (0.275 kg $PM_{2.5}$ for the three-stage vs. 0.218 kg $PM_{2.5}$ for the two-stage, −20%), to OFHH (0.373 kg NOx eq vs. 0.294 kg NOx eq, −21.2%) and OFTE (0.379 kg NOx eq vs. 0.299 kg NOx eq, −21.1%), in favor of the two-stage extraction process. Slightly, smaller differences were recorded for the MRS, where the three-phase generated 0.416 kg Cu eq, −16% compared to the two-phases process (0.348 kg Cu eq) and TAP (1.27 kg SO_2 eq vs. 1.07 kg SO_2) −15% impacts for the two-phases process. Finally, in the case of SOD, the two-phases process produced 6.13×10^{-4} kg CFC11 eq, a difference of −5% compared to the three-phases process (6.46×10^{-4} kg CFC_{11} eq). Therefore, the LCA results showed that the two-step oil extraction process is generally more sustainable than the three-step process.

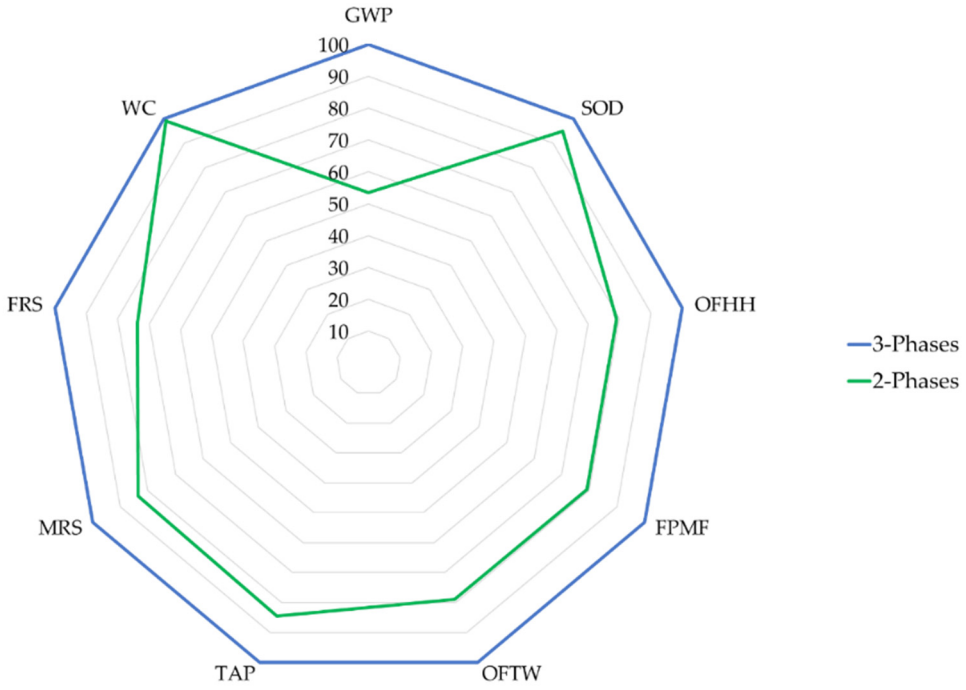

Figure 4. LCIA of the two extraction methodologies compared: three phases vs. two phases (Results characterized). GWP: Global Warming Potential; SOD: Stratospheric Ozone Depletion; OFHH: Ozone Formation—Human Health; FPMF: Fine Particulate Matter Formation; TAP: Terrestrial Acidification Potential; MRS: Mineral Resource Scarcity; FRS: Fossil Resource Scarcity; WC: Water Consumption.

4. Discussions

The LCA results showed that between the two oil extraction processes, the two-stage decanter process displayed the greatest environmental compatibility. These results can be mainly explained by considering the highest water volumes used in the three-step process. In this regard, over-exploitation of freshwater bodies, including groundwater used for water for agricultural/industrial use, can create a water crisis for future generations. Water scarcity is a global problem particularly concerning the countries of the Mediterranean area, affected by the highest level of water stress, with a score of 4.5 out of 5.00, as well as inequitable distribution of water resources. In this context, Italy ranks first in Europe for water withdrawals for drinking (9 billion m^3 per year) [43] and it is placed among European countries with medium to high water stress (with a score of 3.51 out of 5.00) [44]. In many Mediterranean regions, groundwater replenishment depends mainly on rainfall, although in recent decades the reduction in rainfall events hinders the renewal of sufficient water levels. An important aspect related to the use of groundwater is its extraction, which is mainly done through the use of electricity [45], which in turn requires additional water consumption [46]. Water extraction processes are therefore highly energy-intensive and have a high environmental impact due to the significant electricity production [42]. This one is mainly based on the use of fossil fuels and is responsible for the direct emission of

greenhouse gases, such as CO_2 and N_2O, which inevitably affect GWP, as well as FPMP [47]. Table 2 and Figure 3 highlighted that major differences were recorded in the impact categories mainly related to the atmospheric effects (GWP, SOD, OFHH, OFTE, FPMF, and TAP), as well as depletion of abiotic resources (MRS, FRS, and WC). Thus, the production of electricity for water extraction can be considered as the main reason causing the increase in the environmental impact of the process. For example, in the case of GWP, the different electricity mixes produce the major impact on GHG emissions from electricity generation growing to 33.1 GT CO_2 eq in 2018 [48]. Therefore, power plants and electricity generation burning fossil fuels (coal, oil, or gas) are the main sources of GHGs, especially carbon dioxide and nitrous oxide. For example, one Million British Thermal Units of energy is produced by burning anthracite coal resulting in the release of 102 kg CO_2 eq [49]. Therefore, the excessive burning of coal and other fossil resources for electricity generation and power plant operation leads to consequent excessive production of CO_2, but also particulate matter, NO_X (these also greatly influenced by energy intensity) [50] and SO_2, thus affecting GWP, ozone formation, terrestrial acidification, and particulate matter formation. In the LCA perspective of the extra virgin olive oil, the electricity generation required in the gramoling stage for the additional 55 L of water pumping, could lead to an increase in GWP, as well as in FPMF, ozone formation, and TAP. In addition, a high potential for TAP could also result from desulphurisation processes during the processing and production of fossil fuels [51]. On the other hand, in the case of abiotic resources, particularly fossil and mineral resources, their depletion is likely to be related precisely to the reduction in coal and other fossil resources, as well as mineral resources for their extraction. Finally, in the case of water consumption, the difference in impact between the two extraction techniques can be related to a mere difference in quantity, but also the water supply for power plant cooling towers [50]. Additionally, the Ukraine war generated the current energy crisis and the related price raising, emphasizing the importance of energy source management and the transition to eco-friendly resources. Water saving, as well as lower greenhouse gas generation, could be useful strategies for Italy to achieve the goals set by the Water Framework Directive (2000/60/EC), requiring the achievement of the good qualitative and quantitative status of water bodies by 2027 [39,52]. At the same time, they could be a valuable tool to fit the sustainable development goals by 2030, especially sub-goals 6.4.1 (Water Stress) and 6.4.2. (Water Efficiency), the latter being far from fulfilled.

The data obtained from the LCA refer to the milling of 1000 kg of olives, thus to about 187 kg of oil for the two-phases process and 174 kg of oil for the three-phases process. These results, therefore, related to 1 kg of oil, lead to average values of 1.13 kg CO_2 eq per kg of oil for the two-phases process and 2.27 kg CO_2 eq per kg of oil for the three-phases process, showing how the former type of extraction is more sustainable than the latter, both in absolute terms and per kilo of the produced oil. Different LCA studies applied to olive cultivation have been published, following a "from cradle to grave" or "from cradle to farmgate" approach [19,20], mainly highlighting the agricultural phase and thus the contribution of fertilizers, pesticides, and water management. However, there is a paucity of works focusing on the employment of two-phase and three-phase extraction during the olive milling process. Studies investigating the olive milling process are relatively scarce, as most of them considered the entire supply chain, from olive growing to packaging [53]. In these cases, different assumptions and methodological frameworks, such as biomass conversion technologies, were considered [54]. Fernández-Lobato et al. considered both agricultural and industrial activities, based on a "from cradle to gate" approach, in the analysis of specific olive cultivars growing in Tunisia [7]. The same authors assessed the environmental compatibility of a cultivar in Spain, considering 1 kg of virgin oil (from cradle to gate) and an average impact of 1.93 to 3.00 kg CO_2 eq per kg, depending on the yield. [28]. Ben Abdallah et al., on the other hand, evaluated the harvest stage by considering nine production systems and comparing traditional, intensive, super-intensive, conventional, and organic systems [55]. Similarly, Romero-Gámez et al. compare eight traditional systems, three intensive systems, and one super-intensive system, considering

1 ton of olives as FU [36]. Guarino et al. propose an LCA for different production techniques (conventional and organic in the plains and hills), choosing a 0.75 L glass bottle of EVOO as FU and a "cradle to gate" perspective (from olive production to bottling) [10]. Similarly, De Luca et al. considered a cultivar growing in the south Italy area, by assuming a 0.75 L bottle of EVOO as FU and confining the analysis to "from cradle to the milling plant gate," excluding distribution, sale, and use phase [5]. The results of their study range between 0.16–0.18 kg CO_2 eq for the extraction phase and 5.11–5.13 kg CO_2 eq for the agricultural phase. From the analysis of the literature related to LCA application in the olive sector, therefore, a comparison with other studies could lead to results that are not superimposable, mainly due to the different yields and different assumptions. Additionally, our aim was mainly focused on the industrial phase, highlighting the differences in impact between two and three-stage decanters. However, notable among the studies found is that of Iraldo et al., who analysed the environmental impacts of 1 kg of EVOO in Val di Cornia (Italy) with a three-step process, obtaining for the milling process alone a GWP of 3.63 kg CO_2 eq, −59% compared to the results of our study [35]. However, the authors also employ sodium hydroxide and diesel in the milling process, since they also consider transport to the mill, which is excluded in the present evaluation. However, it is important to clarify some limitations of the performed study has led to the need for assumptions. Data for upstream and downstream processes were compiled from secondary data (retrieved from databases or based on global or regional averages), due to the lack of Italian databases, which will see the light of day in 2023 [56]. Thus, these data could not be always truly representative.

5. Challenges and Opportunities in the Elaio-Technical Sector

One of the biggest challenges related to olive oil production is the sustainable disposal of a large number of generated wastes. Specifically, OMWW, OP, and olive pomace hazel generate about 1550 kg of organic pollutants per 1000 kg of produced olive oil [57], thus resulting in an area of 150 m^3 of municipal waste [58]. In the case of three-phase extraction, OMWW is composed by water (83–94% *w/w*), organic compounds (4–18% *w/w*), and inorganic compounds (0.4–2.5% *w/w*) [59], depending on the olive variety, maturity, water content, growing medium, harvest period, climate, and storage time of the fruit [17]. Typical parameters for characterizing OMWWs produced by the three-phase extraction process are chemical oxygen demand (COD), biological oxygen demand (BOD), pH, conductivity, total solids, lipids, and phenolic content, as reported in Table 3 [60]. These parameters highlight a significant amount of organic pollution associated with the triphasic OMWW and usually directly released into the surrounding environment, without any treatment. For example, OMWW, due to its high COD (54–318 g L^{-1}) and BOD (19–134 g L^{-1}) values, is considered one of the most polluting wastewaters [61,62]. In addition, it displayed reduced biodegradability, due to the massive presence of specific antioxidant compounds, such as polyphenol molecules.

Table 3. Physicochemical Characteristics of 3-Phase OMWW.

Parameter	Range of Values
pH	2.24–5.90
Conductivity (μS cm^{-1})	5–81
COD (g L^{-1})	16.5–190.0
BOD (g L^{-1})	13.4–37.5
Dry residue (g L^{-1})	11.5–102.5
Organic matter (g L^{-1})	16.7–81.6
Lipids (g L^{-1})	1.64–9.80
Phenolic compounds (g L^{-1})	0.5–24.0

COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand.

Making a comparison with municipal wastewater, it has been estimated that the polluting power of OMWW is two hundred times higher. Thus, due to the high organic load

and phytotoxic/antibacterial phenolic substances, OMWW is considered one of the main pollutant effluents produced by agro-food industries. In contrast, the pollution parameters of biphasic OMWW are negligible since the production of liquid effluent from this extraction process is minimal (about 3%) [63]. Regarding OP, those derived from the three-phase and two-phase systems, on the other hand, displayed a very different composition, especially in terms of moisture, residual oil, and phenol content. For three-phase OP these parameters are in the following range: moisture, 45–55%; oil, 3.54.5%; and phenolic compounds, 200–300 mg per 100 g; while two-phase OP parameters are in the range 65–75 (moisture), 3–4% (oil), and 400–600 mg per 100 g (phenolic compounds) [64]. Triphasic OP has the consistency of moist soil, while biphasic OP is a dense sludge, with a pasty consistency, difficult to transport, store, and handle. Therefore, sustainable management of these wastes could be important for the sustainable growth of specific sectors of the food industry. Among the three by-products obtained during olive oil production, the reuse of the OP was chosen to be explored. Olive pomace represents more than 50% of the waste obtained in olive oil production [7] and it contains a remarkable added value, useful employment to generate energy from a renewable source, according to Directive 2009/28/EC [65]. Typically, OP was exploited in pomace factories for the extraction of residual oil using organic and hydrophobic solvents (such as n-hexane), but this type of oil has over time lost market to better quality seed oils sold at cheaper prices. This has led to the closure of many pomace factories, while the disposal of the OP has become a significant environmental issue. Therefore, a scenario analysis was accomplished to assess what might be preferable end-of-life for the valorisation of OP to mitigate the impacts of the disposal of waste deriving from the elaio-technical sector. Therefore, two valorisation opportunities were evaluated to be applied in the agriculture (composting) and energy (biogas production) fields, both applied to the two-step and three-step processes.

In these regards, four scenarios are therefore proposed:

1. Two-phases extraction process with final composting of the OP (including compost application) (S1);
2. Two-phases extraction process with final bio-gasification of the OP (S2);
3. Three-phases extraction process with final composting of the OP (including the application of compost) (S3);
4. Three-phases extraction process with final bio-gasification of the OP (S4).

It was assumed not to consider the intermediate process of extracting and refining the OP oil because of the lack of data. Moreover, some of these inputs are considered negligible for the purpose of the analysis. Finally, although the oil extraction would have been more cost-effective, the process is carried out using organic solvents and thus through an unsustainable process [66], contrary to the objective of our study. Further scenarios for the disposal or reuse of OMWW and OP will be considered in a subsequent study.

5.1. S1 and S3: Agricultural Use—Composting of Spent Pomace

Pomace can be used as such or after composting in a mixture with other technically and economically suitable biomasses, such as poultry manure to considerably increase the nitrogen content. However, the soil conditioner must meet standard limits of organic matter > 40%, C/N ratio < 30, pH in the range of 6.0–8.5, absence of pathogens, low levels of heavy metals, and inert and glass materials [67]. The use for agricultural purposes has been mainly favoured by the loss of organic matter in soils, a recurring problem especially in Italy, typically in southern regions, where organic matter decomposes more rapidly. Such depletion can cause profound changes in the physical, chemical, and biological characteristics of the soil, resulting in degenerative phenomena of which erosion and loss of fertility are the most obvious features. Oil by-products due to their high content of potassium, nitrogen, and phosphorus, must be uniformly spread on the ground to replenish the loss of organic matter in the soil.

5.2. S2 and S4: Energy Destination—Biogas Production

Another particularly interesting valorisation scenario for olive pomace could be its bio-gasification to produce biofuel (biogas), consisting mainly of methane and carbon dioxide. Spent pomace, from a regulatory point of view, represents biomass that can be used for energy purposes [65]. As OP takes origin during an exclusively mechanical extraction process, it does not contain additives or foreign chemicals, but only natural organic and inorganic compounds. It follows that its chemical composition is suitable for bio-gasification. Biogas represents an excellent fuel for small-scale valorisation plants aimed to apply a circular economy model and a valuable resource to develop a system to ensure energy autonomy for medium-sized olive oil mills. The use of pomace for biogas production creates a new virtuous and economically viable circuit for a by-product that has lost value over time.

The results of the scenario analysis are depicted in Figure 5. Considering the whole production process, and thus also considering the disposal scenarios, regarding the GWP (Figure 5A) the two-step extraction process (S1 and S2) turns out to be more sustainable than the three-step process (S3 and S4) in both valorisation scenarios. If the OP was to be composted and applied to the soil, in the two-step process (S1) a reduction of 205 kg of CO_2 was recorded, while in the three-step process (S3) this increase was less consistent (140 kg CO_2 eq). However, this reduction could be even greater if the OP was bio-gasified, with a CO_2 reduction of 1745 kg CO_2 eq in the two-step process (S2) and 1199 kg CO_2 eq in the three-step process (S4). The reduction in CO_2 in both disposal processes is mainly related to the use of compost as a soil amendment, avoiding the production of synthetic fertilizers (and related emissions) [68]. On the other hand, biogas production could limit the exploitation of additional fossil fuels and the resulting emissions [69,70].

Regarding SOD, OFHH, FPMF, OFTE, and TA, it is necessary to consider that the emissions of $PM_{2.5}$, SO_2, and NO_X, along with other generated gases (CH_4, hydrocarbons) in the anaerobic digestion and bio-gasification stages, are not vented to the atmosphere.

Although the inventory data in the various databases in SimaPro refer to average processes and technologies, in Italy, composting plants are equipped with bio cells with a suction system. This technical aspect, together with the presence of adequate piping, means that the gases present, along with water vapor, are conveyed to suitable biofilters that have the function of retaining all emissions from the aerobic composting stages [71]. Therefore, the data related to SOD, OFHH, FPMF, OFTE, and TA are to be considered negligible because $PM_{2.5}$, CFCs, NO_X, and SO_2 are not released into the atmosphere.

Regarding the depletion of abiotic resources, for the MRS category (Figure 5B), the results again showed that the preferable scenario is S2 because a reduction of 0.77 kg Cu eq induces greater savings of mineral resources than the other scenarios (−0.49 kg Cu eq for S1, −0.33 kg Cu eq for S3 and −0.53 kg Cu eq for S4). Regarding the FRS category (Figure 5C), similar results were obtained. S2 (−756 kg oil eq) is preferable as compared to the other scenarios (−554 kg oil eq for S1, −380 kg oil eq for S3, and −519 kg oil eq for S4) because of the resources that would be saved for fossil gas production [72]. Further, for WC (Figure 5D), S2 induces less water wastage (0.73 m^3 of water) than the other valorisation pathways. Therefore, the results of the scenario analysis show that in the entire production process, the two-step process is more sustainable than the three-step process, either in the case of composting treatment or in the case of biogas production. In addition, between the two valorisation scenarios, the bio-gasification process appears to be more sustainable than the composting process since it induces greater CO_2 reduction in both scenarios for all impact categories. Therefore, in the elaio-technical sector, the preferable process might be the two-step decanter process, where OP is bio-gasified (S2), while the least preferable, although it still induces a positive benefit for ecosystems, is the three-step extraction with final composting (S4). However, within the two-step extraction process, there are still some challenges that lead olive farmers to prefer the three-step process. The three-stage decanter, although found to be less sustainable than the two-stage decanter, remains the most widely used for several reasons, mainly economic. Most of the oil mill

industries are small and medium-sized companies and it is considered not convenient to remodel their plants concerning the cost incurred in switching the decanter from three to two stages. In this case, the investment would be not justified, due to the difficulty to amortize the expense for a two-stage decanter [53]. In the case of companies with large turnovers, it might be convenient, but the high-water content of biphasic pomace oil could make pomace oil extraction more expensive, difficult, and impactful, due to the high use of organic solvents and energy [73]. In addition, pomace management requires specific facilities (such as storage tanks with special valves, pumps, and tankers) [74]. Therefore, two-phase technology could transfer the problem of the olive waste disposal of the olive mill factory to the seed oil refineries. For these reasons, there is still often a tendency to prefer the three-phase system.

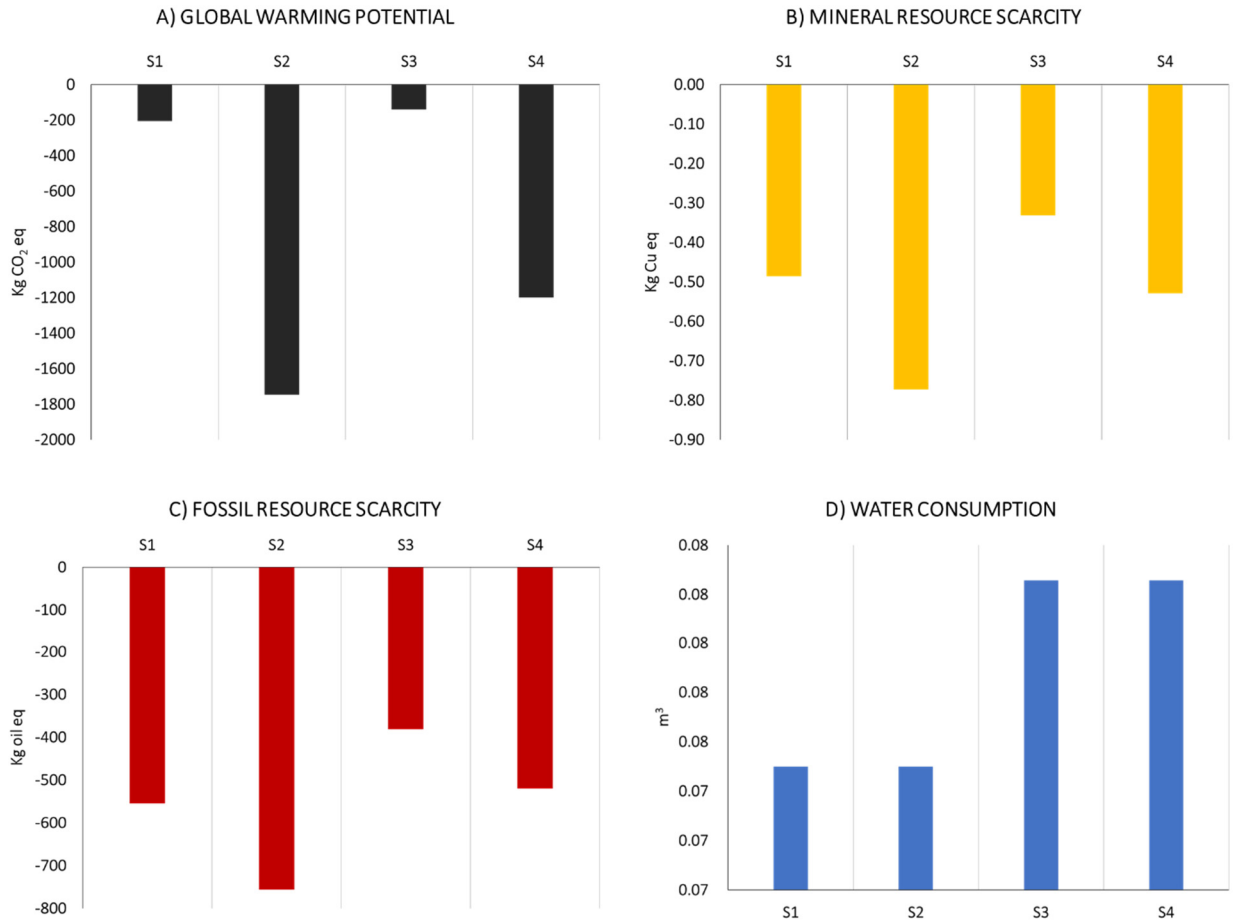

Figure 5. GWP (**A**), MRS (**B**), FRS (**C**), and WC (**D**) for the four scenarios considered. S1: 2-step extraction and composting of olive pomace, S2: 2-step extraction and bio-gasification of olive pomace, S3: 3-step extraction and composting of olive pomace, S4: 3-step extraction and bio-gasification of olive pomace.

6. Conclusions

This research proposes a life cycle assessment approach to compare two different methods of EVOO extraction, a two-phase process vs. a three-phase one. The recorded results show that among the two processes, the two-phases system resulted to be more sustainable than three-phase extractions, with lower values in all considered impact categories. This finding appears mainly related to the extra addition of water, usually required in the three-phase system. Water extraction requires a relevant amount of energy, as well as relevantly increases the volume of the residue to be disposed of. Specifically, the two-phase extraction process induces 46% CO_2 savings (212 kg CO_2 eq) compared to the three-phase

process, and changes in the range of 5–26% in the other impact categories were recorded as well, thus showing preference over the three-phase extraction process. In this framework, it could be worth enhancing greater efforts in terms of energy efficiency and water productivity, thus ensuring a rationalized and sustainable production by a unit of input. In particular, the adoption of a two-phase decanter could advantage mill owners in terms of continuous and automated labour, more compact machinery, OMWW reduction and disposal problems, and better composition and shelf-life of the obtained oil (aspect being also valuable for consumers). All considered, the broader application of the two-phase extraction method should be promoted also with the support of National and/or European funds to sustain the higher costs of adoption. This will result in environmental benefits in the long term and a global collective advantage in terms of resource-saving and reduced environmental pollution. However, one of the main challenges of the sector also includes the management of the by-products with a much higher environmental load compared to the extraction technology itself. To this purpose, some of the most promising solutions could be the possibility of introducing innovative technologies that can improve energy efficiency, and the valorisation of olive oil by-products (e.g., pomace). Bio-gasification and composting technologies were evaluated as possible useful scenarios for the reuse of OP. Specifically, the first one appears the best available technology for bioenergy production, since it induces greater benefits in terms of CO_2 savings. For example, bio-gasification of OP induces savings of −1756 kg CO_2 eq in the two-step compared to the same end-of-life in the three-step process (−205 kg CO_2 eq) and, in turn, greater savings than composting (−140 kg CO_2 eq for the two-step vs. −1199 for the three-step), savings of −0.77 kg Cu eq (two-phase), greater than the −0.49 kg Cu eq of the three-phase, and −756 kg oil eq (two-phase), greater than the −554 kg oil eq of the three-phase. For these reasons, it emerges that bio-gasification is the preferable process. Anyway, it should be underlined that the olive oil sector waste management is a very complex issue, related partly to the chemical features of these by-products, but also to economic, technological, regulatory, and organizational aspects that many times prevent the real adoption of very exciting research purposes by geographically scattered small–medium mills. In this scenario, the LCA approach surely represents an extraordinary tool to evaluate each technology/best practice and its environmental sustainability. However, it should be considered only the starting point leading the way to a more integrated approach involving academia, producers, and decision-makers to switch from a huge problem to an environmental and economic gain.

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3.6 Research article no. 6

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Graphical abstract:

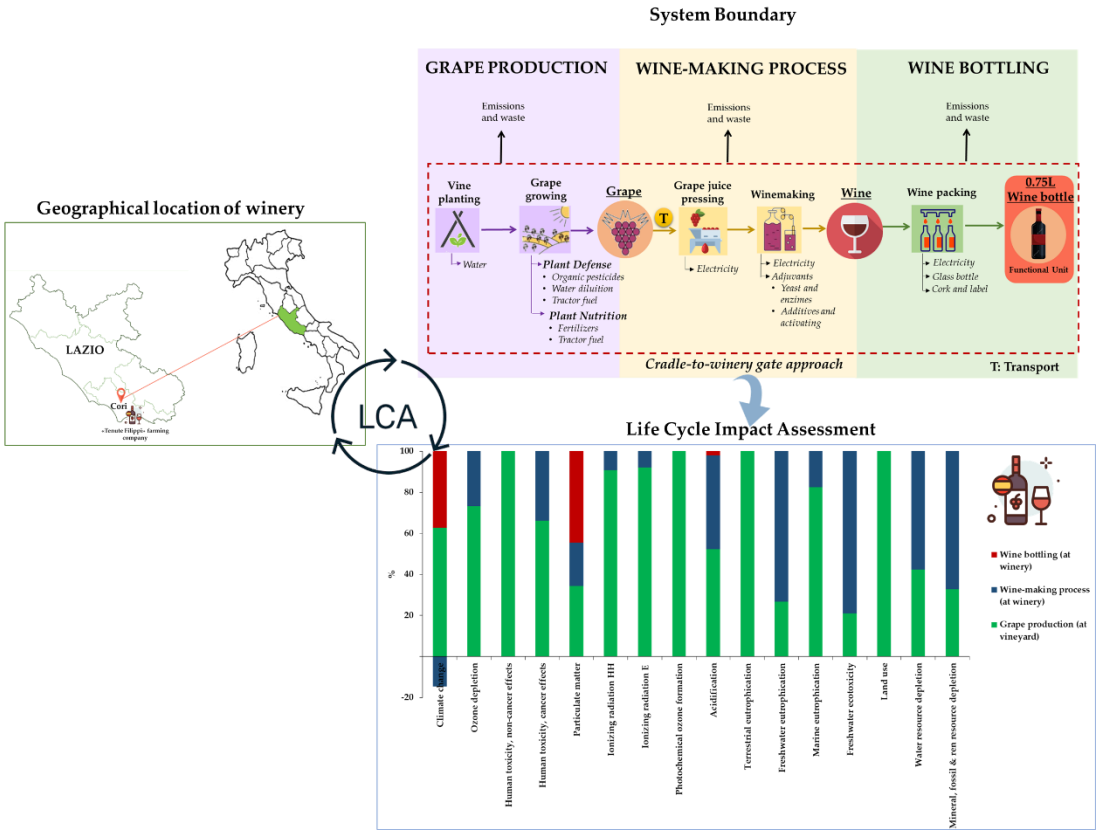

Figure 3.6. Graphical abstract of experimental study no. 6. Source: author's elaboration

sustainability

MDPI

Article

Environmental Impact Assessment of an Organic Wine Production in Central Italy: Case Study from Lazio

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Abstract: Growing awareness of environmental sustainability in the agri-food sector has enhanced the gradual shift toward less-impactful food and organic production systems. In 2021, nearly three million hectolitres of organic wine were produced which accounted for 6% of the whole wine production in Italy (50.2 million hectolitres); thus, registering an increase of almost 60% in the last three years. The economic and cultural importance attributed to Italian wine production worldwide represents a key factor to assess and reduce the environmental burdens associated with the activities of this industry. Furthermore, literature studies have highlighted consumer sensitivity for sustainable winemaking processed, and there is even a trend towards eco-friendly wines. In particular, the bottling stage has been identified as an impactful stage for the environmental performance of the wine life cycle. This study examined the environmental impact assessment of organic wine production in the Lazio region, by performing a "cradle-to-gate" approach according to the life cycle assessment (LCA) methodology. High-quality inventory data for one year of operation was obtained directly from the farming company, "Tenute Filippi" (Cori, Lazio, Italy), and the wine process considered the input from grape cultivation to the winery phases. In these regards, the study also provided an impact assessment for the primary packaging of a 0.75 L wine bottle, with contributions from the different life cycle stages. The results showed a total amount of greenhouse gas emissions (GHGs) of 1.1 kg CO_2 eq, that are responsible for climate change. Referring to the individual production input, the primary packaging phase accounted for 55% of the total GHGs, with 0.86 kg CO_2 eq per bottle, followed by agricultural fuel use for grape production and harvesting activities, with 0.30 kg CO_2 eq. Building on these results, the study provides recommendations on the selection of the most significant and relevant indicators for the environmental life cycle impact assessment, thus, identifying possible hotspots in the wine sector.

Keywords: organic grape production; organic wine; environmental sustainability; life cycle assessment; wine-making; primary packaging; SimaPro software

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1. Introduction

Wine production is one of the oldest economic sectors and, presently, it represents one of the most important agricultural activities worldwide [1,2]. Since viticulture requires a temperate climate, wine production is mainly located in Mediterranean countries, and primarily in Europe, accounting for more than 64% of the global wine production, with 260 million hectolitres of wine produced in 2021 [3]. Out of the global wine production, Italy represents 19.3% (with 50.2 million hectolitres produced in 2021), ranking as the first wine producer in the world. As a result of its geographical conformation and heterogeneous weather conditions, Italian vineyards are characterized by a wide variety of types of wine marketed worldwide. The Italian processed grape market is mainly represented by "Protected Designation of Origin" (PDO) wines (with 23.1 million hectolitres produced in 2021) and Protected Geographical Indication (PGI) wines with 12.3 million hectolitres in 2021.

These certified products mainly designate a product origin, quality, and characteristics that are distinctive for a certain geographical environment, thus, representing a warranty of quality for consumers [4]. In recent years, consumers have also paid attention to purchasing organic products and wine, which is perceived as a choice of quality and safety, as it incorporates a guarantee of control and certification by specialized organizations [5]. In 2020, the European Commission opened a public consultation on organic farming to reach 25% of land cultivated organically by 2030. To achieve this goal, including in the European Green Deal, the Commission in 2021 allocated EUR 40 million to promote organic farming, ensure sustainable soil management, and encourage the purchase of organic products. Organic wine is assuming great importance in the international market; consumers, especially in high-income countries, are increasingly aware of consumer purchasing choices, thus, favoring the purchase of quality and organic wines. In this framework, the Italian wine sector has been characterized by an increase in organic wine production. In 2021, nearly 3 million hectolitres of organic wine were produced and accounted for 6% of the whole wine production in Italy, thus, registering an increase of almost 60% in the last three years [3]. The production of organic wine is regulated by the EU regulations, EC Reg. No. 34/07 and EC Reg. No. 889/08, which mainly concern organic farming and define the management of vineyards and the production of certified organic grapes from an agricultural point of view, and by EC Reg. No. 203/12, which specifically outlines the entire organic wine-making process.

To analyze the Italian wine-growing sector, a SWOT analysis was carried out, thus, providing an understanding of the main strengths (i.e., the internal advantages and opportunities), weaknesses (i.e., the internal risks and dangers), opportunities (i.e., the external advantages and opportunities, and threats (i.e., external risks and dangers) of the sector (Figure 1).

Figure 1. SWOT analysis of the Italian wine sector.

These variables provide an insight into the Italian wine sector concerning the external and internal environment in which it operates, supporting the strategic choices to be implemented. The Italian wine sector has considerable internal strengths that differentiate the sector from its competitors, thus, giving it an important competitive advantage.

The gradual change in consumer purchasing patterns can also be linked to a growing awareness of environmental sustainability in the agri-food sector, which has enhanced the

shift toward less-impactful food production systems, as well as organic production [4]. The wine industry is one of the most impacting sectors worldwide, with significant implications for sustainability issues, as it contributes to a variety of environmental burdens, mainly due to the use of pesticides and fertilizers in the vineyard and the production of glass bottles, as already highlighted in the literature [4,6,7]. Therefore, there is growing pressure in the agri-food sector, both for producers and policymakers, to address the social and environmental impacts within the product life cycle. In this framework, the life cycle assessment (LCA) methodology represents a standardized and valuable tool to quantitatively measure the environmental impacts of a product throughout its life cycle, from cradle (including the supply of raw materials) to grave (end of product life) [8,9].

The sustainability assessment of wine production, from viticulture to the winemaking industry has been addressed by several authors at different levels of temporal resolution, revealing the scientific interest in this emerging topic. The LCA for the operations of an Italian winemaker case study has been proposed by different authors [7,10,11]. These studies assess the system activities, and input for the system (e.g., fertilizers, phytosanitary products, etc.) and quantify some categories of impact, including the global warming potential (GWP), fossil fuel consumption, and resource availability. For example, to improve energy and water efficiency, and to minimize the impacts related to the use of pesticides and chemicals in conventional viticulture, Volanti et al., (2022) investigated the environmental sustainability of organic grapevine crops [4]. Based on three Spanish grape processing systems, they showed a 10% reduction of the total impact of the organic vineyards compared to conventional production, without taking into consideration the wine-making process, and the packaging stage.

Considering the importance of Italian wine production, both in economic and cultural terms, an understanding of the environmental impacts associated with grape cultivation and wine-making activities, could be recommended. In this framework, the study examined the environmental impact assessment of organic wine production in Central Italy (the Lazio region), by performing a "cradle-to-gate" approach. High-quality inventory data for the year 2019 was obtained directly from the farming company, "Tenute Filippi" (Cori, Lazio, Italy), and the wine process considered the input from the grape cultivation (at the vineyard) to the wine-making process (at the winery). In particular, the study also provided an impact assessment for the primary packaging of a 0.75 mL wine bottle, with contributions from the different life cycle stages.

The importance of packaging decisions to reduce the environmental impacts associated with a given product or supply chain has been highlighted by recent studies. The bottling stage in the wine sector, especially glass bottle production, has been identified as an impactful stage for the environmental performance of the wine life cycle [12].

Siracusa et al. [13] used the LCA methodology to demonstrate that the thinning of a plastic film and the adoption of recycled polyamide in food packaging allow a reduction of 25% and 15% of the associated environmental damages, respectively. Indeed, more than 80% of the article considered only the carbon footprint (CF), and water footprint (WF) assessment related to wine products and the confectionary phase. To the best of our knowledge, there are no studies investigating the environmental performances of organic wine production in the Lazio region, considering a cradle-to-gate approach. To this purpose, the present study investigates the environmental sustainability of a 0.75 mL organic bottled-wine, by quantitively assessing 16 impact categories, as well as the energy input, demanding to identify hotspots and to determine best practices to minimize the environmental footprint.

2. Materials and Methods

A life cycle assessment (LCA) is a standardized tool to quantitatively evaluate the environmental impact associated with a product, process, or service [14]. In accordance with the ISOs [8,9], it should involve four phases: i. the goal and scope definition, describing the objective of the study, the functional unit (FU), and the system boundary; ii. a life cycle

inventory (LCI), collecting the data necessary for the environmental assessment; iii. a life cycle impact assessment (LCIA), which is aimed at evaluating the sustainability in terms of the impacts on ecosystems, human health, and resources; and iv. the interpretation of results, in which the LCIA results are interpreted according to the objectives of the study. The SimaPro 9.2.2 (PRè-Sustainability, B.V.) software [15] was used for the evaluation of the environmental impacts of organic wine production in the Lazio region.

2.1. Goal and Scope Definition

The study aimed to assess the environmental impacts of organic wine production, identify possible hotspots in the life cycle, and investigate the most impactful phase from the adopted agricultural organic approach to the primary packaging phase. A cradle-to-gate study was performed: the entire wine production process, from grape cultivation to wine-making, including the bottling and packaging, was considered. The selected functional unit (FU) was one bottle of wine (i.e., 0.75 L of wine) and referred to the grape harvest in 2019. All the production flows analyzed were congruent with the FU examined.

2.2. System Boundaries

The system boundaries were defined on a cradle-to-winery gate approach, including all the input and energy flows associated with grapevine crops, the wine-making process, and bottling (Figure 2); whereas the distribution, retail, and consumption were not taken into account nor the waste management of wine by-products (i.e., stems, lees, and grape pomace), as they were reintroduced as a soil conditioner, constituting a natural fertilizer. The grape production included all the in-field operations, such as trellising, and fertilizer treatments, to prevent the development of fungi, insects, and microorganisms that could damage a harvest.

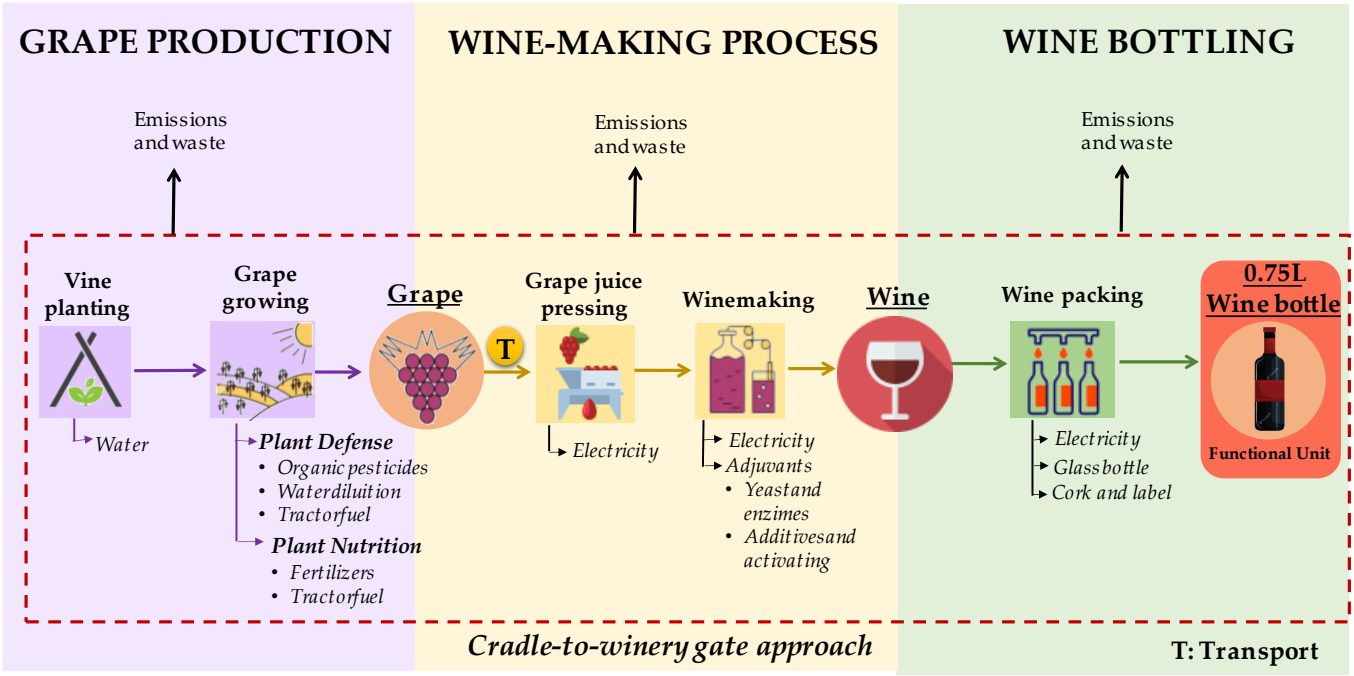

Figure 2. System Boundaries considered in the study according to a cradle-to-winery gate approach.

Considering the winemaking process, the LCI included the use of winemaking equipment (i.e., tannins, plant proteins, diammonium phosphate or DAP, etc.), refrigeration, and primary packaging (e.g., glass bottle, cork, heat shrink capsule, etc.). Farm construction and the production of agricultural machinery and winemaking equipment were not included in the analysis due to i. a lack of data, ii. the exclusion of operating goods in previous LCA

studies on wine [11,16,17], and iii. the assumption that these inputs could be considered negligible because of their minor contribution to a single wine bottle.

2.3. Data Acquisition

The primary data were collected through the administration of questionnaires and face-to-face interviews with the farmers and employees in the farming company, "Tenute Filippi", in the year 2019. These surveys embraced a wide range of inputs for the cultivation sites, such as the fuel use, pesticides, field operations, machinery, or trellises for the grape cultivation, as well as the facilities and operational conditions adopted for the winemaking process, including the primary packaging. The farming company, "Tenute Filippi" is located in Central Italy, in Cori, in the province of Latina, in the Lazio region (Figure 3). The farm covers an area of 15 hectares (ha) in hilly surroundings, of which 7 hectares (ha) are planted with grapevines. On average, the company produces a total of 10 tonnes/year, and about 9300 bottles of wine had been produced in the year 2019.

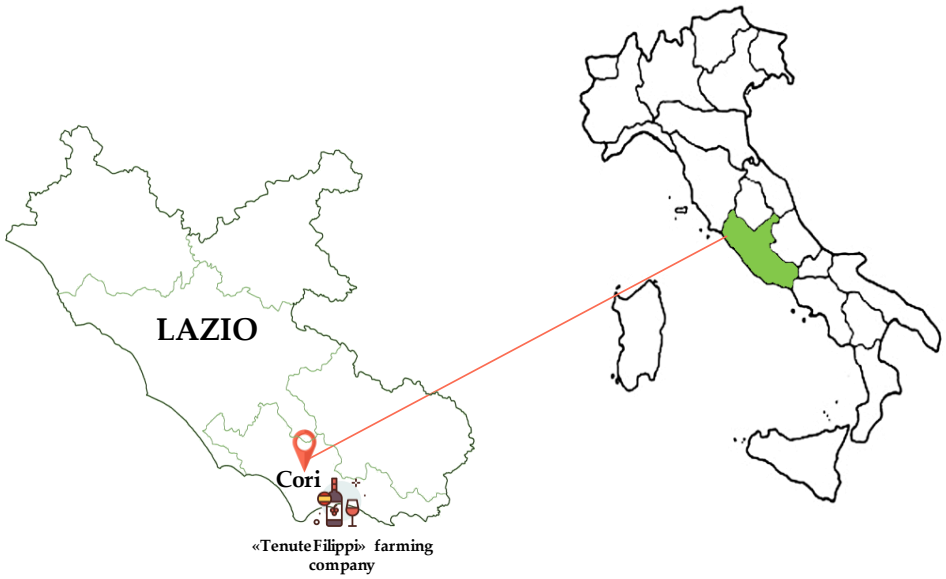

Figure 3. The geographical location of the winery involved in the study.

Direct emissions from field operations, such as the emissions from fossil fuel consumption by agricultural machinery, were estimated based on the characterization factors proposed by "Ente Nazionale per la Meccanizzazione Agricola" (ENAMA) (2005) [18]. Nevertheless, as mentioned in Section 2.2, only the on-field emissions were considered for compost, as the wine by-products (i.e., stems, lees, and grape pomace) were reintroduced as a soil conditioner, constituting a natural fertilizer; therefore, the compost processing stage was excluded from the system boundaries.

The secondary data referred to the input for phytosanitary defense (i.e., zeolite, sulphur, antagonistic fungi, etc.) as well as a trellis or diesel, and they were obtained from the database Ecoinvent v3.8, [19]. The organic fertilizer (i.e., humus, preparation 500, etc.) used in the organic viticulture, as well as adjuvants for the wine-making process (i.e., plant proteins, DAP, etc.), were obtained from the databases, Agribalyse v3.0.1 [20], and World Food LCA Database (WFLDB) [21].

2.4. Life Cycle Inventory (LCI)

Primary input data concerning the in-field operations and the wine-making process are shown in Table 1. These data consist of site-specific data collected through the administration of questionnaires and face-to-face interviews with the farmers and employees in the farming company, "Tenute Filippi", supported by the technical data sheets of the various machines used during the production process. The primary data were subsequently

combined with the secondary data referring to the background production flows (i.e., fertilizers, and energy production) that were obtained from the databases, Agribalyse v3.0.1, Ecoinvent v3.8, and World Food LCA Database (WFLDB), provided in SimaPro 9.2.2.

Table 1. Inventory data used for each wine production stage (data referred to the functional unit—FU: one 0.75 L wine bottle).

Production Stage	Input	Unit	Quantity (0.75 L Wine Bottle)
Grape Production (at Vineyard)			
Plant defense	Copper	g	0.16
	Sulphur	g	0.54
	Zeolite	g	0.32
	Antagonistic fungi	g	0.32
Plant nutrition	Preparation 500	g	0.03
	Hummus	g	107.53
In-field operations	Diesel oil	L	0.04194
Plant water use	Water	L	0.86022
Output			
Grape		kg	1.075
Grape juice pressing (at winery)			
Transport (from vineyard to winery)	Diesel oil	L	0.01724
Crushing, stemming, and pressing facilities	Electricity	kWh	0.06476
Output			
Must		L	0.86
Pips, stalks, and grape skins		kg	0.30913
Wine-making process (at winery)			
Fermentation, clarification, and filtration adjuvants	Plant proteins	g	0.08
	Tannins	g	0.06
	Bioenology S14 A	g	0.015
	Metabisulphite	g	0.04528
	Diammonium phosphate (DAP)	g	0.012
	Yeast	g	0.015
	Activating enzymes	g	0.02275
Fermentation, clarification, and filtration facilities	Electricity	kWh	0.16272
	Water for dilution	L	0.04301
	Cellulose filter	g	0.065
Output			
Wine		L	0.75
Organic solid waste		L	0.02275
Wine packing (at winery)			
Washing bottles, packing, and primary packaging	Water	L	0.12903
	Electricity	kWh	0.00602
	Glass bottle	g	550
	Cork	g	5
	Capsule-PVC	g	1
	Label	g	2
Output			
No. 1 bottled wine		L	0.75

The grape production stage considered the plant production, involving the processes of phytosanitary defence (e.g., zeolite, sulphur, copper, and fungi protection) and plant nutrition (fertilizers) necessary for grape growing after the vine planting. Vine seedlings, corresponding to 0.6 units per bottle of wine, were not included in the life cycle inventory, due to the lack of data. The producers of the "Tenute Filippi" company use organic raw materials to preserve the soil's long-term fertility. In particular, it uses natural products of animal origin for soil fertilization, including humus and Preparation 500, which consists of cattle manure 6 months-aged in horn, and then sprayed on the soil at low concentrations (about 0.1 kg/ha) twice a year [11]. To protect the vineyard from pathogenic molds and toxic compounds, the company uses organic fungicides, including copper, sprayed together with water, sulphur, and finally zeolite. Freshwater consumption is related to the direct water used for irrigation and the dilution volume used for plant protection and nutrition. The diesel oil consumption related to the activities of tractors for the in-field operations (e.g., fertilization and phytosanitary treatments), and the transport distance of 1 km from the vineyards to the winery.

The wine-making process stage involves the grape juice pressing and the wine-making process. During the first phase, the stems, lees, and grape pomace are reused as compost in the fertilization process; therefore, the waste management and emissions from organic waste were not included in the sustainability assessment. The wine-making process included the adjuvants (i.e., additives and activating enzymes), and facilities (i.e., cellulose filters, diesel oil, and water) used for the processes of fermentation, clarification, and filtration. The inputs used in this study for each wine production stage are listed in Table 1. The wine packing stage consists of three processes: washing bottles, packing, and primary packaging. The inputs used for this stage (i.e., glass bottle, cork, PVC capsule, etc.) were obtained from the database Ecoinvent v3.8 [19].

2.5. Life Cycle Impact Assessment (LCIA)

The *ILCD 2011 Midpoint+ V1.11* (European Commission—Joint Research Centre—Institute for Environment and Sustainability 2010) was used for the environmental life cycle impact assessment calculation. The method included 16 environmental impact categories (e.g., climate change, ozone depletion, eutrophication, acidification, human toxicity (cancer and non-cancer related), respiratory inorganics, ionizing radiation, ecotoxicity, photochemical ozone formation, land use, and resource depletion (e.g., materials, energy, and water) [22].

To quantify the energy required for the different stages of wine production, the cumulative energy demand (CED) was calculated according to the CED method V1.11, which has been a widely applied tool to study the energy used by a good or service during its life cycle [23].

2.6. Carbon Footprint (CF)

The carbon footprint (CF) was then calculated based on the LCI and LCIA results. The CF is a measure expressing the greenhouse gas emissions (GHGs) caused by a product, service, or process. It is expressed in kilograms of CO_2 equivalent (kg of CO_2 eq), and according to the Kyoto protocol, the following gases are considered: carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), hydrocarbons, hydrofluorocarbons (HFCs), sulfur hexafluoride (SF_6), and perfluorocarbons (PFCs). Each GHG has a different greenhouse effect; therefore, the CF was calculated according to Forster et al., (2007) [21], based on Equation (1):

$$\text{Carbon footprint} = \sum \text{G.G.}_{\cdot i} \times k_i \quad (1)$$

where $\text{G.G.}_{\cdot i}$ represents the amount of GHGs produced and k_i corresponds to the CO_2 equivalent coefficient for that gas.

The CF was obtained employing the Green Gas Protocol V1.03/CO_2 eq (kg) method (GHGP 2020), by using the SimaPro v.9.2.2. software.

3. Results

The LCIA results for the organic wine production are shown in Table 2. and the life-cycle phases that mostly contributed to the main impact categories have been identified.

Table 2. Environmental impact assessment of organic wine production (data referred to FU: one 0.75 L wine bottle).

Impact Categories	Unit	Grape Production (at Vineyard)	Wine-Making Process (at Winery)	Wine Bottling (at Winery)
Climate change	kg CO_2 eq	2.97×10^{-1}	-6.90×10^{-2}	4.72×10^{-1}
Ozone depletion	kg CFC-11 eq	4.12×10^{-8}	4.32×10^{-8}	5.63×10^{-8}
Human toxicity, non-cancer effects	CTUh	9.21×10^{-7}	2.15×10^{-7}	5.90×10^{-8}
Human toxicity, cancer effects	CTUh	8.83×10^{-9}	1.33×10^{-8}	1.34×10^{-8}
Particulate matter	kg $PM_{2.5}$ eq	2.64×10^{-8}	1.61×10^{-4}	7.68×10^{-4}
Ionizing radiation HH	kBq U_{235} eq	2.05×10^{-2}	1.55×10^{-2}	2.26×10^{-2}
Ionizing radiation E	CTUe	1.57×10^{-7}	1.14×10^{-7}	1.71×10^{-7}
Photochemical ozone formation	kg NMVOC eq	2.59×10^{-3}	1.82×10^{-3}	2.42×10^{-3}
Acidification	molc H^+ eq	2.58×10^{-3}	2.26×10^{-3}	4.94×10^{-3}
Terrestrial eutrophication	molc N eq	9.26×10^{-3}	8.17×10^{-3}	9.08×10^{-3}
Freshwater eutrophication	kg P eq	1.80×10^{-5}	6.75×10^{-5}	1.24×10^{-5}
Marine eutrophication	kg N eq	8.16×10^{-4}	9.89×10^{-4}	7.86×10^{-4}
Freshwater ecotoxicity	CTUe	6.25×10^{-1}	2.97	4.83×10^{-1}
Land use	kg C deficit	2.27	7.45×10^{-1}	1.54
Water resource depletion	m^3 water eq	3.99×10^{-4}	9.42×10^{-4}	1.74×10^{-4}
Mineral, fossil & renewable resource depletion	kg Sb eq	5.57×10^{-5}	1.70×10^{-4}	1.44×10^{-5}

The results obtained for organic wine production showed a variable contribution to environmental impacts at certain phases in the life cycle. The wine bottling phase had the greatest environmental impact in terms of GHGs emissions, followed by the agricultural and wine-making phases.

The characterized results obtained with the ILCD Midpoint+ method for the organic wine production impact assessment are shown in Figure 4.

The wine bottling phase constituted the main contributor to environmental impacts, with a relative contribution ranging from 39% to 67%, in 7 out of the 16 impact categories analyzed. Notably, the wine packing phase mainly involved climate change (4.72×10^{-1} kg CO_2 eq), particulate matter formation (7.68×10^{-4} kg $PM_{2.5}$ eq), acidification (4.94×10^{-3} molc H^+ eq), and ozone depletion (5.63×10^{-8} kg CFC-11 eq) impact categories, accounting on average for 55.6% of the contribution for each impact category. The former also represented a hotspot for non-renewable, fossil energy demand, with 7.71 MJ eq, thus, contributing to 48.1% of the total non-renewable, fossil energy demand, as shown in Table 3, and Figure 5. This is mainly attributable to glass bottle production, which contributed ranging from 35% to 64% of the total impacts of the whole wine production system. Considering the individual production inputs, it can be stated that the glass bottle, whose weight was 550 g, represented the largest carbon footprint, thus, being responsible for generating about 0.54 kg CO_2 (Figure 6). This is in agreement with the results reported in the scientific literature, reporting glass bottles for wine packing as one of the greatest contributors to the global impacts on wine production [6,24,25]. In particular, the closest agreement was found in the study of Masotti et al., (2022), highlighting similar CO_2 emissions (0.5481 kg CO_2 eq) associated with the use of heavy glass bottles weighing between

650 g and 550 g [11]. In these regards, glass bottle production was identified as the highest energy-demanding input in the overall wine production system.

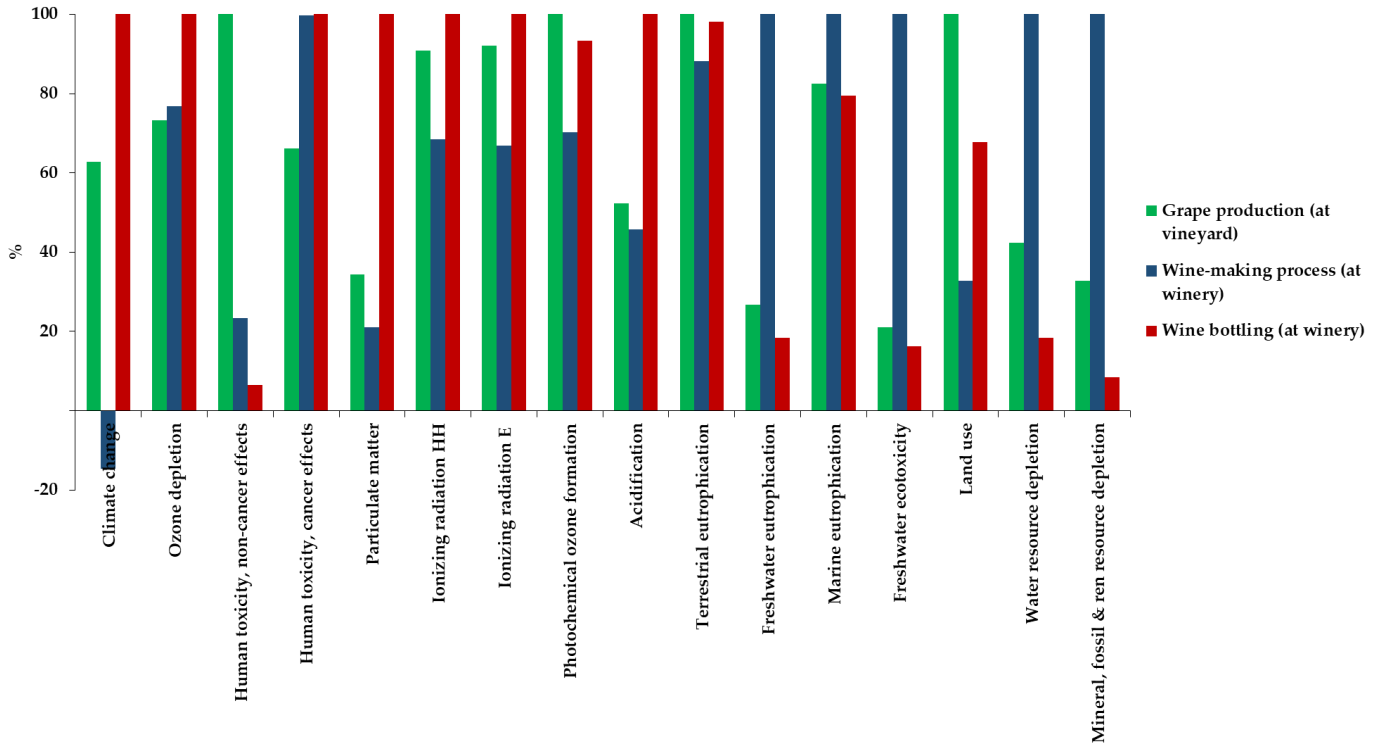

Figure 4. Characterized results of the organic wine production considering the system production from the vineyard (grape production) to the winery (wine-making and wine bottling processes).

Table 3. Results (MJ eq) of Cumulative Energy Demand.

Impact Category	Unità	Grape Production	Wine-Making Process	Wine Bottling
Non-renewable, fossil	MJ eq	4.188581	4.128974	7.706661
Non-renewable, nuclear		0.368467	0.281851	0.431374
Non-renewable, biomass		0.000948	0.000744	0.000317
Renewable, biomass		0.322749	3.393171	1.414292
Renewable, wind, solar, geothermal		0.011911	0.150735	0.055819
Renewable, water		0.205288	0.158478	0.157634

The vineyard phase mostly contributed to 4 out of the 16 impact categories, ranging from 36% to 50% (Figure 4) of the total environmental impacts. In particular, grape production mostly impacted the land use (2.27 kg C deficit), photochemical ozone formation (2.59×10^{-3} kg NMVOC eq), terrestrial eutrophication (9.26×10^{-3} molc N eq), and human toxicity-non cancer effects (9.21×10^{-7} CTUh). These results showed that the emissions arose from diesel oil for in-field operations, thus, being responsible for an average contribution value of 56.74% of the total environmental burdens in most categories. In particular, the diesel burned in agricultural machinery heavily contributed to the human toxicity-non cancer effects (81.6%), land use (59.15%), and climate change (48.4%), thus, being in line with other studies in the literature [6,11,24].

Furthermore, the total impact in the climate change category was mitigated by 89% by the wine-making process, resulting in a reduction of -6.90×10^{-2} kg CO_2 eq. This is attributable to the reduced energy consumption during the wine storage phase, lasting eight months since the wine is stored at room temperature without the use of electricity,

owing to the favorable weather and pedo-climatic conditions of that geographical area. Since electricity is mainly produced from non-renewable fossil fuels, namely, oil and coal, with the consequent CO_2 emissions, a low-energy demanding process could potentially contribute to climate change mitigation [16].

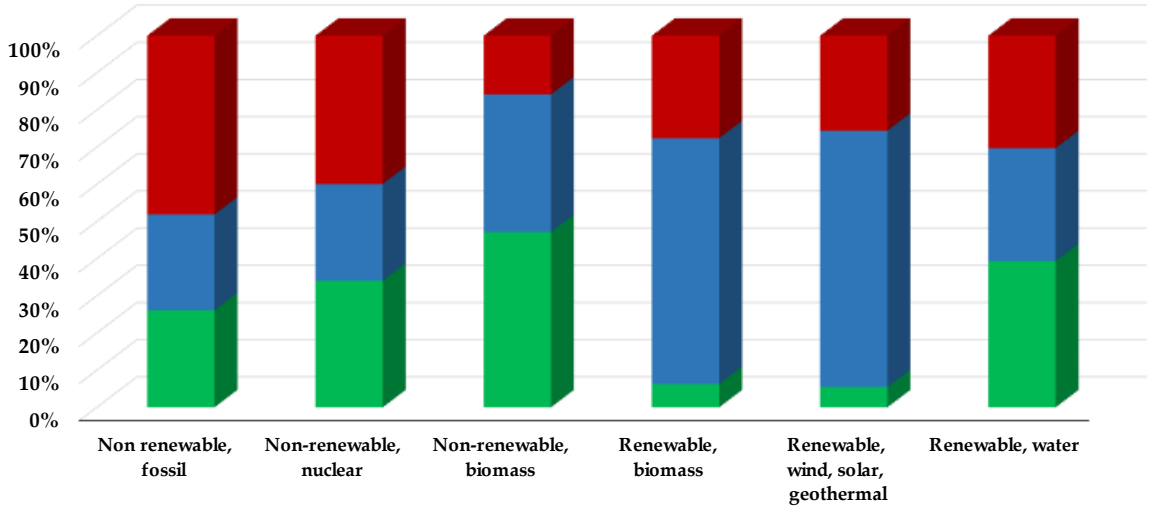

Figure 5. Characterized results for the cumulative energy demand of the wine production system analyzed.

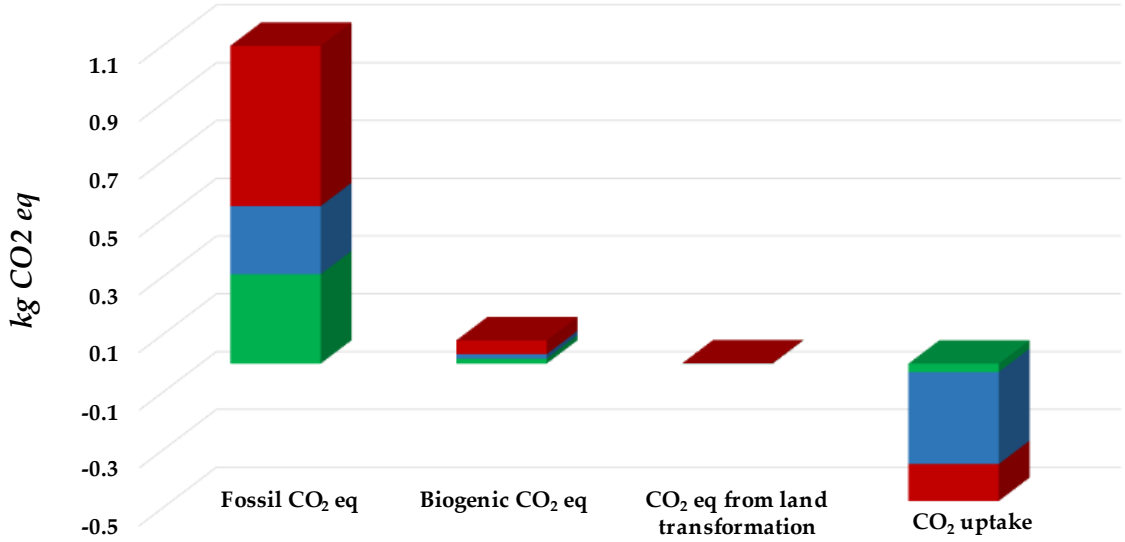

Figure 6. Greenhouse gas protocol results for the carbon footprint related to wine production.

Fossil fuel use for agricultural machinery implies the generation of direct nitrogen oxide emissions (NO_x), SO_2, and particulate emissions, thus, being responsible for photochemical oxidation formation [26]. The use of fertilizers (i.e., hummus, and Preparation 500) and pesticides (i.e., zeolite, sulfur, and antagonistic fungi) for plant nutrition and phytosanitary defense, respectively, represents the second contributor to the vineyard phase, thus, accounting for 6.1% of the total environmental burden in the land use, freshwater ecotoxicity, and climate change impact categories. Analogously to Masotti et al., (2022), also analyzing the organic wine production in Northeast Italy, fertilization and pest management resulted in being the second most impactful phases in the overall production system [11].

In particular, fertilizers can negatively impact on the land use impact category, since they require soil for livestock farming for manure production. The emissions of direct N_2O occurring during the application and production of fertilizers are mainly responsible for

the climate change category, whereas the burdens for freshwater ecotoxicity are mainly related to glyphosate emissions to the air, occurring during the application of pesticides for plant phytosanitary defense. Additionally, as stated by different authors [6,10–27], pesticide treatments, as well as diesel production, are also responsible for hydrocarbons (PAHs), formaldehyde, barium, zinc, and benzene emissions to the air, soil, and water; thus, also representing a burden for human toxicity (with non-carcinogenic effects) [10]. Although the use of fertilizers and pest management were found to be the second most impactful processes in the viticulture phase, their relative contribution range was still significantly lower than the studies in the literature. As different authors have highlighted, fertilization and pest management have provided an average contribution ranging from 30% to 85% of the overall viticulture impacts [27,28]. This was attributable to the fact that the farming company, "Tenute Filippi", adopted natural organic fertilizers, which were produced entirely on-site (i.e., hummus), as well as reusing wine processing by-products (i.e., grape stalks, lees, and seeds) for soil fertilization with organic and natural nutrients.

The wine-making process produces fewer environmental impacts in comparison to the viticulture and wine bottling phases, thus, contributing on average 30% to the overall wine production impacts. In particular, the wine-making process impacted the most on freshwater ecotoxicity (2.97 CTUe), and eutrophication (6.75×10^{-5} kg P eq), the depletion of water resources (9.42×10^{-4} m^3 water eq), marine eutrophication (9.89×10^{-4} kg N eq), depletion of mineral, fossil and renewable resources (1.70×10^{-4} kg Sb eq), and human toxicity with cancer effects (1.33×10^{-8} CTUh). These results are in agreement with those present in the literature, highlighting an average contribution per each cited category between 10% to 13% [6,27]. In particular, the environmental problems caused for freshwater ecotoxicity, and human toxicity are mainly linked to the release of heavy metals (i.e., nickel, and arsenic), and PAHs to the air [27]. Nevertheless, the electricity consumption mainly employed for refrigeration during the fermentation and clarification processes is the first factor contributing from 25% to 69% of the environmental impacts of this phase, as shown in Figure 3. This was mostly related to metal production and end-of-life treatment for electrical supply infrastructure [26].

Subsequently, the cumulative energy demand (CED) and carbon footprint were also calculated for a greater understanding of the results to confirm the LCA results.

As for the CED (MJ equivalent), it was divided into two main categories (renewable and non-renewable) and eight subcategories (fossil, nuclear, biomass, wind, solar, geothermal, and water), as shown in Table 3.

The results highlighted the wine bottling phase as the major contributor, with an average of 55% for the non-renewable fossil (7.71 MJ eq) energy demand. This could be primarily attributable to glass bottle production, which represents, indeed, a high-energy-demanding industrial process in the entire wine production chain [28–30]. In the vineyard phase, the agricultural machinery to cultivate the land for the production of 1.075 kg of grape until its harvest requires a diesel input that accounts for 26% of the CED, thus, representing the second-highest energy-demanding input of non-renewable resources: fossil (4.188581 MJ eq), and nuclear (0.368467 MJ eq). The wine-making process, represented by the storage of wine, makes up 42.4%, leading to the first-highest energy input for renewable resources: the biomass (3.393171 MJ eq), and the wind, solar, and geothermal (0.150735 MJ eq) in the whole wine bottle-life cycle. This was favored by the fact that the farming company, "Tenute Filippi", managed to reduce its energy consumption during the wine storage phase, thus, enabling favorable weather conditions and climate, to store the wine at room temperature without the use of electricity. For this purpose, electricity was produced using non-renewable fossil fuels, especially oil and coal. Furthermore, to minimize negative externalities, the pruning waste, and wine by-products (e.g., the marc, stems, and lees) could be used as biomass to produce energy, reducing the use of fossil fuels [29].

Presently, the increasing sensitivity both of the public authorities and citizens to promote a GHGs reduction target for European municipalities of 50% by 2030 [31], has

allowed the implementation of different quantitative methods to calculate the impact in the global warming category (i.e., carbon footprint). According to ISO 14067, the CF should also consider both fossil and biogenic carbon, for accounting and reporting the financed GHG emissions from real estate operations. In these regards, the Green Gas Protocol V1.03/CO_2 eq (kg) method (GHGP 2020), taking into account both the fossil and biogenic CO_2 emissions as well as the CO_2 uptake, was applied for the carbon footprint assessment. Figure 6. highlights that 1.10 kg CO_2 eq per wine bottle corresponded to the fossil carbon. The study by Laca et al., (2021) [6] provided estimates of the GWP of red wine obtained in around 30 LCA studies worldwide. The authors observed a large variation in the results, ranging from 0.18 to 3.22 kg CO_2 eq per bottle, with an average estimated at 1.74 kg CO_2 eq; therefore, the total GWP obtained in the current study fell well within the reported range. The results were also within the range for the cradle-to-gate GWP, here estimated at 0.86 kg CO_2 eq per bottle. Wine bottling resulted in being the activity most responsible for about 55% of the fossil (0.55676988 kg CO_2 eq), and biogenic CO_2 (0.047892031 kg CO_2 eq) emissions, mainly due to the production of glass bottles [24,25].

Additionally, in wine bottling, some inputs positively affect the CO_2 uptake (−0.12790184 CO_2 eq), thus, mitigating the GWP derived from the whole wine production process. For example, the use of a nomacorc cap has a positive impact on the impact category represented by climate change, as this cap is composed of vegetable biopolymers derived from sugar cane that are recyclable. This leads to the absorption of 0.054 kg of CO_2 eq.

Finally, organic grape production was revealed to be the second contributor to fossil and biogenic CO_2 emissions (i.e., 0.309382193 CO_2 eq, and 0.017594364 CO_2 eq, respectively), thus, representing on average 24.4% of the total GHG emissions. These values were lower in comparison with those reported in the literature, ranging between 55% and 72% [6,27], due to the organic cultivation system applied for grape production.

This system would avoid the GHG emissions mainly derived from the application of synthetic fertilizers and urea, as well as the excessive combustion of diesel by agricultural machinery.

4. Discussion and Improvements

When considering a cradle-to-winery gate approach, it has usually been found that wine bottling and viticulture are the major contributors to the whole wine production system [24]. Currently, the consumer associates the quality of a wine with the weight of the bottle, namely, the heavier the bottle the higher the quality of the wine, and for this reason, the consumer should be sensitized and informed about the environmental impact connected with this choice. To improve the environmental performances of the bottling and packaging phases, different improvement opportunities for the environmental impacts and for settling the energy requirements have been proposed [24,32]. In particular, the use of 30% lighter glass bottles than those currently used by wineries could result in lower energy and resources consumption for glass production; thus, reducing between 2% and 10% of the overall environmental impacts associated with the LCA of a wine bottle [25,33]. The lower weight of the glass bottles could result in a 4% savings in the GWP corresponding to 0.43 kg CO_2 eq per bottle; this is equivalent to around 7000 tonnes of CO_2 eq [25], thus, promoting a lower energy demand [11]. However, the preference for using lighter-weight bottles could not only represent a reasonable improvement action for minimizing the environmental burdens but it could also be replaced with the use of alternative and less-energy-consuming packaging materials, as well as PET, bag-in-box, etc.

Furthermore, focusing on the glass bottle's end-of-life (EoL), it was highlighted that an increase in the glass-recycling rate from 60% to 85% could reduce the environmental impacts associated with the GWP by 11.1% [33]. This was also in accordance with the study of Amienyo et al., (2014), pointing out a reduction of 2% in the GWP by achieving a 10% increase in the amount of recycled glass [25]. This would result in a savings of 0.22 kg CO_2 eq per bottle of wine, thus, corresponding to about 3600 tonnes kg CO_2 eq per year.

Another improvement for the sustainability assessment of wine packing could be glass substitution with different materials. As proposed by Clearly et al., (2013) [34], who studied the application of polyethylene terephthalate for wine bottling, observed an endpoint level impact reduction by 7–9%, in particular for human health (−7.1%), and resources availability (−8.3%). Furthermore, the use of alternatives for packaging, involving the use of sustainable materials for the labels, cork, and capsules (i.e., ultrathin ceramics, paper, and caps from bio-based materials, etc.) [35], could represent an improvement in mitigating the environmental impacts of the wine bottling stage. As a result, for the use of the normacorc cap in this study, a reduction of −5% in the environmental impact related to the GWP was observed, corresponding to 0.054 kg CO_2 eq.

Considering the environmental impacts associated with the agricultural phase for organic grape production, the sources of variability of the results among the literature studies are mainly related to different factors, such as the cultivation systems and agricultural practices (i.e., conventional, organic, and biodynamic), the types of grape, soil and climate conditions of the vineyard, etc., which significantly influence the annual yield of vineyards, and directly impact on the wine production [24,26]. The study of Villanueva-Rey et al., (2014) showed how the adoption of alternatives and more sustainable agricultural practices (i.e., organic and biodynamic), can reduce up to 50% of the environmental impacts of viticulture, thus, highlighting cultivation systems as the first discriminant for the wine production impact assessment [17]. It is also worth noting that in the LCA of agricultural systems, variations in the soil carbon stocks represent a key factor for biodiversity and soil quality improvement, as different authors have stated [17,36]. Furthermore, when considering the wine-making process, it is worth highlighting that reduced use of energy during the wine storage process could mitigate the impacts related to CO_2 emissions by about 89%. In this sense, possible improvements to reduce the GHG emissions during the winemaking phase caused by electricity could include the use of an energy source (i.e., photovoltaic panels), with the creation of an Off Grid winery, disconnected from the national energy distribution network, with a view to sustainability. A possible solution aimed at minimizing the use of electricity could be the installation of inverters and variable speed drives on the machinery, adapting the energy consumption to the actual needs per process, thus, achieving greater energy efficiency [37].

Improvement opportunities for the viticulture environmental performance could, therefore, consider the optimization of upstream processes for the use of bio-diesel for in-field operations, pesticides, and fertilizers composed of organic and bio-based ingredients [33,38,39], as well as acting on downstream processes, reintroducing, for example, the processing of by-products as other inputs for a new process to valorize the agricultural chain [40].

The management of organic and solid waste from wine production (i.e., grape pomace, lees, stalks, etc.) is also a determining factor in the wine-making process, thus, influencing the environmental impacts in variable percentages, between 10% and 30%, depending on the winery and wine type [41]. In this framework, many studies have evaluated different alternatives, for the management and waste valorization, such as composting stalks and wastewater sludge for the production of organic fertilizers [42], the design of adsorbent materials for heavy metals [43,44], as well as the recovery of bioactive compounds from wine wastewater for formulating functional foods [45].

5. Conclusions and Future Perspectives

In the wine sector, adopting a proactive and sustainable approach is a valuable action, as it is most affected by climate change; consequently, introducing such models contributes to mitigating global temperature increases. A focus on sustainability in this sector was here presented, through the cultivation of organic grapes and the implementation of sustainable practices during the winemaking process. A LCA was applied concretely, to study the production cycle of a 0.75 L bottle of wine, produced by the "Tenute Filippi" farming company. Considering a cradle-to-gate approach, it was possible to identify the

hotspots of wine production comprehensively, both by considering their environmental impact assessment as well as the energy input demand. The LCIA results showed that the wine bottling stage represented the main contributor ranging from 39% to 69% of the environmental impacts, mainly due to the glass production process, as well as the use of fertilizers and pest management that were found to be the second most impactful processes in the vineyard phase. Based on the hotspot analysis, several options were identified to reduce the environmental impact of the wine industry, among which the use of a 10% lighter glass bottle could be beneficial to the environment, thus, saving at least 0.43 kg CO_2 eq per wine bottle. Furthermore, the reuse of biomass from pruning the grapes as natural organic fertilizers, as well as wine processing by-products (i.e., grape stalks, lees, and seeds) represented a potential alternative for soil fertilization with organic and natural nutrients.

However, a limitation of this study resides in the exclusion of some upstream activities (i.e., transportation and production of phytosanitary products, corks, labels, and caps), as well as the downstream activities, such as waste management, a bottle's EoL, and the transport, storage and distribution phases of the wine bottles to consumers. This was due to two main reasons: (i) a lack of available data, and (ii) the negligible aspect of these activities, as confirmed by other studies in the literature [27,33]. In this framework, it would be worth carrying out a more complete study, covering more wine production life cycle phases, and revealing each one's relative importance.

Furthermore, the present study does not provide a comparison with other cultivation systems and agricultural practices (i.e., conventional, organic, and biodynamic), and it should be considered as the first reference for further studies to evaluate the environmental sustainability in a comprehensive form, thus, developing a harmonized study for the comparison among LCA results for further implementation by the same farming company.

Therefore, based on the results obtained, it is desirable to rationalize and sustainably manage resources in wine production, acting both on the upstream and downstream levels. The former should provide resource efficiency, and increased productivity in the production process, thus promoting the use of renewable resources, and orienting production toward "*zero* waste". Meanwhile, downstream activities should promote sustainable management, and valorisation, and reintroduction of wine by-products into the economic system, to reduce synthetic fertilizers, improving their efficiency of use through the introduction of new technologies (i.e., precision agriculture) and new technical means (i.e., bio-stimulants). As in the automotive industry, there should be greater interest in electrifying agricultural machinery, reducing the potential environmental impacts associated with fossil fuels.

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3.7 Research article no. 7

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Graphical abstract:

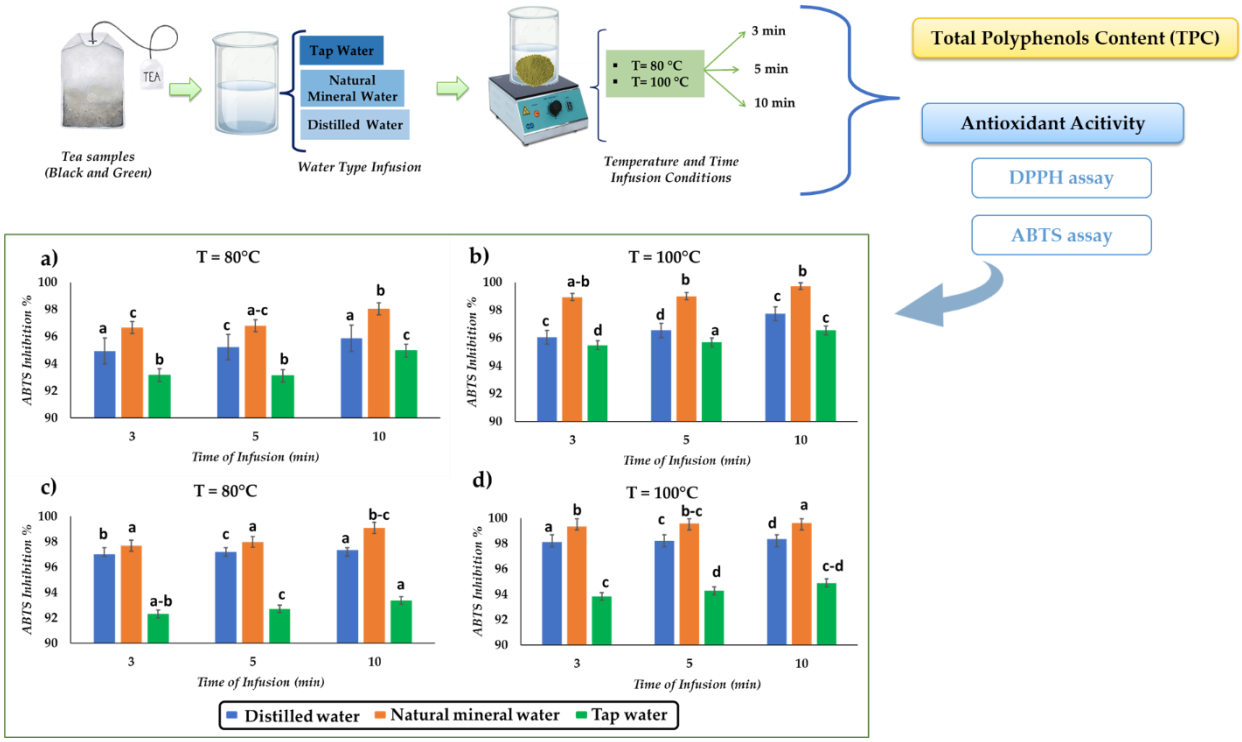

Figure 3.7. Graphical abstract of experimental study no. 7. Source: author's elaboration

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Article

The Influence of Green and Black Tea Infusion Parameters on Total Polyphenol Content and Antioxidant Activity by ABTS and DPPH Assays

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Abstract: Tea contains about 230 chemical bioactive compounds, of which polyphenols represent the most considerable fraction (30% of total dry weight). These compounds have relevant nutritional and pharmacological effects on human health, exerting antioxidant activities against oxidative stress-induced damage. The industrial processes applied in tea production can lead to qualitative and quantitative changes in the phenolic content and composition and in antioxidant properties, thus influencing their potential biological activities. Meanwhile, the procedure for tea preparation may influence the quantity of the extracted phenolic compounds. In this study, the effects of different infusion parameters, such as the water type used for infusion (tap water, distilled water, and natural mineral water), time (3, 5, and 10 min), temperature (T = 80 °C and 100 °C), and pH (ranged between 3 and 9) were considered. The optimal infusion variables resulting from the study were obtained by extracting phenolic compounds at T = 100 °C for 10 min, both for green (916.12–1169.81 mg GAE/g) and black (932.03–1126.62 mg GAE/g) bagged tea samples, respectively.

Keywords: green and black tea; psychoactive beverages; infusion tea; extraction conditions; total polyphenols; antioxidant properties

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1. Introduction

Tea (*Camellia sinensis*) is one of the oldest beverages and widely consumed worldwide [1]. Since tea plant cultivation needs a humid and hot climate, production is mainly concentrated in tropical and subtropical regions. Hence, most tea is produced in large estates in East Africa and Southeast Asia [2]. From 2004 to 2019, world tea production has risen from 3.15 million metric tons (MMT) to 6.1 MMT, with a total trade of $7.44 B. China alone produced around 2.8 MMT in 2019, leading the market as the primary producer and leading exporter ($1.77 B) [3]. The other leading largest countries for the production of tea in 2019 were India (1.4 MMT), Kenya (459 thousand tons), Sri Lanka (300 thousand tons), and Indonesia (129 thousand tons) [4].

Tea production starts with leaf collection, which then undergo processing. During the transformation phases, tea leaves undergo oxidative and hydrolysis processes due to endogenous enzymes (e.g., polyphenol oxidase and peroxidase) in leaf cells [5]. Depending on the level of fermentation and processing methods, it is possible to distinguish between six main kinds of tea (Figure 1): yellow, white, and green tea (non-fermented), oolong tea (semi-fermented), black tea (fully fermented), and dark tea (post-fermented) [6,7]. In green tea, the leaves are rolled and steamed to inactivate polyphenol oxidase, thus reducing oxidation before drying; while, in black tea production, leaves are rolled to allow the rupture of cellular constituents, thus facilitating contact between phenolic compounds and polyphenol oxidase.

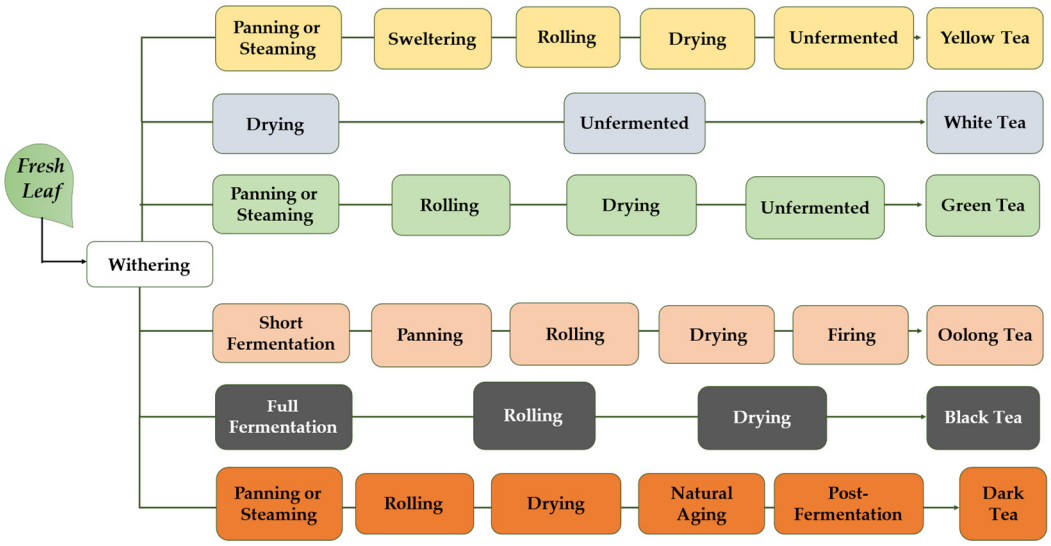

Figure 1. Tea processing technologies [7].

Tea is considered a psychoactive beverage, similar to coffee and chocolate drinks, because of their stimulating effects that are mainly derived from methylxanthines (e.g., theophylline, theobromine, caffeine) [8]. Dried tea leaves contain about 230 chemical bioactive compounds [9], of which polyphenols represent the largest fraction (30% of the total dry weight, TDW), and they are considered to have beneficial effects on human health [10]. It is worth noting that tea consumption can have different health-promoting effects, such as anticarcinogenic and antioxidant activities and cardiovascular and metabolic disease prevention [5,8]. However, polyphenol profiles are markedly different among teas based on their diverse potential biological activities. The most beneficial effects of green tea are credited to green tea polyphenols, mainly catechins, which make up 25–35% of the TWD of tea leaves. The remaining part is mainly composed of caffeine (approximately 3.5% of the TDW), theobromine (0.15–0.2%), theophylline (0.02–0.04%) and other methylxanthines, lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%), and other pigments. Green tea has been shown to exert chemoprotective, antimicrobial, cardioprotective, and immunostimulatory functions [11]. In contrast, black tea polyphenols are mainly derived from the polymerization of catechins into theaflavin (0.3–2% of TDW) and thearubigins (10–20% of TDW) produced during fermentation [12]. These compounds, found in tea leaves and infusions, can exert antioxidant activities against free radicals, thus protecting the human body from oxidative stress-induced damage.

Over the years, several works concerning the antioxidant activities of infusions of *Camellia sinensis* have been published. These works show the great interest of scientific researchers in the antioxidant compounds of tea. In the literature, the polyphenol content in green and black tea infusions is well documented [13–22]. Different studies have investigated the application of different solvents to the extraction of phenolic compounds in green and black tea extracts [14,16,21]. Otherwise, they considered only a single type of water (e.g., distilled water) as an extractive solvent, performing the extraction at 100 °C for 45 min [17,18]. However, few studies simultaneously considered the optimization of different infusion parameters of green and black teas [12,20,22]. McAlpain et al. [20] and Zargar et al. [12] studied tea infusions obtained in water by varying only the time of infusion (1–10 min) while keeping the temperature fixed.

Individual preferences for the consumption of tea change according to the country of origin. Generally, tea is usually consumed after an infusion in water between 95 °C and 100 °C for a determined time [23]. In Western countries, the consumption of bagged black tea leaves after a short infusion time (<3 min) in water at 100 °C is preferred. In India, Pakistan, and some Middle Eastern Countries, black leaves are boiled in a pot for longer before consumption. In China and Japan, tea is prepared by steeping green leaves in hot, not

boiling, water, and only the second and subsequent infusions are consumed [23]. Therefore, although the procedure for preparing tea is not the same worldwide, it is essential to keep its active principles intact. To deepen the beneficial effects derived from tea consumption in the laboratory setting, it is essential to understand how the total polyphenol content varies when varying the kind of water used for the infusion and the temperature/time conditions. Currently, most research focused on steeping times often used by consumers reported that over 50% of polyphenols were released in the initial 5 min of infusion time [8,24]. Studies have only investigated a few steeping times, such as an extended timeframe (e.g., beyond the first 5 min of infusion), or have only examined a specific type of tea at a fixed temperature [23–25].

The objective of the study was to evaluate the total polyphenol content and the antioxidant activity by means of the Folin–Ciocâlteu method and the Diammonium salt (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays in black and green tea samples after considering various infusion parameters (time, temperature, pH, type of water used for infusion: tap, natural mineral, and distilled water). The ability to directly analyze aqueous green and black tea extracts could replace and limit the use of organic solvents (e.g., methanol, ethyl acetate), which are often harmful to human health and the environment. To date in literature, no studies have compared the application of different types of water in the extraction of polyphenols, but they have performed a comparison between organic solvents and only distilled or tap water [14,16,21].

2. Materials and Methods

2.1. Chemicals

Folin–Ciocâlteu reagent ($H_3[P(W_3O_{10})_4/H_3[P(Mo_3O_{10})_4]$, gallic acid ($C_7H_6O_5$), sodium carbonate ($Na_2CO_3$), Eriochrome Black T (MB 11), ammonium chloric (NH_4Cl), ethylenediaminetetraacetic acid (EDTA), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate ($K_2S_2O_8$), sodium phosphate dibasic (Na_2HPO_4), phosphate-buffered saline (PBS), methanol (CH_3OH), and ultrapure water were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Instead, acetic acid (CH_3COOH), sodium acetate (CH_3COONa), chloridric acid (HCl), and sodium hydroxide (NaOH) were purchased from Carlo Erba, Milan, Italy.

2.2. Instruments

The following instruments were used: Bandelin Sonorex RK100H water and ultrasonic thermostatic bath, Hanna Instruments pH211 Microprocessor pH Meter (Sigma Aldrich, Milan, Italy)), and UV–Vis spectrophotometer (Jenway, Stone, (UK)).

2.3. Samples

Twenty commercial tea bags of two varieties (green and black ones) were collected from local markets and tea shops. For each tea variety, n = 10 samples of three different brands were purchased. Samples were stored in darkness at T = 15–20 °C until the day of analysis. The results of the analyses are presented as the average of the samples for each type of commercial tea: green and black tea bagged samples.

2.4. Aqueous Tea Extract Preparation

Before conducting polyphenol aqueous extraction, water hardness was measured as follows: 50 mL of sample was placed in a flask, to which 4 mL of NH_4Cl/NH_3/EDTA and 0.2 g of MB11 were added. The solution was then titrated under stirring with 0.01 M EDTA solution. The results were calculated as follows:

$$\text{Hardness } (^\circ\text{F}) = \frac{V_3 \times M \times 10}{V_4}$$

where V_3 = volume (mL) used for titration, M = molarity of EDTA, V_4 = volume (mL) of the sample tested.

According to Das et al. [21], extraction of total polyphenols from the different tea samples was performed as follows: commercial tea bags, which weighed between 1.5 and 2 g were opened, and 2 g of sample was weighed for each aliquot. The sample was then placed into a glass flask with 200 mL of water for infusion: tap water (TW), high hardness (33.5 °F) distilled water (DW), and natural mineral water (NMW), low hardness (13.3 °F). Table 1 reports the physicochemical characteristic of water used for the analysis.

Table 1. Physiochemical characteristics of natural mineral, tap, and distilled water.

Parameter	Unit	NMW	TP	DW
Electrical conductivity at 20 °C	µS/cm	668	571	6.8
pH	-	7.06	7.50	7.00
Fixed residue	mg/L	440	408	<1
Hardness	F°	13.3	33.5	<0.01
Calcium (Ca^{2+})	mg/L	124	104.0	-
Magnesium (Mg^{+})	mg/L	29.4	18.70	-
Sodium (Na^{+})	mg/L	4.0	4.1	-
Potassium (K^{+})	mg/L	1.2	0.97	-
Bicarbonate (HCO_3^{-})	mg/L	498	399	-
Sulfates (SO_4^{2-})	mg/L	17.2	16.60	-
Chlorides (Cl^{-})	mg/L	6.6	6.5	-
Nitrates (NO_3^{-})	mg/L	2	2.99	-
Nitrites (NO_2^{-})	mg/L	<0.002	<0.01	-

The extraction of total polyphenols from the different tea samples was performed for different infusion times (3, 5, 10 min) and at different temperature ratios (T = 80 °C and 100 °C), respectively (Figure 2). These time and temperature conditions were chosen to replicate the usual homemade preparation conditions in the laboratory setting. For each sample, analyses were performed in triplicate.

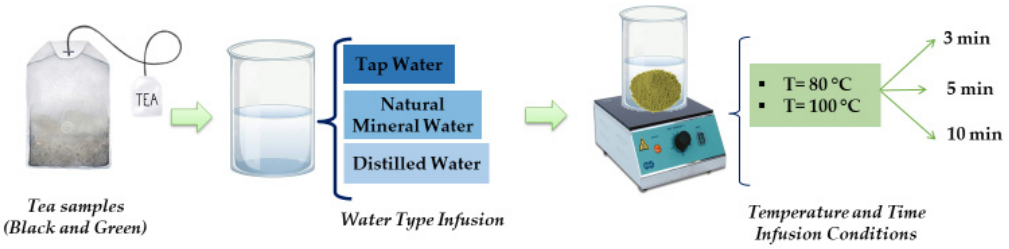

Figure 2. Experimental and water extraction conditions for tea samples.

2.5. Polyphenols Aqueous Extraction at Different pH

To evaluate the influence of pH on the extraction of polyphenols, two types of water were considered: natural mineral water and distilled water. The effect of pH on the extraction capacity of total polyphenols in tea samples was examined, considering the best conditions for polyphenol extraction (T = 100 °C for 10 min). pH values ranging between 3 and 9 were analyzed. This pH range was considered for the analysis as it reflects the pH values of most commercial beverages (e.g., soft drinks, fruit juices) [26]. DW and NMW were acidified by adding HCl (0.1 M) and alkalized by the addition of NaOH (0.1 M). In addition, different buffer solutions at different pH were considered. Buffer solutions were obtained between pH 3 and 6 using CH_3COOH/CH_3COONa (0.1 M) buffer, while for the buffer solutions at basic pH (7–9), the Na_2HPO_4/HCl (0.1 M) buffer was used. After the aqueous solution and buffer solution preparation, polyphenol extraction was conducted

on commercial tea bag samples to which 200 mL of acidified aqueous solution, alkaline aqueous solution, and buffer solution was added. All analyses were performed in triplicate.

2.6. Determination of Total Polyphenol Content (Folin–Ciocâlteu)

Total polyphenol content (TPC) was measured by spectrophotometric analysis using the Folin–Ciocâlteu method [27]. The TPC method was modified for tea infusion analysis: 1 mL of tea infusion sample was added to 0.25 mL of Folin–Ciocâlteu reagent (2.0 N). After 3 min, 0.5 mL of Na_2CO_3 (7.5%, *w*/*v*) was added and brought to a final volume of 10 mL with distilled water. The tea samples were left for 45 min in the dark at room temperature. The absorbance was measured at λ = 750 nm in cuvettes with a 1 cm path length relative to the aqueous solution. The total content of phenols was expressed as milligrams of gallic acid equivalent (GAE) per gram of bagged tea samples. The results were obtained through a calibration curve ranging from 50 to 500 mg/L of gallic acid solution ($y = 0.0005x - 0.0023$; $R^2 = 0.9905$).

2.7. Determination of Antioxidant Activity (ABTS and DPPH Assays)

Antioxidant activity was determined through ABTS and DPPH assays, according to a previously reported method of Thaijpong et al. [28]. The free radical scavenging activity of the aqueous tea extracts was assessed by measuring the decrease in absorbance at 515 nm for $DPPH^{\bullet}$ and at 734 nm for $ABTS^{\bullet+}$ radical cation. Absorbance was measured in cuvettes with a 1 cm path length relative to aqueous solution (distilled, tap, and natural mineral water, respectively), using a UV–Vis spectrophotometer (Jenway, Stone, UK). Results were expressed as inhibition percentage (I%) and were calculated based on Equation (1):

$$I\% = \frac{A_0 - A_f}{A_0} \times 100 \quad (1)$$

where A_0 is the initial absorbance of the radical cation and A_f is the absorbance after the addition of tea sample extract.

2.8. Statistical Analysis

The statistical package SPSS, v.27, was used (SPSS Inc. a.s., 2000, Bologna, Italy) to calculate significant differences between the tea samples in all the analyses. Results were evaluated with one-way analysis of variance (ANOVA) and *p*-values of <0.05 were considered significant.

3. Results and Discussion

3.1. Effect of Infusion Variables on TPC

A comparison of the polyphenol content of tea samples infused in different types of water (tap water, natural mineral water, and distilled water) is proposed for the first time in this manuscript.

The Folin–Ciocâlteu assay has been extensively used for the determination of total polyphenol content (TPC) in different food matrices [27,29,30], including tea [20,31]. However, no studies have been simultaneously considered different infusion temperatures (80 °C and 100 °C), different infusion times (3, 5 and 10 min). and different types of water (distilled, tap and natural mineral water). Figure 3 shows the total phenolic content (TPC) of green tea infusion considering the different time/temperature conditions and different types of water used for the infusion. There was a clear increase in TPC with a longer steep time for each type of water investigated. Moreover, an increase in TPC was observed in green tea samples extracted at T = 100 °C for 10 min (Figure 3b). Among water types, it was found that NMW for all three infusion times (3, 5, and 10 min) resulted in higher TPC values (916.12–1169.81 mg GAE/g).

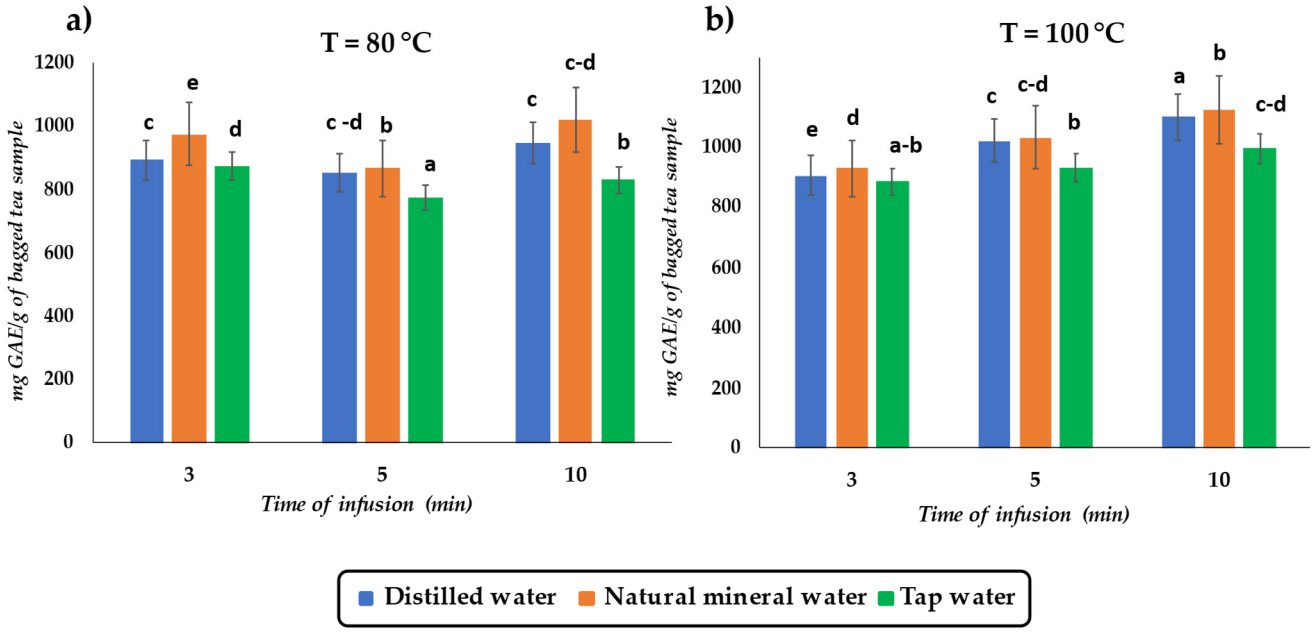

Figure 3. Total phenolic content (TPC) in green tea samples (mg GAE/g). (**a**) TPC for green tea infusion at T = 80 °C for different infusion times and types of water; (**b**) TPC for green tea infusion at T = 100 °C for different infusion times and types of water. Error bars are ± standard deviation. Same letters indicate a significant difference according to the ANOVA test ($p < 0.05$).

For black tea samples (Figure 4), the TPC is lower than that of green tea samples. However, black tea infusion also displays the same trend as green tea, with a higher TPC for the infusions extracted at a temperature of 100 °C (Figure 4b) than those extracted at a temperature of about 80 °C (Figure 4a). In addition, even for black tea, NMW extracts more polyphenols than tap water and distilled water.

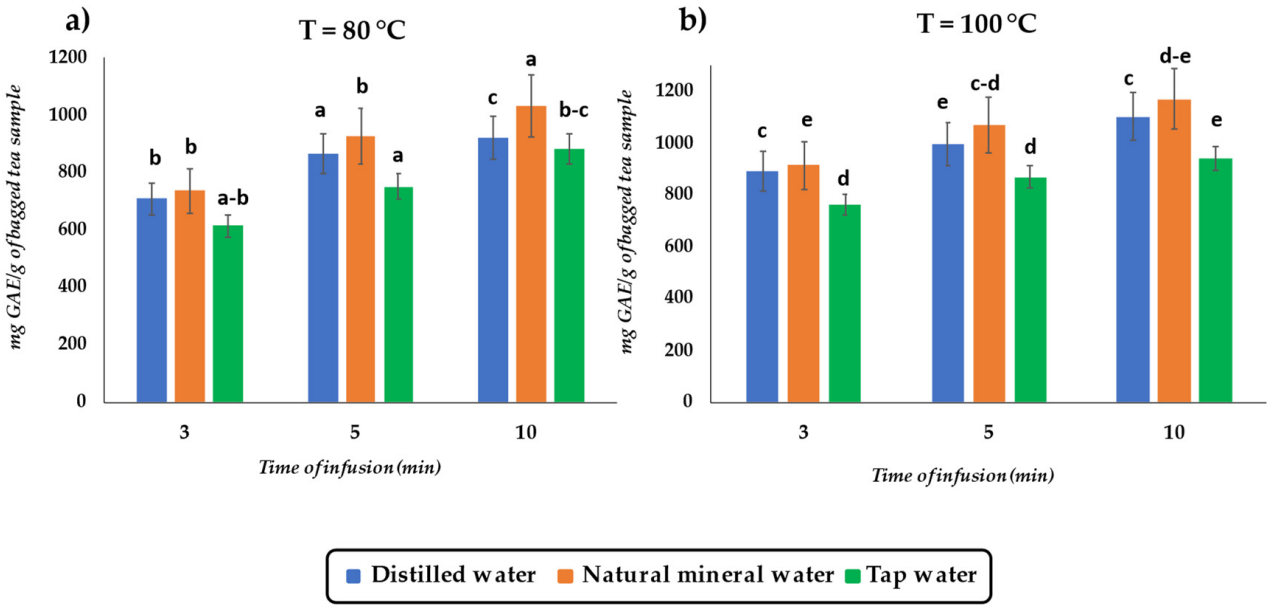

Figure 4. Total phenolic content (TPC) in black tea samples (mg GAE/g). (**a**) TPC for black tea infusion at T = 80 °C for different infusion times and different types of water; (**b**) TPC for black tea infusion at T = 100 °C for different infusion time and different types of water. Error bars are ±standard deviation. Same letters indicate a significant difference according to the ANOVA test ($p < 0.05$).

The greater TPC of green teas than black tea could result from their production processes. According to Astill et al. [23], during the production of green tea, the primary polyphenols (i.e., catechins) remain relatively intact during the process. This could be attributable to the deactivation of enzymes that can catalyze the oxidative polymerization of catechins by heat treatment (pan-roasting or steaming) immediately after harvesting. In contrast, black tea production involves a leaf-breaking step to promote the enzymatic oxidation of catechins, thus decreasing the polyphenol content.

The parameters that most influence the concentration of TPC are the infusion time and the type of water used for the infusion preparation. Indeed, the increase in temperature from 80 °C to 100 °C is accompanied by a slight increase in the TPC. Infusion time is the parameter that mainly influences the extraction of polyphenols, according to the literature [12,20,22]. For commercial packs of tea, the recommended infusion time is 2–3 min; this is recommended because an excessive concentration of polyphenols could influence the taste of the product [32].

Therefore, the results showed that the best extraction conditions for antioxidant compounds in tea is using NMW with an extraction time of 10 min at 100 °C (1126.62 mg GAE/g). These infusion procedures could be applied to homemade preparations to keep its active principles intact, thus strengthening the beneficial effects of its consumption [13].

3.2. Effect of pH of Aqueous Solution on TPC

In addition to the infusion time/temperature conditions, the effect of pH on the extraction of total polyphenols from green tea samples was performed when using the best conditions for polyphenols extraction (T = 100 °C for 10 min). In addition, only green tea was used for this analysis, as it was the sample with the highest polyphenol content (890.09–075.01 mg GAE/g). Figure 5 shows the values of TPC for green tea infusions in distilled water, buffer solutions, and natural mineral water at different pH values. It is possible to notice a decrease in total polyphenol content between pH 3 and pH 9. This decrease of about 20% in the TPC of green tea prepared at neutral or alkaline pH is probably due to the stability of some phenolic compounds in tea, such as catechins. It has been shown that these compounds have greater stability at acidic pH and tend to change epimer conformation as the pH increases, thus triggering the polyphenol degradation reactions in tea that lead to the formation of lower molecular weight compounds and, consequently, the loss of phenolic compounds [33]. In addition, it should be considered that, despite the different starting pH of the distilled water, during the infusion, the pH changed until it reached a value of 4.7 in all infusions, regardless of the initial pH value. This is in line with the study of Vuong et al. [34], which reported a similar phenomenon in black tea samples extracted with distilled water at different pH values. Indeed, using buffer systems, it was possible to maintain a constant pH throughout the infusion time. A similar trend was found at different pH values than distilled water, but the observed decrease reached almost 40% going from pH 3 to pH 9. In this case, it is possible to assume that it arises not from an increase in degradation reactions as a decrease in the extraction efficiency of TPCs at the different pH considered, which is in line with the study of Gadkari et al. (2015) [35], who found a decrease in extraction efficiency in phosphate buffers in the pH range between 6 and 8 in green tea samples.

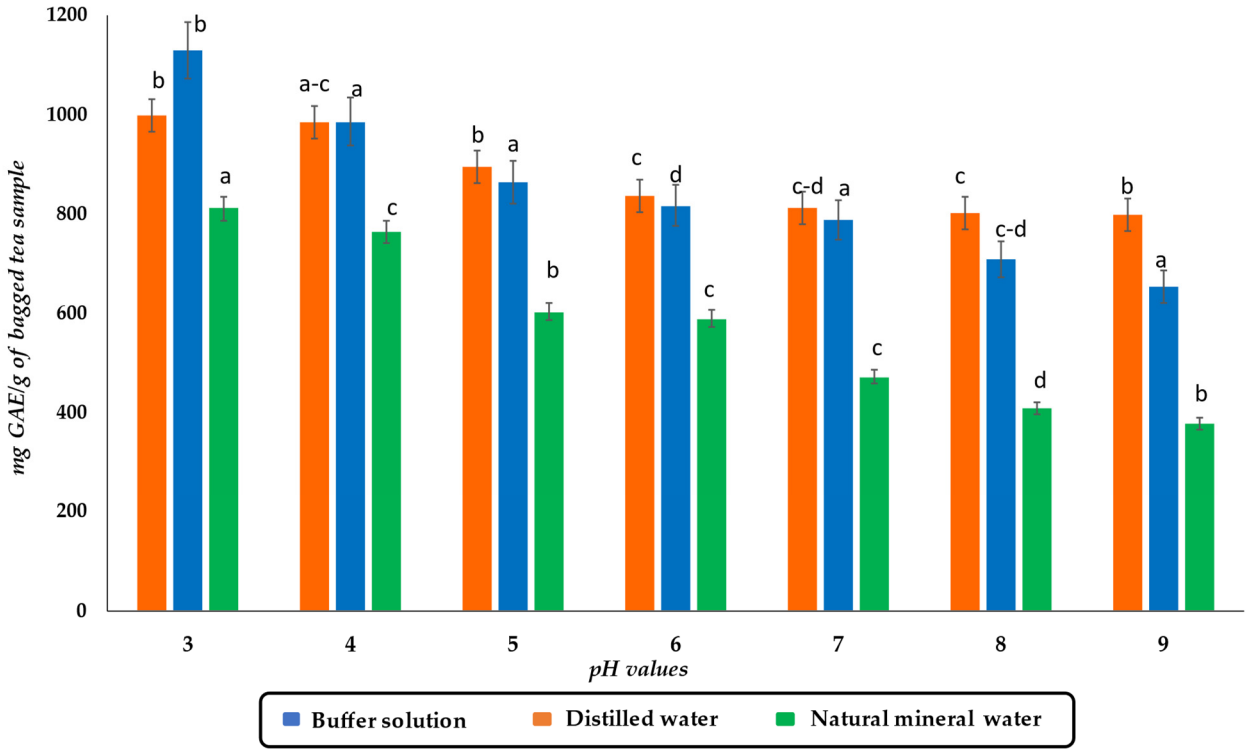

Figure 5. TPC of green tea infusion (mg GAE/g) prepared at different pH values between 3 and 9, in distilled water; in buffer solution, and in natural mineral water at 100 °C for 10 min. Error bars are ±standard deviation. Same letters indicate a significant difference according to the ANOVA test ($p < 0.05$).

As pointed out by Ananingsih et al. [33], TPC values can be related to the presence of the ions within the mineral water. It has been reported by [36] that some metal ions (e.g., $Fe^{2+,}$ Fe^{3+}, and Cu^{2+}) form complexes that catalyze the oxidation of some phenolic compounds (e.g., catechins). Therefore, the reduction in the TPC of green tea samples could be attributable to the composition of the mineral water used (hardness: 13.3 °F). In addition, the concentration of calcium ions in the natural mineral water (33.7 mg/L) could be the major contributor to the observed decrease. The composition of tap water and water with a high level of mineralization includes large amounts of dissolved mineral salts, especially calcium and magnesium salts, and hydrogen carbonate ions, which can inhibit the extraction process and react with the polyphenolic compounds [18,36], lowering the antioxidant properties of the infusion [37]. In addition, extracts of phenolic compounds from plants may contain various contaminants and interfering substances [21]. pH is also an essential factor in the extraction of phenolics. Usually, a low pH in the extraction solution can prevent the oxidation of phenolics, although they can also be eliminated through chelation with metal ions [21]. Hence, it can be assumed that tea polyphenols are more stable under acidic conditions and weaker under alkaline conditions. This is confirmed because by alkalizing the mineral water at pH 9, a precipitate ($Ca(OH)_2$ or $CaCO_3$) was formed. When performing the analysis on a filtered aliquot of the sample, the TPC was higher than that obtained at pH 3, thus confirming the possible interaction between polyphenols and calcium ions present in the water.

3.3. ABTS Assay of Tea Infusions

In Figure 6, the ABTS radical scavenging capacity of different green and black tea infusions obtained with different waters (DW, NMW, and TW) at different steeping times/temperatures. In the green tea infusion, the highest ABTS activity is observed in the samples prepared with NMW at 100 °C for 10 min (99.73, I%), while the lowest is at

80 °C for 3 min with TW (93.10, I%). The trend found in green tea is also observed for black tea infusions (Figure 6).

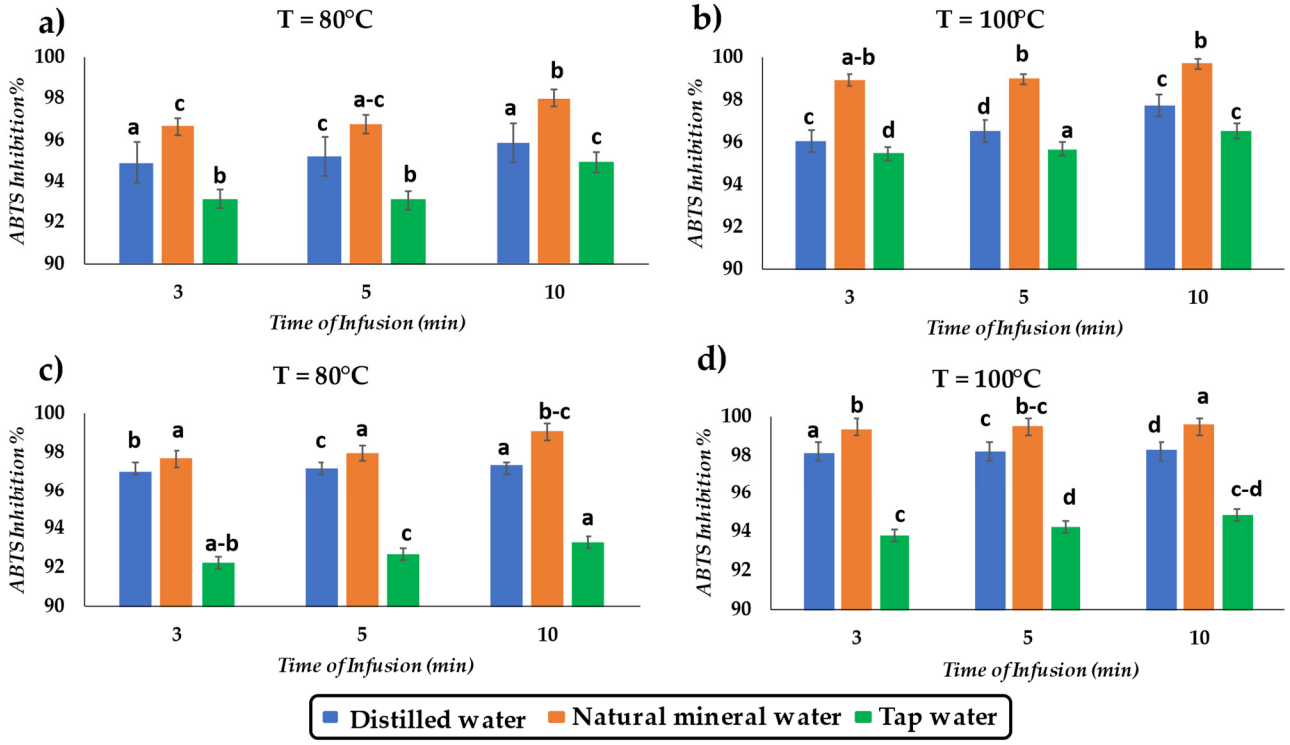

Figure 6. ABTS radical scavenging activity of green tea infusion, at T = 80 °C (**a**) and at T = 100 °C (**b**); and of black tea samples, at T = 80 °C (**c**) and at T = 100 °C (**d**); in distilled water; in natural mineral water, and in tap water. Error bars are ±standard deviation. Same letters indicate a significant difference according to the ANOVA test ($p < 0.05$).

Polyphenols from green tea (unfermented) and black tea infusion (fermented) are both effective in scavenging the ABTS radical, but the differences in scavenging activities may be due to the decrease in polyphenol concentrations during the fermentation process [12]. In addition, the water temperature does not negatively affect the antioxidant capacity, probably creating the assumption that the chemical structure of polyphenols maintains efficiency even at high temperatures [19,21]. In addition, the higher antioxidant activity may be related to the total polyphenol content of the tea infusions. Indeed, higher antioxidant activity was also found for this assay in tea infusions prepared with NMW [38].

3.4. DPPH Activity of Tea Infusions

The results show that DPPH activity increases with increasing steeping temperature and decreases with increasing infusion time (Figure 7). The highest DPPH activity in green tea infusions was obtained at 100 °C for 3 min (95.01, I%), whereas in black teas the highest antioxidant activity was obtained at 80 °C for 3 min (77.29, I%). Moreover, it was observed that DW is the water with the greatest extraction capacity for polyphenols for both green and black teas; this is probably due to the absence of calcium and magnesium salts dissolved in it [37].

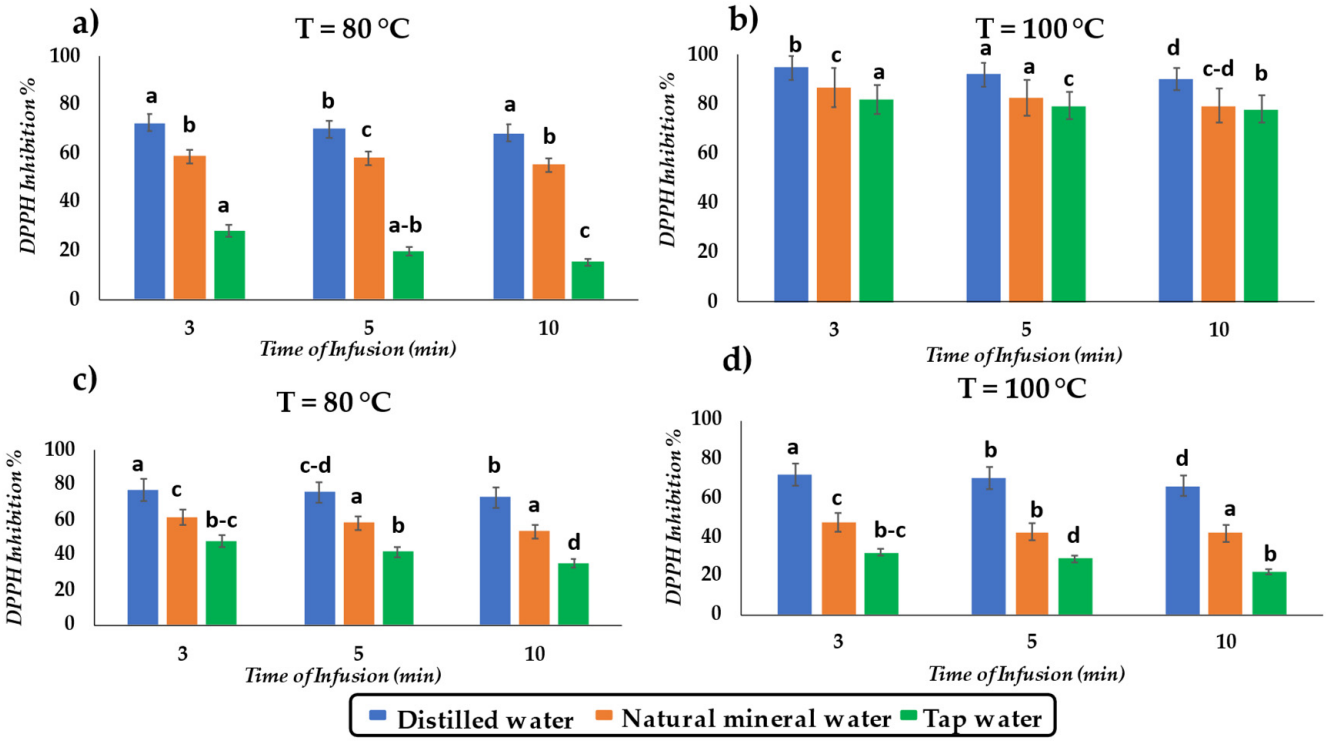

Figure 7. DPPH radical scavenging activity of green tea infusion (**a**,**b**), at T = 80 °C (**a**) and at T = 100 °C (**b**); and of black tea samples (**c**,**d**) at T = 80 °C (**c**) and at T = 100 °C (**d**); in distilled water, in natural mineral water, and in tap water. Error bars are ±standard deviation. Same letters indicate a significant difference according to the ANOVA test ($p < 0.05$).

Among the different infusions of tea obtained with three different types of water, there is a reduction in antioxidant activity probably related to the concentration of Ca^{2+} and Mg^{2+} ions. In addition, a precipitate is observed during the analysis of samples obtained with tap water (33.8 °F), probably because during the infusion phase, Ca^{2+} ions interact with polyphenols to form a polyphenol/calcium complex that is deposited on the bottom. An aliquot of tea infusion was subjected to centrifugation and then analyzed. This procedure revealed an antioxidant capacity higher than that obtained without centrifugation, confirming the possible interaction between calcium ions and polyphenols [36,37].

Furthermore, the increase in antioxidant activity may be due to the total polyphenol content and may be related to steeping time, leaf size, and the porosity of tea bags [12,32].

Nevertheless, the results of the DPPH assay differ from those of the ABTS assay; this is probably related to the different type of reagents used. The DPPH reagent is a stable nitrogen radical that interacts with peroxidic radicals involved in lipid peroxidation, while ABTS reacts with hydrophilic and lipophilic compounds. Therefore, the reactivity of DPPH is limited to the lipophilic fraction [39].

4. Conclusions

In this study, we evaluated the influence on polyphenol extraction from in green and black tea matrices, of different types of water (tap, distilled, and natural mineral water) with different hardness, different infusion times (3, 5, 10 min) and temperatures (80 °C and 100 °C). The optimal infusion variables were obtained by extracting phenolic compounds at T = 100 °C for 10 min, both for samples of green tea (916.12–1169.81 mg GAE/g) and black tea (932.03–1126.62 mg GAE/g) in natural mineral water. In addition, it has been shown that, under the same infusion conditions, acidic solutions have a higher capacity for polyphenol extraction, probably because acidic solutions stabilize polyphenols, limiting their oxidation [35]. Therefore, the optimal infusion variables (time, temperature, water types, and pH) could be considered for the preparation of domestic and industrial teas, as

well as those indicated on the label, in order to obtain infusions with higher polyphenol content and antioxidant activity and achieve greater benefit from the health-promoting effects. Moreover, this study could be a starting point for future research, examining the number and commercial tea types present on the market and investigating their polyphenolic profile.

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3.8 Research article no. 8

Title: Quality markers evaluation in chocolates with different cocoa content.

Year of publication: 2022

Journal: International Journal of Health Sciences

Authors: Prencipe S.A., Maddaloni L., Ruggieri R., Vieri S., Vinci G.

Research macro-area: **Food Quality (FQ), Food Safety (FS**)

Graphical abstract:

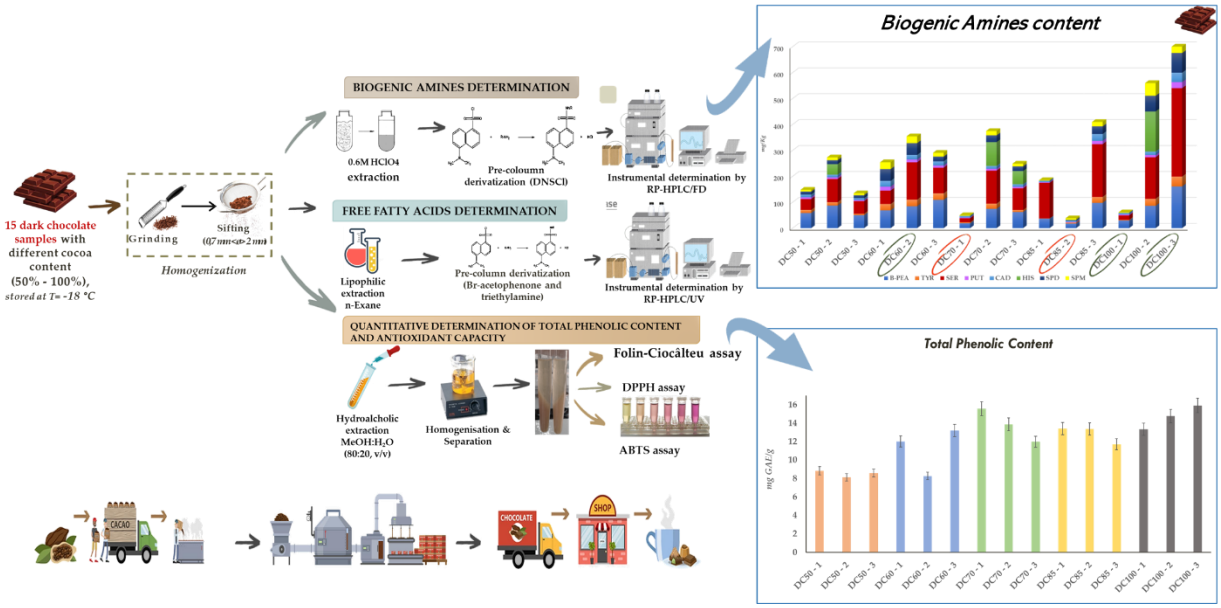

Figure 3.8. Graphical abstract of experimental study no. 8. Source: author's elaboration

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Quality markers evaluation in chocolates with different cocoa content

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Abstract---Quality assessment of cocoa-based food products (i.e., chocolate) reached increasing interest by scientific research, as it is rich in bioactive compounds (BCs) (i.e., catechins, methylxanthines, serotonin, etc.), which can have beneficial effects on human health (cardiovascular, immunomodulatory, mood regulator, etc.). Thus, the cocoa content may influence the quality of chocolate products. In this regard, the study aimed at a comprehensive evaluation of different BCs: biogenic amines (BAs), free fatty acids (FFAs), and antioxidant compounds (Total Polyphenols Content, TPC and Antioxidant Activity, AA), in chocolate samples with different cocoa contents (50%, 60%, 70%, 85%, 100%). By means of HPLC-UV/RF analysis, chocolates samples with higher cocoa content showed the highest concentration of serotonin, which represented the 25% of the total BAs

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concentration. Considering the total FFAs, unsaturated fatty acids (linoleic, linolenic and oleic) accounted for 30 to 50% in all chocolate samples analyzed. Antioxidant assays showed a great variability within samples, mainly highlighting a gradual increase of TPC and AC as a function of higher cocoa content. The findings of this study provide useful insights into cocoa-based products, showing that cocoa content influences the BCs concentration, thus improving the nutritional characteristics and the quality of the final product.

Keywords---antioxidant compounds, biogenic amines, chocolate, fatty acids, quality markers.

Introduction

Chocolate is a cocoa-based product widely consumed worldwide, which is obtained by processing and transforming seeds of the *Theobroma cacao* L. Chocolate can be a very nutritional component in food and the knowledge of its various medicinal properties represents a stimulus to those involved with its production, processing and consumption. Quality and flavor of cocoa-based products are mainly influenced by the various stages of cocoa processing [1,2]. These processes generally consist of fermentation, drying, roasting, grinding of cocoa beans and manufacturing of cocoa product by mixing of different ingredients (cocoa butter, cocoa mass and powder, sugar and other optional ingredients). According to Directive 2000/36/EC, dark chocolate is defined on the minimum 43% of cocoa content, depending on the percentage of total dry cocoa solids [3].

In recent years, interest in the consumption of dark chocolates with higher cocoa content has increased as a result of a major interest in food quality and safety issues. Eating chocolate, as part of a healthful balanced diet, could potentially provide a beneficial way to improve wellbeing, thus exerting cardio- and neuro-protective effects, modulating inflammatory markers and immune responses, cognitive and mood elevation [4,5]. Furthermore, it also has psychoactive functions based on its composition [1,6]. Therefore, in order to increase the value of the final product, the evaluation of food quality, is highly advisable. In this regard, quality assessment can be carried out through the determination of molecular markers, typical for a sample, which can establish the origin of the sample or the good state of storage and preservation. [7].

Cocoa and its based products (i.e., chocolate) are considered rich in different bioactive compounds (BCs), such as serotonin [8], polyphenols [6], free fatty acids, alkaloids, methylxanthines, etc. [9,10]. Within these compounds, biogenic amines (BAs) are widely considered as food safety markers because of their presence in food and their effect on the human organism. BAs are produced by microbial enzymes that decarboxylate amino acids; however, their occurrence in food can also be related to spoilage and poor preservation. In addition, BAs can induce several physiological reactions, and investigating their content in foods can be a quality and safety index for consumer health and diet formulation [11]. Serotonin is a bioactive monoamine with a broad activity in human brain, playing

a crucial role in modulating mood, appetite as well in muscle contraction and blood pressure regulation [8]. At the same time, the consumption of food containing high concentrations of some biogenic amines (histamine, putrescine, cadaverine, etc.) can cause undesirable toxicological effects, that are similar to those of food poisoning (spasms, nausea, scombroid syndrome, allergic reactions, etc.), and, in extreme cases, cerebral hemorrhage, anaphylactic shock and death [12].

Cocoa beans and cocoa products consist of 12-18% (total dry weight, TDW) of antioxidant compounds (i.e., polyphenols), a broad class of organic compounds produced from the secondary metabolism of plants. They may have antioxidant, anti-inflammatory, antibacterial functions, also providing indications about oxidation and degradation status of food [6, 9]. Furthermore, the nutritional value of cocoa – based products could be also influenced by the composition of cocoa butter [1]. It represented about 45-53% of TDW, in cocoa beans, consisting mainly of saturated (palmitic and stearic acids), monounsaturated (oleic acid), and polyunsaturated (linoleic and linolenic) fatty acids, which were largely investigated in food science, for nutritional labelling, quality control, nutrition and health purposes [4].

However, the chocolate manufacturing processes and the content of cocoa solids may influence the amount of BCs in cocoa-derived products. In this study, a quality marker evaluation in dark chocolates as function of different cocoa content was proposed. The content of eight BAs was evaluated in dark chocolate samples with different cocoa content by means of high-performance liquid chromatography with fluorescence detection (HPLC-FD) and pre-column derivatization with dansyl-chloride. The BAs studied as polyamines were putrescine (PUT), and cadaverine (CAD), spermine (SPM) and spermidine (SPD); whereas, β-phenylethylamine (β-PEA), HIS, SER, and TYR were studied for monoamines. The content of six free fatty acids (FFAs), Myristic, Stearic and Palmitic for saturated FFAs, and Linolenic (ω-3), Linoleic (ω-6) and Oleic were studied for unsaturated FFAs. Thereafter, the evaluation of total polyphenols content (TPC) by means of Folin-Ciocâlteu, and antioxidant activity (AA) by means of ABTS and DPPH assays was carried out through UV-Vis spectrophotometric analysis.

Materials and Methods

Chemicals

HIS, SER, SPM, SPD, PUT, β-PEA, CAD, and TYR and dansyl chloride were purchased from Supelco (Bellefonte, PA, USA). The six FFAs – Myristic, Stearic, Palmitic, Oleic, ω-3 and ω-6 acids, and the derivatizing agents, Br-acetophenone and triethylamine were supplied by Sigma-Aldrich (Milan, Italy). Methanol (CH3OH), n-Hexane (C6H14), water and acetonitrile (ACN) for HPLC, Folin–Ciocâlteu reagent (H3[P(W3O10)4]/H3[P(Mo3O10)4]), ABTS (diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium persulfate ($K_2S_2O_8$), sodium bicarbonate (NaHCO3), gallic acid (C7H6O5), perchloric acid 70% (HClO4), sodium hydroxide (NaOH), sodium carbonate (Na2CO3), ammonium hydroxide (NH4OH), and acetic acid (CH_3COOH) were supplied by Sigma-Aldrich (St. Louis,

MO, USA).

Instruments

The following instruments were used: Bandelin Sonorex RK100H ultrasonic thermostatic bath, G-Therm AG-System heater, and Whatman (PTFE) 0.45 μm 100 syringe filters (Sigma Aldrich, Milan, Italy), UV-Vis spectrophotometer (Jenway, Stone, UK), NEYA 10R refrigerate centrifuge (Exacta Optech, Modena, Italy). Chromatographic analysis was performed using an ATVP LC-10 HPV binary pump with an RF-10° XL fluorimetric (FD) detector (Shimadzu, Kyoto, Japan) operating to $\lambda_{emission}$=320 nm, and $\lambda_{excitation}$= 523 nm. A Supelcosil LC-18 column (250 mm × 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column were used for the determination of BAs. An SPD-10AVP UV detector (Shimadzu, Kyoto, Japan) operating to λ = 254 nm, and a Supelcosil LC-18 column (150 mm × 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column were used to determine FFAs.

Sampling

Dark chocolate samples were with different cocoa content (50%, 60%, 70%, 85% and 100%) were purchased from local supermarkets. After acquisition, samples were homogenized by grinding and sifting by a sieve with a 0.7 ÷ 2mm diameters holes. The obtained particle size fraction was collected and stored at refrigerated temperature, T= -18 °C until the day of analysis.

BAs Determination in Dark Chocolate samples

The BAs determination was carried out according to a previously reported method with some modifications [13]. About 2.5g of chocolate sample was extracted with 7 mL of 0.6 M $HClO_4$, homogenized for 3 min at 200 rpm with a magnetic stirrer, and centrifuged at 2900× g for 10 min, at T= 4 °C. The supernatant was collected in a flask. The residue was added with 7 mL of 0.6 M $HClO_4$, mixed, and again centrifuged for 10 min. Then, the second extract was added to the first one, and filtered through a 0.45 μm membrane syringe filter. The final volume was adjusted to 20 mL with 0.6 M $HClO_4$. An aliquot of 1 mL of the final extract was then derivatized by adding 200 μL of 2 M NaOH, 300 μL of saturated $NaHCO_3$ solution, and 2 mL of dansyl chloride solution (10 mg/mL in acetone). After shaking, the samples were left in the dark for 60 min at 45 °C. About 100 μL of NH_4OH was added to stop the derivatizing reaction. The final volume was adjusted to 5 mL by adding ACN. The dansylated extract was filtered using 0.45-μm filter (Whatman® Puradisc filters, Sigma Aldrich, Milan, Italy), injected into the HPLC system, and analyzed with a previous standardized method [13].

For the chromatographic determination of the BAs, a volume aliquot of 20 μL (loop 20 μL) was injected. Analyses were performed by using a Supelcosil LC-18 column (250 mm × 4.6 mm, 5 μm), Supelco, Bellefonte, PA, USA) coupled with an FD detector ($\lambda_{emission}$=320 nm, and $\lambda_{excitation}$=523 nm). The analyses were carried out maintaining a fixed temperature of 30 °C. The solvents used for chromatographic elution were: (A) purified water and (B) ACN. The elution program started with 3 min of isocratic elution (50% A; 50% B) reaching 100% B

after 18 min and ending with another 3 min of isocratic elution. Finally, it also required 5 min to return to the initial isocratic conditions (50% A; 50% B). The flow rate was then kept constant at 1.2 mL/min, for a total analysis time of 30 min. The results were achieved by linear regression through calibration curve for each BAs ranging from 0.1 and 25 mg/L.

FFAs Determination in Dark chocolate samples

FFAs content in chocolate samples was determined according to Fratoddi et al., with some modifications [14]. Briefly, 4 ml of n-hexane was added to 0.1g – homogenized sample, sonicated for 5 min at room temperature and then centrifuged at 2900× g for 10 min, at T= 25 °C. The supernatant was collected in a flask. The residue was added with 2 mL of n-hexane, mixed, and again centrifuged for 10 min. Then, the second extract was added to the first one, and then dry-filled under nitrogen (N_2) flow. The dried state – organic extract was resuspended in 2 ml of n-hexane and filtered through 0.45µm PTFE syringe filters. An aliquot of 50 µL of the final extract was then derivatized by adding 50µl of triethylamine solution (25 mg/ml in acetone), and 50µl of bromo-acetophenone (20 mg/ml in acetone), in a glass tube. The closed tube was placed in an oven at T=100°C for 15 minutes. The sample was then cooled and 80µl of a solution of glacial acetic acid (10 mg/mL in acetone) was added, and placed again in the heater at T=100°C for 15 minutes. Thereafter, the tube was cooled and the contents brought to dryness under nitrogen flow. The residue was recovered in 250µl of an aqueous acetonitrile solution (ACN:H_2O, 70:30 *v/v*) and sonicated in the ultrasonic bath for 15 minutes. The blank was prepared by derivatizing 50µl of n-hexane, the fatty acid extracting solvent.

For the chromatographic determination of the FFAs, a volume aliquot of 20 µL (loop 20 µL) was injected. Analyses were performed by using a Supelcosil LC-18 column (150 mm × 4.6 mm, 5 µm), Supelco, Bellefonte, PA, USA) coupled with an UV detector (λ= 254 nm). The analyses were carried out maintaining a fixed T= 43 °C. The solvents used for the chromatographic separation were: (A) water purified and (B) acetonitrile. The elution program started with 3 min of isocratic elution (70% A; 30% B) reaching 100% B after 28 min and ending with another 3 min of isocratic elution. Finally, it took 5 min to reinstate the initial isocratic conditions (70% A 30% B). The flow rate was mantained constant at 0.8 mL/min, for a total analysis time of 32 min. The final results were achieved by linear regression with calibration curves different for each fatty acid analyzed: Linolenic acid (0.002 – 0.04 mg/ml), Linoleic acid (0.012 – 0.4 mg/ml), Myristic acid (0.005 – 0.2 mg/ml), Palmitic acid (0.005 – 0.4 mg/ml), Oleic acid (0.08 – 1 mg/ml), Stearic acid (0.1 – 1 mg/ml).

Determination of TPC and AA in Dark chocolate samples

Sample extraction for TPC and antioxidant were prepared according to a previously published method with some modification [13]. After removing the organic fraction (*Section 2.5*), the residue was extracted with 5 ml of methanol in aqueous solution (60:40, *v:v*), homogenized in a ultrasonic bath for 5 min at room temperature, and centrifuged at 2900× g for 10 min, at T= 25 °C. The supernatant was collected in an amber vial. The extraction procedure of total polyphenols was

repeated twice. TPC was determined by the Folin-Ciocâlteu method [14], modified for chocolate samples as follows: 1 mL of methanolic extract was added to 0.25 mL of Folin-Ciocâlteu reagent and 0.5 mL of aqueous Na_2CO_3 solution (7.5%, w/v) in a 10 mL volumetric flask. The final volume was achieved with purified water. Spectrophotometric analysis was carried out at λ= 750 nm after 45 min of incubation in the dark at room temperature. The total polyphenols content was expressed as milligrams of gallic acid equivalent (mg GAE) per kg. The final results were obtained through a calibration curve ranging from 5 to 100 mg/l (R^2 = 0.9998). Antioxidant activity was determined by means of DPPH and ABTS assays, according to a previously reported method of Preti et al. [13]. The free radical scavenging activity DPPH and ABTS of the chocolate hydroalcoholic extracts was assessed by measuring the absorbance decrease at 515 nm (DPPH), and 734 nm (ABTS). Absorbance was measured in 1 cm path-length cuvettes against methanol in aqueous solution (60:40, v:v), by using a UV-Vis spectrophotometer (Jenway, Stone, UK). Results were expressed as inhibition rate and were calculated based on Equation:

$$\mathbf{I\% = (A0 - Af/A0) \times 100}$$

where A_0 is the radical cation's initial absorbance, and A_f is the absorbance after the addition of sample extract.

Results and Discussions

Biogenic Amines Content in Dark chocolate samples

In this study, contents of eight BAs were determined in dark chocolate (DC) samples as function of different cocoa content (50%, 60%, 70%, 85% and 100%). The quantification of BAs in chocolate samples was summarized in Table 1. Chocolates samples showed a great variability in BAs content. BAs detected in all samples at high concentrations were SER (15.8 – 340.98 mg/kg), β-PEA (17.62 – 68.52 mg/kg), SPD (0.36–75.92 mg/kg), and SPM (1.74–49.05 mg/kg), thus agreeing with results from literature [15]. DC with 60% of cocoa content presented the highest amount of PUT (8.45–15.36±0.02 mg/kg). HIS was present only in three samples (50%, 70% and 100%), within the limit established by European Regulation [16], thus reducing the potential risk to human health. An important highlight should be done about Serotonin, which was detected in all chocolate samples, thus representing the 25% of the total BAs concentration. Dark chocolate samples with higher cocoa content (100%) showed the highest concentration of SER (159.96 – 340.54 ± 0.93 mg/kg). This could be related to the chemical composition of chocolate samples, thus explaining the plausible physiological mechanism between chocolate and mood.

Results from previous research reporting that many kinds of chocolate, rich in sugar and with low protein, can stimulate the synthesis of serotonin, affecting mood [8,17]. The most diverging data are related to dark chocolate with 60% of cocoa content, presenting the highest amount of CAD (24.42–25.28 ± 0.1 mg/kg); other studies, for the same cocoa content chocolate samples, never contained levels of CAD higher than 5.3 mg/kg [15]. In this sense, it should be considered that CAD content, considered as a food spoilage maker, could be affected by many

parameters related to hygienic conditions, temperature, pH values of the raw materials or the manufacturing process, as well as the handling and storage conditions.

Fatty acid content in Dark chocolates

The content of six FFAs was investigated in dark chocolates with different cocoa content (50 ÷ 100%). Results are shown in Table 2. Values are expressed as % of FFAs on the % of total free fatty acids (TFFAs) analyzed in this study. Considering the total FFAs, Palmitic (10.9–31.9% ± 0.54 of TFFAs), and Stearic (10.3-40% of TFFAs) were the most abundant saturated FFAs in all tested chocolate samples; while Linolenic (n.d – 8.30% ± 0.52), Linoleic (1.97 – 11.40% ± 0.25), and Oleic (11.97 – 24.48% ± 0.31). The prevalence of C14:0 and C16:0 fatty acids common in almost foods including many oils and fats, as well as in cocoa-based products.

Table 1
Quantitative results of biogenic amines in chocolate samples (mg/kg) ± standard deviation

	Dark Chocolate (50%)	Dark Chocolate (60%)	Dark Chocolate (70%)	Dark Chocolate (85%)	Dark Chocolate (100%)
SER	41.91–89.39 ± 0.25	98.91–143.88 ± 0.34	15.80–127.03 ± 0.1	n.d.–203.18 ± 0.2	16.86–340.98 ± 0.18
TYR	5.37–13.10 ± 0.06	24.42–25.28 ± 0.1	3.15–20.16 ± 0.03	0.76–22.96 ± 0.02	2.60–38.01 ± 0.05
β-PEA	50.07–86.81 ± 0.18	68.52–109.1 ± 0.12	19.23–74.46 ± 0.07	17.62–97.52 ± 0.04	29.64–160.83 ± 0.25
PUT	4.35–7.14 ± 0.01	8.45–15.36 ± 0.02	2.59–7.21 ± 0.01	2.33–13.07 ± 0.02	3.17–23.4 ± 0.01
CAD	7.56–10.32 ± 0.03	16.18–22.16 ± 0.12	6.20–11.1 ± 0.01	5.24–26.31 ± 0.1	6.43–36.20 ± 0.03
HIS	n.d.–40.37 ± 0.11	n.d.	n.d. –91.84 ± 0.21	n.d.	n.d.–154.61 ± 0.07
SPD	11.29–15.33 ± 0.05	18.3–45.44 ± 0.11	0.65–26.16 ± 0.03	0.36–29.46 ± 0.04	0.36–75.92 ± 0.09
SPM	7.04–10.81 ± 0.06	14.71–26.22 ± 0.05	2.45–16.47 ± 0.02	1.74–17.11 ± 0.02	1.74–49.05 ± 0.08

SER: serotonin; TYR: tyramine; β-PEA: β-phenylethylamine; PUT: putrescine; CAD: cadaverine; HIS: histamine; SPD: spermidine; SPM: spermine; n.d.: not detectable

**The ranges obtained from triplicate analysis, referred to the average of the n= 3 samples of DC 50%; n= 3 samples of DC 60%; n= 3 samples of DC 70%; n= 3 samples of DC 85%; n= 3 samples of DC 100.*

Considering the fatty acid profiles of chocolates, palmitic acid (C16:0, 3.37-20.13g/100 g), stearic acid (C18:0, 4.10-29.09 g/100 g) and oleic acid (C18:1, 4.10-29.09 g/100 g) were the most abundant [18], thus implying the dependence of the chocolate fatty acid profile on cocoa beans as a raw material. Cocoa beans are the main source of cocoa butter (45-53% of Total Dry Weight, TDW), consisting mainly of saturated (palmitic and stearic acids) and monounsaturated (oleic acid) fatty acids. Furthermore, the determination of the fatty acid profile could be considered as a quality marker for the technological characteristics and the desired nutritional characteristics of cocoa beans and their manufacturing process [19]. Myristic acid was only detected in 50%, 60% and 100% cocoa – dark chocolate (0.04 – 0.97% ± 0.09), thus representing the lowest FFA among the total of FFAs analyzed. This result was in agreement with previously published results of chocolate FFAs content, especially for dark chocolate samples [20]. For polyunsaturated fatty acids, (Linoleic, and Linolenic) accounting for 30% TDW in all chocolate samples analyzed, it is important to highlight, that an increased consumption can have beneficial effects on human health, thus exerting anti-inflammatory, antioxidant, anticancer and cholesterol lowering properties. Therefore, dark chocolate relatively rich in polyunsaturated fatty acids could be of interest for incorporating these bioactive lipids into novel foods designed to produce certain health-related benefits [18].

Total Polyphenols and Antioxidant Activity evaluation

Cocoa beans and cocoa derived–products consist of 12-18% TDW of antioxidant compounds (i.e., polyphenols), which are a broad class of organic compounds produced from the secondary metabolism of plants. In cocoa-derived products such as dark chocolate, polyphenols are widely studied for their beneficial effects (i.e., antioxidant, anti-inflammatory, antibacterial, cardiovascular, etc.) on human health [6]. The TPC assay was carried out to determine the total polyphenols content in the hydroalcoholic fraction [13]. The antioxidant activity was assessed by means of two different in vitro antiradical tests - ABTS and DPPH [13]. In addition, these two radicals have specific free radical scavenging abilities and reducing power that are sensitive to different types of antioxidant compounds occurring in plant food extracts. Consequently, the combining use of these two assays provided an effective assessment of antioxidant activity in chocolate samples. The results are shown in Figure 1.

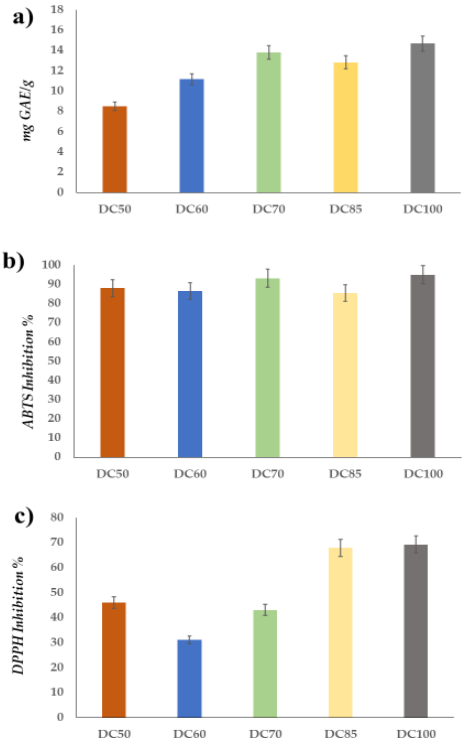

Figure 1. Evaluation of antioxidants in chocolate samples. Histograms of TPC (a) expressed in mg GAE/g ± standard deviation; Antioxidant Activity by means of ABTS (b) and DPPH (c) expressed as Inhibition % ± standard deviation. DC50: Dark Chocolate with 50% of cocoa content; DC60: Dark Chocolate with 60% of cocoa content; DC70: Dark Chocolate with 70% of cocoa content; DC85: Dark Chocolate with 85% of cocoa content); DC100: Dark Chocolate with 100% of cocoa content

Table 2
Quantitative results of free fatty acids in dark chocolates with different cocoa content. Values are expressed as % of FFAs on the % of total free fatty acids (TFFAs) analyzed ± standard deviation

	Dark Chocolate (50%)	Dark Chocolate (60%)	Dark Chocolate (70%)	Dark Chocolate (85%)	Dark Chocolate (100%)
Linolenic (C18:3)	6.84–6.88±0.2	n.d.–5.27±0.13	n.d–3.99±0.61	n.d.–8.30±0.56	n.d–1.02±0.07
Linoleic (C18:2)	5.11–5.45±0.4	4.47–11.40±0.23	1.97–8.12±0.48	5.73–9.79±0.08	1.46–2.97±0.62
Myristic (C14:0)	n.d.–0.97±0.09	n.d.–0.33±0.08	n.d.	n.d.	0.04–0.49±0.17
Palmitic (C16:0)	10.84–21.15±0.5	12.49–31.90±0.54	18.43–29.87±0.22	22.98–27.36±0.15	14.68–26.88±0.39
Oleic (C18:1)	11.97–21.74±0.7	18.43–29.87±0.32	17.02–37.54±0.18	24.48–30.72±0.42	12.51–31.38±0.05
Stearic (C18:0)	15.65–19.03±0.4	10.38–18.60±0.41	10.38–18.60±0.27	15.53–20.19±0.34	22.44–39.99±0.54

**The ranges obtained from triplicate analysis, referred to the average of the n= 3 samples of DC 50%; n= 3 samples of DC 60%; n= 3 samples of DC 70%; n= 3 samples of DC 85%; n= 3 samples of DC 100*

The results obtained from triplicate analysis referred to the average of the n= 3 samples of DC 50%; n= 3 samples of DC 60%; n= 3 samples of DC 70%; n= 3 samples of DC 85%; n= 3 samples of DC 100. For TPC (Fig. 1a), dark chocolate with the highest cocoa content was found to be rich in polyphenols, although each chocolate samples were different in polyphenol content. Dark Chocolate (DC) with 100% showed the highest TPC values (13.33 – 15.91 ± 0.53 mg GAE/g), followed by DC with 70÷85% and 50% of coca content (11.71 – 15.56 ± 0.34 mg GAE/g, and 8.13 – 8.83 ± 0.1 mg GAE/g respectively). However, differences between dark chocolates can be ascribed to geographical and plant factors such as soil type, cropping and fermentation conditions, climate and cultivar. As polyphenols are mainly concentrated in non-fat cocoa solids, chocolates with a higher cocoa content are considered to be a better source of these compounds, which have a greater antioxidant activity. [21]. The trend of TPC results agreed with ABTS radical scavenging assay (Fig. 1b) for antioxidant activity, thus providing a high radical inhibition % (I%) by the different cocoa content – chocolates.

The highest result was achieved by DC with 85% of cocoa content (99.20, I%). A similar result was reported by Godočiková et al., [22] who established that the highest antioxidant activity measured by ABTS assay was shown by the sample of chocolate with 90% of cocoa solids, positively correlating with the total polyphenol content. However, the DPPH assay results (Fig. 1c) were significantly lower than those obtained with ABTS assay, for all DC samples analyzed. The results obtained were in consonance with the result of Belšcak et al., [23], where DPPH showed lower results than ABTS test, because of its reactivity only with lipophilic antioxidants. In addition, DPPH radical does not react with phenolic acids and, therefore, the antioxidant capacity determined by DPPH and ABTS methods are partially different.

Conclusions

The study investigated quality markers in chocolates as function of different cocoa content. By means of chromatographic analysis, chocolates samples showed a great variability in BAs content. Dark chocolate samples with higher cocoa content (100%) showed the highest concentration of BAs (159.96 – 340.54 ± 0.93 mg/Kg), thereof SER amount (159.96 – 340.54 ± 0.93 mg/kg) represented the

25% of the total BAs concentration. HIS was present only in three samples (50%, 70% and 100%), within the limit established by European Regulation. Considering the total FFAs: palmitic (10.9-31.9% Total of analyzed FFAs), and stearic (10.3-40% of TFFAs) were the most abundant in all chocolate samples; while unsaturated fatty acids (linoleic, linolenic and oleic) accounted for 30 to 50% in all chocolate samples analyzed. Therefore, dark chocolate relatively rich in polyunsaturated fatty acids could be of interest for incorporating these bioactive lipids into functional foods intended to exert certain health promoting benefits (anti-inflammatory, antioxidant, anti-cancer, etc.). Even though there were individual differences in the polyphenol content in dark chocolate samples, a good correlation between the antioxidant potency and the declared cacao content was observed, mainly highlighting a gradual increase of TPC and AC as a function of higher cocoa content. Therefore, the chosen bioactive compounds (BCs) resulted to be suitable markers for chocolate quality assessment, showing that cocoa content influences the BCs concentration, thus determining the quality and nutritional characteristics of the final products.

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3.9 Research article no. 9

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Journal: International Journal of Health Sciences

Authors: Gobbi L., Maddaloni L., **Prencipe S.A.,** Vinci G.

Research macro-area: **Food Quality (FQ), Food Safety (FS)**

Graphical abstract:

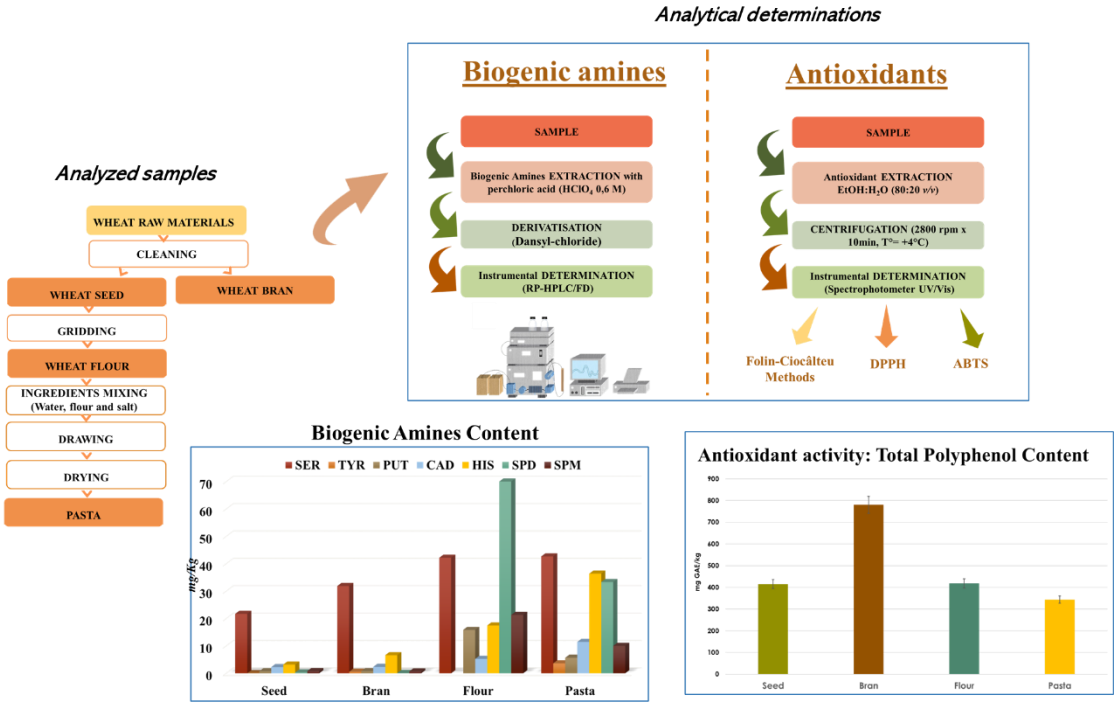

Figure 3.9. Graphical abstract of experimental study no. 9. Source: author's elaboration

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Evaluation of biogenic amines, phenolic and antioxidant compounds in "Senatore Cappelli" durum wheat products

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Abstract---"*Senatore Cappelli*" durum wheat products have attracted much interest in recent years because of their nutritional and quality characteristics, as they are a source of different bioactive compounds (i.e., polyphenols, serotonin, etc.). This study examines the content of biogenic amines (BAs), ß-phenylethylamine, putrescine, cadaverine, histamine, tyramine, serotonin and spermine, total polyphenols content (TPC) and antioxidant capacity (AC) of different products of the "*Senatore Cappelli*" durum wheat chain (SCDW): seeds, chaff, flour, and pasta. BAs were detected and quantified by HPLC-RF. While UV-Vis spectrophotometer was applied for the determination of TPC and AC. All BAs investigated were found in the samples analyzed at different concentrations. Particularly, the presence of serotonin (21.71 ± 0.15 - 42.66 ±0 .03 mg/kg), an BA with positive effects on human health, was found in all SCDW products. In addition, a higher concentration in SCDW pasta of histamine (36.37 ± 0.01 mg/kg) and cadaverine (11.43 ± 0.36 mg/kg), which are the main BAs involved in

allergic processes, compared to the other SCDW products was found. TPC was significantly higher in bran samples (780.35 ± 1.4 mg GAE/kg); while pasta had a lower content (343.1 2± 3.35 mg GAE/kg) than flour and seeds. This information could be important for the wheat industry to obtain products with higher nutritional and functional characteristics. The chosen compounds were found to be a suitable marker for assessing the quality of SCDW products.

Keywords---antioxidant compounds, biogenic amines, durum wheat, food quality, food safety.

Introduction

Senatore Cappelli (*Triticum turgidum* ssp. *durum*) (SC) is an autumnal durum wheat cultivar obtained by geneticist Nazareno Strampelli, by selecting individual dwarf genes from Italian, North African, and Syrian – Palestinian landraces [1]. Selection procedures have been carried out to obtain intensive crop management – resistant genotypes, performing wide adaptability, and good agronomic performances also in border areas. In this regard, Senatore Cappelli is generally considered an *ancient grain*, because of its characteristic of never undergoing modern plant breeding programs of intensive farming. This could be of strategic importance in the selection of low environmental impact agricultural cultivars with high yield and better technological quality (i.e., gluten quality) [2, 3]. Moreover, breeding programs have always focused on improving yield and technological properties, without considering the nutritional and nutraceutical importance of wheat consumption in the human diet [2].

Cereals and cereal products are placed at the base of the food pyramid, accounting for more than 55% of total consumption in the Mediterranean Diet [4]. The ancient grain SC is generally used in mixture as raw material to produce cereal-based products, such as pasta, flours, etc., of which Italy represents the first world producer, with the highest pro capita consumption ratios [5]. For this reason, a quality assessment on its nutritional value is highly recommended. Durum wheat products (*T. turgidum* ssp. *durum*) are a rich source of bioactive compounds, presenting an excellent amount of dietary fiber, proteins, and antioxidants [2], which can exert beneficial effects on human health (i.e., chronic diseases prevention, cholesterol-lowering properties, anti-inflammatory, antioxidant properties, etc.) [6]. However, the production process to which cereal products are subjected is determined by the quality of the final product. In this regard, food markers (i.e., phenolic compounds, biogenic amines, etc.) are allowed to provide corrective action in the event of noncompliance with product safety or quality standards. Biogenic amines are ubiquitous bioactive compounds, which originate from microbial decarboxylation of amino acids. They are widely used as food safety markers as their presence in foods could be either associated with physiological and health-promoting functions (i.e., nucleic acid regulation, membrane stabilization, etc.) or negative inflammatory reactions, such as “Histamine poisoning” [7]. In grains, these markers can be formed from endogenous enzymes or microorganisms contained in the raw material or added during processing [8], allowing food production to be monitored at all stages of

processing and storage.

Moreover, grain processing leads to the production of by-products (such as chaff), which are often not used, but could be applied in alternative productions and other supply chains (Fig. 1). To address this gap, over the last decade the EU has adopted a shift towards a circular economic model by 2030 for the food and beverage industry and aims to avoid processing waste going to landfills as much as possible [9]. In Europe, wheat chaff could provide an annual biomass potential of about 54.8 Megatons (Mt). However, advances in harvesting technology play a key role in transforming unexploited by-products into valuable raw materials [10]. Some studies have examined the potentialities of the valorization of durum wheat by-product production [9–11]. Most of them only provided a microbiological assessment of mycotoxin contamination of commercial-scale cereal grain cleaning operations [11, 12]. Since the low availability of literature research focusing on the nutritional and quality features of SC durum wheat products, this study aimed to provide a quality and safety assessment of the Senatore Cappelli durum wheat chain (SCDW) (seeds, flour, pasta, and chaff), through the evaluation of biogenic amines, phenolic and antioxidant compounds.

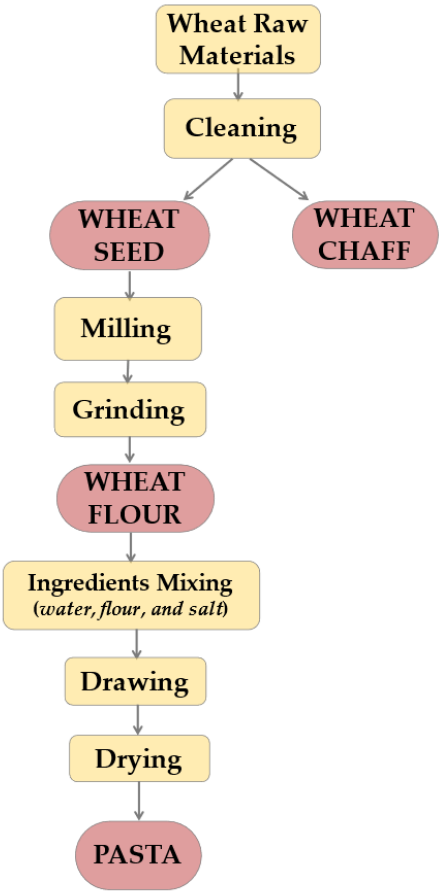

Figure 1. The production process of durum wheat products

Eight Bas was evaluated in SCDW samples by high-performance liquid chromatography with fluorescence detection (HPLC-FD) with pre-column derivatization. The BAs studied were putrescine (PUT), cadaverine (CAD), spermine (SPM), and spermidine (SPD), for polyamines, whereas β-phenylethylamine (β-PEA), SER, TYR, and HIS were studied for monoamines. Thereafter, the evaluation of antioxidant compounds by means of Folin-Ciocâlteu, ABTS and DPPH assays was carried out by UV-Vis spectrophotometric analysis.

Materials and Methods

Chemicals

β-PEA, PUT, CAD, HIS, TYR, SER, SPM, SPD were supplied by Supelco (Bellefonte, PA, USA) as well as the derivatizing agent, dansyl chloride. Ethanol (C_2H_5OH), methanol CH_3OH), water (HPLC grade), acetonitrile (HPLC grade), Folin–Ciocâlteu reagent (H3[P(W3O10)4]/H3[P(Mo3O10)4]), ABTS (2,2-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium persulfate, sodium bicarbonate (NaHCO3), gallic acid (C7H6O5), perchloric acid (HClO4), sodium hydroxide (NaOH), sodium carbonate (Na2CO3), ammonium hydroxide (NH4OH), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instruments

Bandelin Sonorex RK100H water and ultrasonic thermostatic bath, IKA T18 digital Ultra–Turrax (IKA-group, Saufen, Germany), and Whatman 0.45 μm 100 (PTFE) syringe filters (Sigma Aldrich, Milan, Italy), UV-Vis spectrophotometer (Jenway, Stone, UK), NEYA 10R refrigerate centrifuge (Exacta Optech, Modena, Italy). Chromatographic analysis was performed using an ATVP LC-10 HPV binary pump with an RF-10° XL fluorimetric (FD) detector (Shimadzu, Kyoto, Japan) operating to $\lambda_{emission}$=320 nm, and $\lambda_{excitation}$= 523 nm. A Supelcosil LC-18 column (250 mm × 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) pre-column were used for the determination of BAs.

Sampling

Different Senatore Cappelli durum wheat products were analyzed: seeds, flour, pasta and chaff. The samples were purchased from a cereal farm located in the Campania region, in Italy. After acquisition, all samples were homogenized by grinding and sifting with a 0.7 ÷ 2 mm diameters holes – sieve. The obtained particle size fractions were collected and stored at refrigerated temperature, T= -18 °C until the day of analysis.

Determination of Biogenic Amines

BAs detection was performed according to a previously optimized method with some modifications [13]. Approximately 1 g of durum wheat sample was extracted with 12 mL of 0.6 M $HClO_4$, homogenized with an Ultra-Turrax T-18 tissue homogenizer at 3000x g for 3 min, and centrifuged at 2700×g for 10 min, at T= 25 °C. The supernatant was collected in a flask. The residue was added with 12 mL

of 0.6 M $HClO_4$, mixed, and centrifuged again for 10 min. Then, the second extract was added to the first one and filtered through a syringe filter with a 0.45 µm membrane. The final volume was adjusted to 25 mL with 0.6 M $HClO_4$. An aliquot part of 1 mL of the final extract was then derivatized by adding 200 µL of 2 M NaOH, 300 µL of saturated $NaHCO_3$ solution, and 2 mL of dansyl chloride solution (10 mg/mL in acetone). After stirring, the samples were left in the dark for 60 min at 45 °C. To stop the dansyl-chloride reaction about 100 µL of 25% NH_4OH was added. The final volume was adjusted to 5 mL by adding acetonitrile. The dansylated extract was filtered using 0.45-µm filter (Whatman® Puradisc filters, Sigma Aldrich, Milan, Italy), injected into the HPLC system, and analyzed with a previous standardized method [13].

For the chromatographic detection of BAs content in SCDW samples, a volume aliquot of 20 µL (loop 20 µL) was injected. Analyses were performed by using a Supelcosil LC-18 column (250 mm × 4.6 mm, 5 µm), Supelco, Bellefonte, PA, USA) coupled with an FD detector ($\lambda_{emission}$=320 nm, and $\lambda_{excitation}$=523 nm). The analyses were carried out maintaining a fixed temperature of 30 °C. The solvents used for the chromatographic separation were: (A) purified water and (B) acetonitrile. The elution program started with 3 min of isocratic elution (50% A; 50% B) reaching 100% B after 18 min to finish with a further 3 min of isocratic elution. Finally, it took 5 min to restore the initial isocratic conditions (50% A 50% B). The flow was kept constant at 1.0 mL/min, for a total analysis time of 30 min. The results were obtained through a calibration curve for each BA, ranging from 0.1 and 25 mg/l.

Total Phenolic Content (TPC) and Antioxidant Activity (ABTS and DPPH) evaluation

Sample extraction for TPC and antioxidant activity were carried out according to a previously published method with some modifications [14]. Briefly, 1g of each homogenized durum wheat sample (seeds, flour, pasta, and chaff), was extracted with 4 ml of refrigerated–ethanol (T= 4 °C) in an aqueous solution (EtOH:H_2O 80:20, *v:v*), homogenized in an ultrasonic bath for 5 min at room temperature, and centrifuged at 2800×*g* for 10 min, at T= 4 °C. The supernatant was collected in an amber vial. The residue was added with 4 ml of refrigerated EtOH:H_2O (80:20, *v:v*), homogenized and centrifuged again for 10 min. The second extract was collected with the first one, and adjusted to a final volume of 10 ml with EtOH:H_2O (80:20, *v:v*). The final extracts were then filtered by 0.22 µm membrane syringe filter, and stored at T= -20 °C, until the day of analysis.

The Folin-Ciocâlteu method was used for the determination of TPC [15], some modifications were made for SCDW samples as follows: 1 ml of hydroalcoholic extract was added to 0.25 mL of Folin–Ciocâlteu reagent and 0.5 mL of Na_2CO_3 water solution (7.5%, *w/v*) in a 10-mL volumetric flask. Purified water was added to arrive at the final volume. Spectrophotometric analysis was performed at λ= 750 nm after 30 min of incubation in dark at room temperature. TPC was expressed as milligrams of gallic acid equivalent (GAE) per kg. The results were obtained through a calibration curve ranging from 10 to 100 mg/l (R^2 = 0.9997). Antioxidant activities were determined by means of DPPH and ABTS assays, according to the methods of Preti et al. [15]. Free radical scavenging activity of

SCDW hydroalcoholic extracts was evaluated by measuring the decrease in absorbance at 515 nm (DPPH), and 734 nm (ABTS). The absorbance was measured in 1-cm path length cuvettes against ethanol in aqueous solution (80:20, *v:v*), through a UV-Vis spectrophotometer. Results were expressed as inhibition percentage (I %) and were calculated based on Equation 1:

$$I\% = \frac{Ao - Af}{Ao} \; x \; 100 \qquad (1)$$

where A_0 is the radical cation's initial absorbance, and A_f is the absorbance after the addition of hydroalcoholic SCDW sample extracts.

Results and Discussion

Biogenic Amines Content in Senatore Cappelli Durum Wheat products

Numerous studies showed that BAs concentration in cereal-based products (i.e., flour, pasta, etc.) are largely dependent on varietal features, such as cultivar, pedoclimatic conditions, cultivation, and post-harvest treatment, including wheat milling and processing [6, 8, 16]. In this study, Bas content were determined in SCDW products by means of HPLC-FD. The quantification of BAs in SCDW samples was summarized in Table 1. Among wheat products, the highest total BAs contents were detected in flour (172.72 mg/kg), and pasta (143.08 mg/kg). Among SCDW products, HIS has always been detected with the highest amounts in pasta (36.37 ± 0.01 mg/kg) and flour (17.45 ± 0.02 mg/kg), nevertheless within the limit established by European Regulation 2073/2005 [17]. These processed durum wheat-based products also presented the highest concentrations of SPD (70.71 ± 0.01mg/kg in SCDW flour, and 33.32 ± 0.02 mg/kg in SCDW pasta), and SPM (21.30 ± 0.01 mg/kg in flour, and 10.01 ± 0.01 mg/kg in pasta), respectively. SPD and SPM are natural polyamines, which synthesis is endogenous in all animals and plants, including cereals [18]. Different authors reported that the occurrence of these polyamines is significantly affected by the transformation processes of cereal, such as milling [8, 16]. This could be a positive feature since a high polyamines (SPD and SPM) intake could be linked with a decreased risk of allergic reaction [19].

The lowest amount of TYR, PUT, and CAD was detected in SCDW seeds (0.01 ± 0.01 mg/kg, 0.73 ± 0.01 mg/kg, and 2.25 ± 0.01 mg/kg, respectively), thus enhancing good preservation status [7]. After all, an interesting remark should be done about SER content, which represented the 36% of total BAs detected in all SCDW samples, with the highest amount in pasta (42.66 ± 0.03 mg/kg), and flour (42.17 ± 0.15 mg/kg), followed by chaff (31.84 ± 0.12 mg/kg). Durum wheat seeds presented the lowest content (21.71 ± 0.03 mg/kg). These results agreed with previously published results [8], founding a similar trend in SER content only in durum wheat pasta (40 – 90 mg/kg) and semolina (0 – 130 mg/kg). However, the excellent SER amounts in Senatore Cappelli durum wheat products, may induce some health-increasing effects, such as stress and mood modulation, muscle contraction and blood pressure regulation [8, 20].

Total Phenolic Content and Antioxidant evaluation in Senatore Cappelli Durum Wheat products

Phenolic compounds are considered as important givers to antioxidant activity in durum wheat, due to the presence of hydroxyl groups that react and stabilize free radicals [16]. In this regard, a remarkable highlight has been given to the health-promoting effects of introducing these bioactive components into the daily diet, leading to renewed interest in selecting varieties, including ancient cultivars, for their nutritional potential. Table 2 summarized antioxidant evaluation in SCDW products, by means of Folin-Ciocâlteu reaction, ABTS and DPPH assays. TPC was evaluated by means of Folin-Ciocâlteu assay. This assay establishes the total amount of decreasing substances by measuring the change in color due to the reduction of metal oxides operated by phenolic antioxidants, and other reducing substances, such as nitrogen compounds and proteins [21]. This approach would also provide indication of antioxidant capacity for free or bound substances present in the extracts. Among all samples, durum wheat chaff showed the highest amounts of TPC with 780.35 ± 2.7 mg GAE/kg, followed by SCDW flour and seeds (418.17 ± 1.6 mg GAE/kg, and 415.35 ± 3.2 mg GAE/kg, respectively); while SCDW pasta presented the lowest TPC values (343.61 ± 1.4), showing a 20% reduction in total phenolic content, compared to flour and seeds. De Pula et al., [22]. also showed similar TPC trends in barley flour and pasta, with 808.01 ± 26.45 mg/kg, and 735.9 ± 21,59 mg/kg, thus highlighting a reduction of 9% in TPC content in pasta compared to flour. Compared to cereals, the lower polyphenol content in pasta may be attributable to the pasta production process, which promotes the bond interference between the phenolic compounds and the food matrix components, thus facilitating the extraction of phenolic.

The antioxidant activity was also evaluated by means of free radical scavenging, ABTS and DPPH assays [23]. The trend of TPC resulted in agreement with ABTS radical scavenging activity, proving the highest radical inhibition % (I%) in Senatore Cappelli Durum Wheat chaff (96.57, I%), and in SCDW seeds (66.89, I%). The lowest result was achieved by pasta (12.14, I%). A similar trend was reported by Fares et al., [24], who comparing semolina and durum wheat pasta observed a decrease in phenolic and antioxidant contents. This could be attributable to the oxidative degradation of antioxidants induced by heat treatment, and drying conditions during pasta processing [22]. Nevertheless, the values obtained with DPPH assay were significantly lower than those of ABTS assay, even if the trends of I% among SCDW products were similar: chaff (75.76, I%) > seeds (52.18, I%) > flour (13.46, I%) > pasta (8.17, I%). This could be related to the different scavenging activity of these two *in vitro* antis–radical assays. In fact, ABTS is mainly oxidized by peroxyl radicals and is soluble in both aqueous and organic solvents, thus reacting both with hydrophilic and lipophilic compounds. While DPPH reagent is a stable nitrogen radical that bears no resemblance to the peroxyl radicals involved in lipid peroxidation. Therefore, its reactivity is limited to the lipophilic fraction [25]. The highest presence of antioxidant compounds in chaff could be of interest for the wheat industry, thus valorizing a product destined to become 'waste', giving it the role of a 'new resource' to be reused in other production chains, such as agri-food, cosmetics, pharmaceutical, agro-industrial, environmental sectors, etc. [10].

Table 1
Biogenic amines contents (mg/kg) ± standard deviation values in *Senatore Cappelli* durum wheat products

	SCDW Seeds	SCDW Flour	SCDW Chaff	SCDW Pasta
ß-PEA	*n.d.*	*n.d.*	*n.d.*	*n.d.*
SER	21.71±0.03	42.17±0.15	31.84±0.12	42.66±0.03
TYR	0.01±0.01	*n.d.*	0.56±0.01	3.63±0.16
PUT	0.73±0.01	15.84±0.01	0.75±0.01	5.67±0.16
CAD	2.25±0.01	5.25±0.02	2.33±0.01	11.43±0.36
HIS	3.18±0.02	17.45±0.02	6.61±0.01	36.37±0.01
SPD	0.32±0.01	70.71±0.01	0.08±0.01	33.32±0.02
SPM	0.79±0.01	21.30±0.01	0.67±0.01	10.01±0.01
Total BAs	28.98	172.72	42.83	143.08

β-PEA: β-phenylethylamine; SER: serotonin; TYR: tyramine; PUT: putrescine; CAD: cadaverine; HIS: histamine; SPD: spermidine; SPM: spermine; Total BAs: Total amount of biogenic amines; n.d.: not detectable.

Table 2
The results of evaluation of antioxidant compounds in Senatore Cappelli Durum Wheat (SCDW) products. Values are expressed as mg GAE/kg for TPC, and Inhibition % for ABTS and DPPH assays ± standard deviation

	SCDW Seeds	SCDW Flour	SCDW Chaff	SCDW Pasta
TPC (mg GAE/kg)	415.35 ± 3.2	418.17 ± 1.6	780.35 ± 2.7	343.61 ± 1.4
ABTS (I%)	66.89 ± 0.76	27.44 ± 0.31	96.57 ± 0.12	12.14 ± 0.54
DPPH (I%)	52.18 ± 1.6	13.46 ± 0.11	75.76 ± 0.71	8.17 ± 0.16

TPC: Total Phenolic Content; GAE: Gallic Acid Equivalent; ABTS: diammonium salt; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

Conclusions

The study aimed at assessing the quality of Senatore Cappelli Durum Wheat products (seeds, flour, chaff, and pasta), through the quantitative determination of biogenic amines content and antioxidant properties. The results showed a great variability of BAs content among all samples analyzed. Among SCDW products, the highest total BAs content was detected in processed cereal-based products, flour (172.72 mg/kg), and pasta (143.08 mg/kg, because of their transformation processes. These processed durum wheat-based products also presented the highest concentrations of SPD (33.32–70.71 mg/kg), and SPM (10.01–21.30 mg/kg in flour), thus being associated with a decreased risk of food allergy. Meanwhile, an interesting remark should be made about SER content (21.71 – 42.66 mg/kg), which represented 56% of the total BAs detected in all samples.

Furthermore, the highest TPC and antioxidant compounds in chaff, which represented 51% of the total phenolic content, with 780.35±2.7 mg GAE/kg for TPC and 96.57, ABTS I%, respectively, could be of interest for the wheat industry both for nutritional and technological aspect. Senatore Cappelli Durum Wheat products relatively rich in these beneficial BA could be of interest to wheat producers these bioactive compounds in functional foods are designed to produce certain health benefits (anti-inflammatory, anti-allergenic, mood regulator, etc.). Likewise, enhancing and re-using by-products could be an opportunity for wheat industries to reduce 'waste', giving it the role of a 'new resource' to be reused in other production chains, such as agri-food, cosmetics, pharmaceutical, agro-industrial, environmental sectors, etc.

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3.10 Research article no. 10

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Research macro-area: **Food Quality (FQ), Food Safety (FS)**

Graphical abstract:

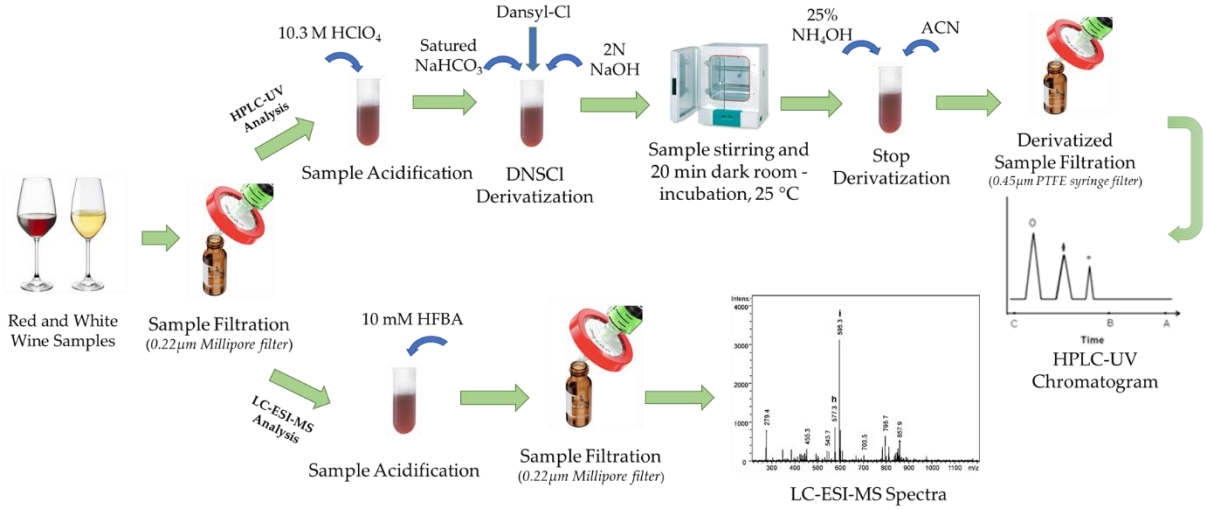

Figure 3.10. Graphical abstract of experimental study no. 10. Source: (Vinci, G.; Maddaloni, L.; and Prencipe, S.A.; Ruggieri, R., 2021)

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Natural Contaminants in Wines: Determination of Biogenic Amines by Chromatographic Techniques

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Abstract: Biogenic amines (BAs) are natural contaminants of wine that originate from decarboxylase microorganisms involved in fermentation processes. The primary relevance of biogenic amines in food could have both toxic effects on consumers' health (i.e., allergic reactions, nausea, tremors, etc.), if present at high concentrations, and concurrently it can be considered as a remarkable indicator of quality and/or freshness. Therefore, the presence of nine biogenic amines [Tryptamine (TRP), ß-phenylethylamine (ß-PEA), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonin (SER), tyramine (TYR), spermidine (SPD), and spermine (SPM)] was investigated in red and white wine samples, which differed in the winemaking processes. The qualitative-quantitative determination of BAs was carried out by chromatographic methods (HPLC-UV/Vis and LC-ESI-MS). The analysis showed that both winemaking processes had all the nine BAs considered in the study at different amounts. Data showed that red wines had a higher concentration of PUT (10.52 mg L^{-1}), TYR (7.57 mg L^{-1}), and HIS (6.5 mg L^{-1}), the BAs most involved in food poisoning, compared to white wines, probably related to the different type of fermentation (alcoholic and malolactic).

Keywords: contaminants; red wines, white wines; food quality; biogenic amines; winemaking processes; food safety; microorganisms; alcoholic fermentation, malolactic fermentation

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1. Introduction

Biogenic amines (BAs) are a class of organic, basic, and low-molecular weight compounds with heterocyclic (histamine, tryptamine), aliphatic (spermine, spermidine, putrescine and cadaverine) and aromatic (tyramine, phenylethylamine) structures [1,2]. They can be endogenous or exogenous in plants, as well as in animal and microorganisms, where they play, at lower concentration, an important role in physiological and metabolic functions—e.g., membrane stabilization, nucleic acid regulation, and protein synthesis [3]. The most amines occurring in food originate from proteolytic processes that make available large quantities of amino acids, which are the ideal substrate for enzymatic decarboxylation reactions. In addition, BAs may also be synthetized from the amination and transamination of aldehydes and ketones by the amino-acetic transaminases. BAs can be synthesized both in perishable and fresh food (i.e., fruits and vegetables, meat, fish, etc.), which are exposed to decarboxylase-positive microorganisms [4], as well as in fermented and/or processed food (i.e., wine, beer, coffee, chocolate, etc.), as a direct consequence of their transformation process—e.g., alcoholic and malolactic fermentation. The primary relevance of biogenic amines in food could have both toxic effects on consumers' health, if present at high concentrations, and concurrently it can be considered as a remarkable indicator of quality and/or freshness. From the toxicological point of view, BAs have been widely investigated as human harmful compounds, since their excessive food-mediated intake and a reduced or absent catabolism may induce symptoms that are

similar to those of food poisoning: migraine headaches, gastric disorders, nausea, cardiac palpitations, and psychoactive effects [4,5]. The toxic effect most attributable to the ingestion of BAs is scombroid syndrome (Scombrotoxin Fish Poisoning, SFP), also called "Histamine poisoning", as this amine is the one responsible for this intoxication. However, the other BAs enhance the effects of histamine. Therefore, the toxicity of BAs in food or beverages is mainly due to a synergistic effect of several BAs [5]. Table 1 shows the physiological and pathological effects of BAs on human health. Below physiological conditions, BAs can be metabolized by three different enzymes, present in the gastrointestinal tract [diamino oxidase (DAO), monoamine oxidase (MAO), and histamine-N-methyltransferase (HMT)], which have the function of inactivating BAs by oxidizing the amino groups. Through these three enzymes, the human body has the ability to inactivate BAs that are normally taken with food and beverages [4,6]. Furthermore, in alcoholic beverages, it has been shown that the presence of ethanol and acetaldehyde, its catabolite, inhibits the enzymatic activity of BAs detoxifying enzymes (DAO, MAO, and HMT) and increases the biogenic amines permeability in the gastrointestinal wall, consequently increasing its toxic effects. This aspect is of relevant importance in alcoholic beverages, such as wine, which are eaten on its own or paired with other foods containing high concentrations of BAs (i.e., cheese, fish, meat, etc.) [7].

However, the toxic effects of BAs are dose-dependent, and the severity of the toxicity response is also influenced by personal sensitivity to these compounds. The symptoms, similar to those of food poisoning (nausea, vomiting, respiratory dysfunctions, itching, skin rash, etc.), have a variable duration between 8–12 h and they can therefore occur with more or less serious consequences even in subjects with a correct functioning of the enzymatic activity [4,8]. The daily dose of BAs acceptable to the human body is not yet known, as the toxic effects of the individual BAs are correlated and enhanced by their co-presence in food. Based on this, the EFSA (European Food Safety Authority) has defined that the dose, referred to histamine, for which the human body is able to activate the defense mechanisms is equal to about 25–50 mg and that the poisoning occurs following the intake of BAs equal to about 70–300 mg.

However, although their potential toxicity is known, to date, there is no legislation that allows for limiting the sale of products with high BA content. Currently, European legislation regulates only the presence of histamine in fish and fishery products (Reg. 2073/2005), while, as regards wine, only some European Countries (Germany, France, The Netherlands, Belgium, and Austria), arbitrarily, they proposed limits for histamine, ranging from 2 to 10 mg L^{-1} [5].

The formation of BAs therefore presupposes the co-presence of three factors: (i) a precursor, that is a specific amino acid for each specific BAs; (ii) contaminating microorganisms with decarboxylase activity; (iii) favorable environmental conditions (i.e., pH, temperature, water activity, etc.). Wine is an excellent substrate for BAs synthesis while ensuring the presence of amino acids, microbial populations with decarboxylating activity and a generally favorable environment for microorganism growth. Therefore, the BAs determination in wines is not only important to safeguard the consumers' health, but also for food quality assessment, because the presence of biogenic amines may influence the organoleptic characteristics of the finished product. The winemaking processes (Figure 1), which occur following different biochemical and metabolic pathways by microorganisms (bacteria, fungi, and yeasts), can lead to the formation of BAs [2,6].

Table 1. Physiological and pathological effects of the major BAs in food.

Biogenic Amines	Amino Acid Precursor	Physiological Effects	Pathological Effects	Ref.
Histamine	Histidine	Release of adrenaline and noradrenaline, Allergic processes, Stimulation of the smooth muscles of the uterus, intestine, and respiratory tract, Stimulation of sensory and motor neurons Control of gastric secretion	Allergic reaction (nausea, burning in the mouth, flushing of the face and body, abdominal cramps, diarrhea, swelling of the face and tongue)	[9–13]
Tyramine	Tryptamine	Peripheral vascularization Increase in cardiac output Increased lacrimation and salivation Increased breathing Increased blood sugar levels Noradrenaline release of the sympathetic nervous system Migraine	High blood pressure, Rapid heart rate, Tremors, Seizures, Hyperthermia	[11,14–17,18]
Putrescine	Ornithine	Hypotension Bradycardia Lockjaw Extremity paralysis	Cytotoxicity, Rule in tumors growth, Enhancement of the toxicity of other amines	[11,18–20]
Cadaverine	Lysine			
Tryptamine	Tryptophan	Increase in blood pressure	Relaxations, Mild euphoria, Hallucinogens	[15,21,22]
ß-Phenylethylamine	Phenylalanine	Noradrenaline release of the sympathetic nervous Increase in blood pressure Migraine	Migraine	[11]
Spermidine	Methionine	Hypotension, Bradycardia	Acute decrease in blood pressure, Respiratory symptoms, Nephrotoxicity, carcinogenesis, tumor invasion, and metastasis, Enhancement of the toxicity of other amines	[15,23–26]
Spermine				
Serotonin	Tryptophan	Modulation of anger, aggression, mood and sexuality, appetite, Physiological homeostasis Muscle contraction Blood pressure regulation	Altered behavior and neurochemical activities, cognitive decline, muscular inflammation, and immune activation	[24,25]

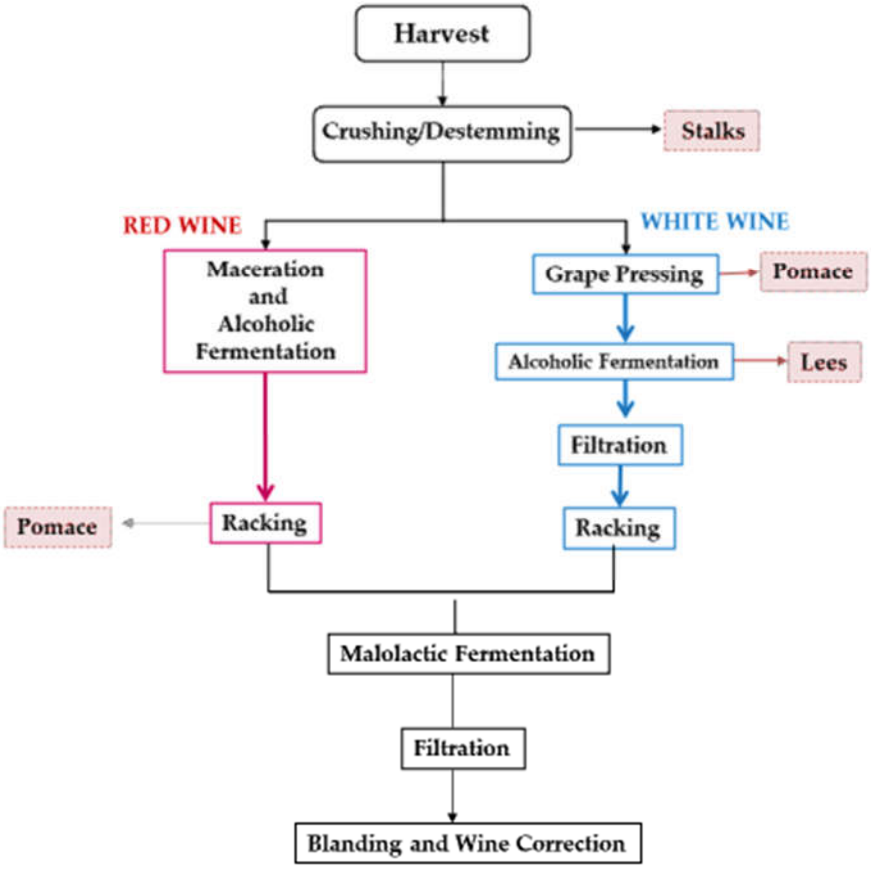

Figure 1. White and red vinification process**.**

The presence of these compounds in wine occurs at different points in the winemaking process. Especially, it can be influenced by the conservation conditions of grapes bunches, by their degree of ripeness and by the pedoclimatic conditions in which the wine is cultivated. The main step involving the BAs formation is the fermentation phase, in fact, depending on the type of microorganism involved and the type of fermentation (alcoholic and malolactic), not only the concentration but also the type of BAs present can be influenced in the finished product [1]. Table 2 shows the main microorganisms involved and the main BAs synthesized for the two types of fermentation.

Two different winemaking processes, red and white ones, were taken into consideration as they are the most representative wine classes of the Italian market. In this study, to evaluate safety and quality of wines samples, nine biogenic amines were analyzed (TRP, HIS, TYR, β-PEA, CAD, PUT, SER, SPD, and SPM) and the BA profile was studied to identify the difference between red and white wines. The content of BAs has been analyzed by two chromatographic methods (HPLC UV/Vis and LC-ESI-MS).

Table 2. Biogenic amines and microorganisms involved in wine fermentation processes.

Fermentation	Microorganism	Biogenic Amines	Ref.
Alcoholic	Spontaneous	HIS, MET, ETH, TYR, β-PEA, PUT, CAD, SPD, SPM, AGM	[8,26–33]
	Saccharomyces cerevisiae	AGM, ETA, ETH, PUT, TYR, CAD, β-PEA, HIS	[15,30]
	Dekkera/B. bruxellensis	ETA; MET; AGM; TRY; β-PEA; PUT; CAD; HIS; SPM	[28,29]
	Kloeckeraapiculata; Candida stellata; Metschnikowiapulcherrima	ETA; MET; AGM; TRY; β-PEA; PUT; CAD; HIS	[28]
	Kluyveromycesthermotolerans; Schizosaccharomyces pombe V2. Selected S. pombe; Non-Selected S. pombe	HIS; TYR; β-PEA; PUT; CAD	[31,32]
Malolactic	Spontaneous, *Oenococcus oeni, L. plantarum* DSM 4361; Yeast	PUT; SPD; SPM; AGM; CAD; SER; HIS; TYR; β-PEA	[8,33–35]
	Commercial malolactic bacteria	HIS; MET; ETH; TYR; β-PEA; PUT; CAD	[36]

HIS = Histamine; MET = Methionine; ETH = Ethylamine; TYR = Tyramine; β-PEA = β-Phenylamine; PUT = Putrescine; CAD = Cadaverine; AGM = Agmatine; ETA = Ethanolamine; TRP=Tryptamine; SPD = Spermidine; SPM = Spermine; SER = Serotonin.

2. Materials and Methods

2.1. Chemicals

Tryptamine (TRP), ß-phenylethylamine (ß-PEA), putrescine (PUT), cadaverine (CAD), histamine (HIS), serotonin (SER), tyramine (TYR), spermidine (SPD), and spermine (SPM) with a purity of 99%, the internal standard 1.7 diaminoheptane (IS), derivatizying agent (dansyl chloride) and heptafluorobutyric acid (HFBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) used for derivatization were purchased by Sigma-Adrich (Milan, Italy). The following solutions were used for the sample preparation and the chromatographic determination of the compounds: Acetonitrile (HPLC grade, Merck, Darmstadt, Germany), Methanol (Carlo Erba Reagenti, Milan, Italy), Perchloric acid ($HClO_4$) 65% (Merck,Darmstadt, Germany), Ammonium hydroxide (NH_4OH) 25% (Carlo Erba Reagenti), distilled water purified using a Milli- Q system (Millipore, Bedford, MA, USA) and ultrapure water (Millipore, USA).

2.2. Instruments

The following instruments were used: Sartorius model 1712 analytical balance, ALC 4236 centrifuge, homogenizer Universal Laboratory Aid MPW-309, Bandelin Sonorex RK100H water and ultrasonic thermostatic bath, FALC model F60 magnetic stirrer, Whatman 0.20 μm 100 (PTFE) syringe filters, Sigma Aldrich (Milan, Italy). Chromatographic analysis was performed using an ATVP LC-10 HPV binary pump with an SPD-10AVP UV detector (Shimadzu, Kyoto, Japan) operating to λ = 254 nm. A Supelcosil LC-18 column (250 mm × 4.6 mm, 5 μm) with a Supelguard LC-18 (Supelco, Bellefonte, PA, USA) precolumn was used for the determination of BAs. The following instrumentation was used for the analysis with the LC-ESI-MS system: Thermoquest (Manchester, UK) model P2000 with Alltima column (Alltech, IL, USA) C18 in reverse phase (250 mm × 4.6 mm id, dimension of particles 5 μm). Regarding mass spectrometry, the Finnigan AQA single quadrupole bench-top mass spectrometer was used. Instrument control, data acquisition and

processing were carried out with Mass Lab (version 2.22) of Thermoquest Finnigan (Manchester, UK).

2.3. Standard Solution

For each BAs (TRP, ß-PEA, PUT, CAD, HIS, SER, TYR, SPD and SPM), individual standard solutions were prepared at 1 mg mL^{-1} in purified water and kept in the dark at 4 ± 1 °C. In addition, the standard solution containing all nine BAs (MIX 9) was obtained with 1 mL of each standard solution of the individual BAs and diluted in 25 mL with purified water. Different aliquots of these standard solutions were applied to obtain the standard solutions necessary for the construction of the calibration curves and for the execution of recovery experiments. The standard solutions were added with HFBA in order to obtain a final solution with an acid concentration of 5 mM. The concentrations of BAs injected for the construction of the calibration lines were 0.1, 0.4, 0.8, 4.0, 8.0, and 16.0 mg L^{-1}. Furthermore, all solutions contained the IS, at the same concentration of 0.8 mg L^{-1}.

2.4. Samples

Forty-four wine samples (24 white wines and 20 red wines) produced in different regions of Italy were purchased in local supermarkets. The wine samples were stored at room temperature and protected from light until the day of analysis. The wine samples were chosen to be as representative as possible of the Italian wine market and of the red and white winemaking processes.

2.5. Biogenic Amines Extraction

The extraction of biogenic amines from the wine samples was carried out by applying the method described in a previous article [37]. The wine samples were previously filtered using a 0.20 μm Millipore membrane filter. Subsequently, for HPLC-UV/Vis analysis, 10.3 M $HClO_4$ was added to 25 mL of the filtered wine samples to obtain an acid solution at 0.2 M. Instead, for LC-ESI-MS analysis, HFBA was added to 25 mL of the filtered wine samples to obtain an acid solution at 5 mM. After a second filtration, an aliquot of 50 μL of the wine samples was injected into a chromatographic column.

2.6. HPLC-UV/Vis Method

Before performing the HPLC-UV/Vis analysis, the samples were subjected to derivatization, obtained through the use of dansyl-chloride [5-(dimethylamino) naphtalene1-sulfonyl chloride]. The derivatization reaction was carried out by adding 200 μL of 2N NaOH, 300 μL of saturated $NaHCO_3$ solution, and 2 mL of dansyl-chloride solution (15 mg mL^{-1} in acetone) to 1 mL of extract. After stirring, the samples were left in the dark at room temperature for 20 min. To stop the reaction, 100 mL of 25% *v*/*v* NH_4OH are added at the end and the final volume was brought to 5 mL with acetonitrile. The derivatized sample was subsequently filtered with 0.45 μm PTFE syringe filter. For the chromatographic determination of the BAs, a volume aliquot of 50 μL (loop 50 μL) was injected. Analyses were performed by use of a Supelcosil LC-18 column (150 mm × 4.6 mm, 5 μm), Supelco, Bellefonte, PA, USA) coupled with an UV detector (254 nm). The analyses were carried out maintaining a fixed temperature of 25 °C. The solvents used for the chromatographic separation were: (A) water purified and (B) acetonitrile. The elution program started with 3 min of isocratic elution (50% A; 50% B) reaching 10% A and 90% B after 20 min to finish with a further 3 min of isocratic elution. Finally, it took 4 min to restore the initial isocratic conditions (50% A 50% B). The flow was kept constant at 1.2 mL/min, for a total analysis time of 35 min.

2.7. LC-ESI-MS Method

During the LC-ESI-MS analyses, the ESI unit operated at 4.0 kV, the capillary was heated to 200 °C and as desolvation gas (300 L/h), as well as for the nebulizer (5 L/now), nitrogen was used. The ESI-MS system was set up to operate in positive ionization (PI) mode. Diagnostic fragment ions were obtained by in-source collisop-induced dissociation (CID) of the protonated molecule $[M + H]^+$ after optimization of the skimmer cone voltage. Selected Ion Monitoring (SIM) was applied for scheduled analyte recording. The mobile phase solvents, which were applied for LC-ESI-MS analysis, were (A) methanol (10 mM heptafluorobutyric acid) and (B) water (10 mM heptafluorobutyric acid), respectively. The mobile phase flow was set at a flow rate of 1 mL/min. The column was kept at room temperature and the eluted analytes using an initial linear gradient program from 10% solvent A to 85% in 15 min, then going from 85% solvent A to 100% in 1 min., followed by a 100% isocratic elution of A for 3 min. An additional 10 min have been added to reach initial conditions. The injected volume, both of standard solutions and of samples, was 50 μL. The data acquisition parameters are shown in Table 3.

Table 3. Data acquisition parameters used in LC-ESI-MS for BA detection.

Biogenic Amines	MW	Channel, m/z (Relative Abundance)	Cone Voltage (V)	Retention Window (min)
Tyramine	137.2	121.2 (30), 138.3 (100)	30	0–12.85
ß-Phenylethylamine	121.2	105.1 (10), 122.3 (100)	30	12.85–16.00
Putrescine	88.2	89.3 (100)	40	0–12.85
Cadaverine	102.2	86.2 (10), 103.3 (100)	30	0–12.85
Histamine	111.1	95.2 (30), 112.1 (100)	40	0–12.85
Serotonin	176.2	160.3 (10), 177.2 (100)	40	0–12.85
Tryptamine	160.2	144.3 (40), 161.2 (100)	30	12.85–16.00
Spermidine	145.2	112.3 (10), 129.2 (10), 146.3 (100)	40	12.85–16.00
Spermine	202.3	129.2 (20), 112.3 (10), 203.4 (100)	40	12.85–16.00

2.8. Descriptive Analysis

All measurements were conducted in triplicate. The data obtained were analyzed mathematically and graphically using Microsoft Excel (Microsoft, Redmond, DC, USA). Data were expressed as mean ± standard deviation (SD). ANOVA tests were performed, and significantly different means were compared with the Turkey's pairwise test ($p < 0.05$) for all data collected. In addition, PCA (Principal Component Analysis) was applied to highlight a natural grouping of winemaking samples depending on their BA amounts. All the computations were performed using R-based Chemometrics Software (http://www.gruppochemiometria.it/index.php/software/19-download-the-r-based-chemometric-software, accessed on 15 July 2021).

3. Results and Discussion

3.1. Wine Samples Analysis by HPLC-UV/Vis and LC-ESI-MS

HPLC-UV/Vis and LC-ESI-MS methods were optimized for the detection of biogenic amines in red and white wine samples. Scheme of BAs isolation from wine samples is shown in Figure 2. Both for HPLC-UV/Vis and LC-ESI-MS determination, wine samples were previously filtered with 0.22 μm Millipore filters and then acidified with 10.3 M $HClO_4$ and 5 mM HFBA, respectively (Section 2.5.).

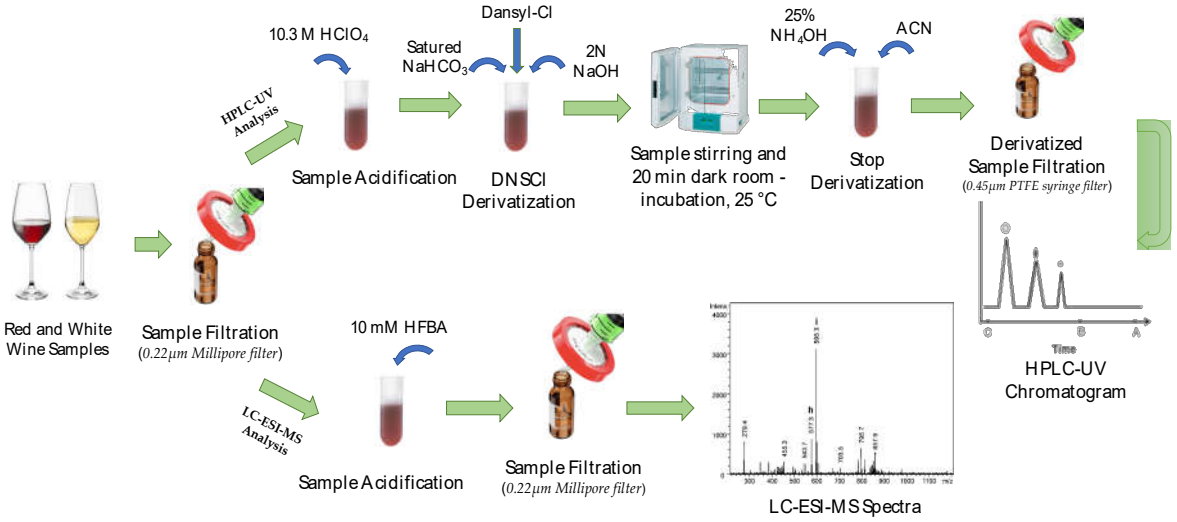

Figure 2. Scheme of biogenic amines determination in wine samples by HPLC-UV and LC-ESI-MS Analysis.

3.1.1. Optimization and Performance Characteristics of the HPLC-UV/Vis Method

Before BA determination, the derivatization conditions were optimized as reported in Vinci et al. [38] and Vinci et al. [39]. Three different parameters have been optimized: pH, temperature, and time of reaction. The method optimization conditions are reported in supplementary materials. The performance characteristics of the HPLC-UV/Vis method are shown in Table S1. In Figure S1A, a chromatographic plot of the BA standard solution is shown. Calibration curves of nine biogenic amines are shown in Figure S1B.

3.1.2. Optimization and Performances of the LC-ESI-MS Method

Initially, the LC-ESI-MS method was performed to investigate the fragmentation behavior of the nine BAs, based on their mass/charge ratio. In order to carry out this evaluation, standard solutions of the single column-less BAs were injected in full scan mode. It was shown that these analytes, having a low relative molecular mass, split into a very small number of fragments [38,39]. The optimized LC-ESI-MS conditions to obtain the maximum fragments are summarized in Figure S2A,B.

To evaluate the performance of the method, linearity was taken into consideration, which was evaluated using standard solutions of the 9 BAs in 5mM HFBA acid solution [38,39]. The test results are summarized in Table S2.

3.2. Biogenic Amines Determination in Wine Samples

Forty-four wine samples (24 white and 20 red) were analyzed using both HPLC-UV and LC-ESI-MS methods under the selected experimental conditions. Three replicates were performed for each determination. Table 4 shows the BA amounts and their total concentration obtained for each wine sample. By comparing the reported values of the total BA amounts, it resulted in the total concentrations of BAs being much higher in red wines than in white wines. Data, showed in Figure 3A,B, can be explained by the fact that red wines are generally less acidic than white wines, and it is known in literature that BAs are produced in high quantities at high pH [27]. Furthermore, high values of BAs in wines are not only related to high pH values but also to the complexity of the bacterial microflora. Optimal growth conditions and greater bacterial diversity are mainly observed in red wines, which, therefore, show a higher content of BAs [8]. The significant differences observed in the content of BAs reported for the samples (Table 4) are probably attributable

to the fact that the presence of BAs in wines is strongly dependent on different winemaking processes, which are characterized by different pH values of wines, the duration of fermentation, the aging time, and the soil and climatic conditions under which the wines are grown [30]. The data obtained show a high PUT content in both white (nd-4.22 mg L^{-1}) and red (nd-10.52 mg L^{-1}) wines. In particular, it was shown that red wines also had high concentrations of HIS (nd-7.57 mg L^{-1}) and TYR (nd-6.59 mg L^{-1}). Although these two amines have physiological functions, their excessive intake can cause food poisoning in consumers. Furthermore, their toxic effect is enhanced by the simultaneous intake of ethanol and its catabolites present in wines. Therefore, it is essential to determine HIS and TYR simultaneously, as they present a high risk of causing toxic effects due to their vasoactive and psychoactive properties [14]. Red wines also have a higher content of TRP (nd-2.50 mg L^{-1}) and SER (nd-3.80 mg L^{-1}), compared to white wines which are almost absent. This is probably due to the fermentation processes to which the grapes are subjected; in fact, it has been seen that wines that also undergo malolactic fermentation have higher concentrations of BAs [27].

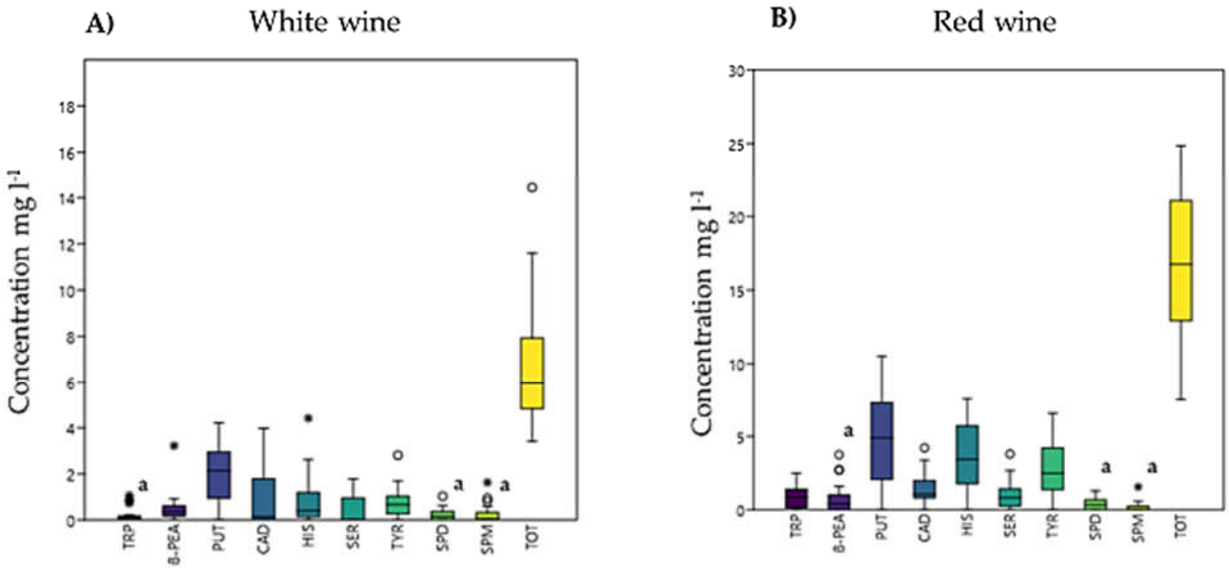

Figure 3. BoxPlot of Biogenic amines amount in white (**A**) and red (**B**) wine. Values are the mean sum of all samples. Bars indicate the minimum and maximum values of the BA amount. Mean values with the same letters are not significantly different according to the ANOVA test ($p < 0.05$).

The analysis of variance (ANOVA) showed significant differences ($p < 0.05$) among individual and total BA values. For this reason, all the biogenic amines are considered for multivariate analysis.

Table 4. Biogenic amines (mg L^{-1}) amount in white and red wines.

Wine	Sample	TRP	ß-PEA	PUT	CAD	HIS	SER	TYR	SPD	SPM	Total BAs
WHITE	Wine 1	0.72 ± 0.03	0.13 ± 0.17	0.90 ± 0.13	1.54 ± 0.31	2.61 ± 0.05	0.55 ± 0.20	nd	1.03 ± 0.08	0.94 ± 0.01	9.74 ± 0.98
	Wine 2	nd	nd	1.17 ± 0.19	1.79 ± 0.19	nd	1.78 ± 0.11	0.57 ± 0.09	0.62 ± 0.16	nd	6.49 ± 0.74
	Wine 3	0.23 ± 0.02	0.16 ± 0.09	1.83 ± 0.11	2.76 ± 0.23	1.52 ± 0.07	0.96 ± 0.15	2.81 ± 0.04	nd	0.41 ± 0.04	11.59 ± 0.75
	Wine 4	nd	0.24 ± 0.01	nd	0.51 ± 0.21	nd	0.34 ± 0.02	nd	0.52 ± 0.08	nd	7.37 ± 0.32
	Wine 5	0.16 ± 0.05	3.22 ± 0.16	1.40 ± 0.12	3.97 ± 0.31	**4.42 ± 0.40**	nd	0.32 ± 0.11	0.42 ± 0.07	0.29 ± 0.02	14.44 ± 1.32
	Wine 6	nd	nd	1.70 ± 0.15	2.73 ± 0.26	0.14 ± 0.03	1.05 ± 0.13	0.26 ± 0.09	nd	0.22 ± 0.10	6.87 ± 0.76
	Wine 7	0.83 ± 0.02	nd	0.89 ± 0.12	nd	nd	0.95 ± 0.20	0.10 ± 0.03	0.21 ± 0.04	nd	5.16 ± 0.49
	Wine 8	0.89 ± 0.15	0.85 ± 0.17	0.86 ± 0.19	2.36 ± 0.24	0.51 ± 0.08	1.37 ± 0.13	0.67 ± 0.18	nd	0.58 ± 0.13	9.03 ± 1.14
	Wine 9	nd	0.92 ± 0.23	**2.57 ± 0.34**	nd	2.28 ± 0.29	1.37 ± 0.21	0.24 ± 0.09	0.26 ± 0.05	1.63 ± 0.14	9.79 ± 1.35
	Wine 10	1.07 ± 0.17	0.66 ± 0.12	0.96 ± 0.25	1.80 ± 0.12	nd	nd	0.21 ± 0.02	nd	0.80 ± 0.21	6.76 ± 0.89
	Wine 11	nd	0.88 ± 0.07	0.65 ± 0.21	nd	1.49 ± 0.16	nd	0.88 ± 0.14	0.52 ± 0.11	nd	5.02 ± 0.69
	Wine 12	nd	0.27 ± 0.04	2.95 ± 0.33	nd	1.10 ± 0.24	0.88 ± 0.12	1.04 ± 0.18	0.35 ± 0.03	nd	7.57 ± 0.94
	Wine 13	nd	0.41 ± 0.07	2.51 ± 0.27	nd	0.16 ± 0.01	nd	0.33 ± 0.09	nd	nd	3.41 ± 0.44
	Wine 14	0.02 ± 0.01	0.33 ± 0.09	**3.51 ± 0.34**	nd	0.41 ± 0.11	nd	0.85 ± 0.27	0.10 ± 0.03	0.03 ± 0.01	5.25 ± 0.86
	Wine 15	nd	0.24 ± 0.08	2.81 ± 0.39	0.32 ± 0.11	0.50 ±0.17	nd	0.67 ± 0.12	0.15 ± 0.02	0.05 ± 0.02	4.74 ± 0.91
	Wine 16	nd	0.38 ± 0.15	2.70 ± 0.41	0.30 ± 0.09	0.40 ± 0.13	nd	1.00 ± 0.09	0.08 ± 0.02	nd	4.86 ± 0.89
	Wine 17	nd	0.22 ± 0.02	3.10 ± 0.23	0.08 ± 0.03	0.22 ± 0.04	nd	0.88 ± 0.14	0.15 ± 0.05	0.05 ± 0.01	4.70 ± 0.52
	Wine 18	nd	0.60 ± 0.21	2.96 ± 0.32	nd	0.45 ± 0.10	nd	1.20 ± 0.23	0.18 ± 0.09	0.06 ± 0.02	5.45 ± 0.97
	Wine 19	0.03 ± 0.01	0.58 ± 0.18	**4.22 ± 0.39**	nd	0.50 ± 0.13	nd	1.38 ± 0.28	0.22 ± 0.06	0.08 ± 0.02	7.01 ± 0.78
	Wine 20	0.04 ± 0.01	0.15 ± 0.07	3.03 ± 0.44	nd	0.27 ± 0.03	nd	1.69 ± 0.12	0.08 ± 0.03	nd	5.26 ± 0.70
	Wine 21	nd	0.55 ± 0.11	2.41 ± 0.21	nd	0.25 ± 0.07	nd	0.34 ± 0.06	0.12 ± 0.07	0.20 ± 0.05	3.87 ± 0.57
	Wine 22	0.10 ± 0.02	0.42 ± 0.21	1.87 ± 0.19	0.20 ± 0.04	0.13 ± 0.02	nd	1.03 ± 0.17	nd	0.10 ± 0.03	3.85 ± 0.68
	Wine 23	1.28 ± 0.11	1.58 ± 0.27	2.09 ± 0.23	nd	1.85 ± 0.25	2.41 ± 0.35	1.37 ± 0.22	nd	0.51 ± 0.11	12.26 ± 1.54
	Wine 24	0.77 ± 0.21	2.75 ± 0.38	2.76 ± 0.27	4.22 ± 0.43	**3.25 ± 0.35**	nd	**3.71 ± 0.35**	0.20 ± 0.02	0.17 ± 0.05	18.82 ± 2.16
RED	Wine 25	nd	nd	1.57 ± 0.21	1.75 ± 0.19	0.51 ± 0.21	1.51 ± 0.17	0.38 ± 0.09	0.33 ± 0.05	nd	8.00 ± 0.92
	Wine 26	nd	2.68 ±0.37	**3.39 ± 0.47**	nd	**6.51 ± 0.52**	0.80 ± 0.13	**6.59 ± 0.59**	0.72 ± 0.31	0.39 ± 0.15	24.31 ± 2.54
	Wine 27	nd	nd	1.65 ± 0.12	1.90 ± 0.23	1.23 ± 0.17	2.57 ± 0.37	nd	nd	nd	8.41 ± 0.89
	Wine 28	0.47 ± 0.12	0.29 ± 0.01	2.47 ± 0.19	1.84 ± 0.19	nd	3.80 ± 0.31	**2.68 ± 0.29**	0.42 ± 0.12	0.56 ± 0.11	14.11 ± 1.34
	Wine 29	1.41 ± 0.17	3.75 ± 0.38	**7.59 ± 0.56**	2.91 ± 0.31	**3.10 ± 0.39**	nd	1.99 ± 0.21	nd	nd	24.25 ± 2.02
	Wine 30	1.83 ± 0.21	nd	1.52 ± 0.11	2.25 ± 0.21	1.52 ± 0.21	2.66 ± 0.19	1.20 ± 0.32	0.58 ± 0.21	0.30 ± 0.07	13.14 ± 1.53
	Wine 31	nd	0.35 ± 0.07	nd	2.80 ± 0.31	nd	0.84 ± 0.23	**2.22 ± 0.27**	0.55 ± 0.12	1.56 ± 0.23	7.51 ± 1.23
	Wine 32	0.80 ± 0.21	nd	1.96 ± 0.37	3.37 ± 0.27	**3.61 ± 0.39**	0.33 ± 0.11	0.86 ± 0.18	nd	nd	12.09 ± 1.53
	Wine 33	0.15 ± 0.13	nd	**4.42 ± 0.21**	0.83 ± 0.21	2.22 ± 0.37	nd	**5.19 ± 0.45**	1.00 ± 0.13	0.21 ± 0.04	14.02 ± 1.54

Wine 34	2.49 ± 0.36	1.06 ± 0.31	**10.52 ± 1.23**	1.09 ± 0.19	**7.57 ± 1.05**	0.80 ± 0.24	1.33 ± 0.16	nd	nd	24.86 ± 3.54
Wine 35	0.10 ± 0.02	0.34 ± 0.10	**5.88 ± 0.41**	1.10 ± 0.29	**3.25 ± 0.57**	1.40 ± 0.35	**4.28 ± 0.74**	0.77 ± 0.21	nd	17.12 ± 2.69
Wine 36	1.05 ± 0.19	nd	**5.23 ± 1.23**	nd	**4.54 ± 0.87**	nd	**3.38 ± 0.28**	1.28 ± 0.38	nd	15.48 ± 2.95
Wine 37	0.85 ± 0.25	0.75 ± 0.18	**6.76 ± 0.97**	0.75 ± 0.43	**4.03 ± 1.31**	0.95 ± 0.23	**2.29 ± 0.19**	nd	nd	16.38 ± 3.56
Wine 38	1.24 ± 0.29	0.93 ± 0.23	**8.54 ± 1.25**	0.93 ± 0.27	**5.61 ± 0.98**	1.23 ± 0.24	**2.87 ± 0.24**	0.57 ± 0.15	0.08 ± 0.01	22.00 ± 3.66
Wine 39	2.50 ± 0.12	0.18 ± 0.09	**7.22 ± 1.11**	0.81 ± 0.19	**7.11 ± 1.23**	1.00 ± 0.36	**4.61 ± 0.46**	0.33 ± 0.29	nd	23.76 ± 3.05
Wine 40	1.64 ± 0.23	0.24 ± 0.06	**6.66 ± 1.03**	0.88 ± 0.35	**3.93 ± 0.59**	0.78 ± 0.20	**3.70 ± 0.34**	0.24 ± 0.16	0.05	18.12 ± 2.96
Wine 41	0.57 ± 0.12	0.54 ± 0.24	**10.04 ± 1.07**	1.15 ± 0.37	**6.06 ±0.65**	0.84 ± 0.24	1.55 ± 0.37	nd	nd	20.75 ± 3.06
Wine 42	1.33 ± 0.34	0.61 ± 0.31	**4.97 ± 0.87**	0.89 ± 0.41	2.90 ± 0.37	nd	**1.90 ± 0.23**	1.11 ± 0.14	nd	13.71 ± 2.9
Wine 43	0.88 ± 0.27	0.47 ± 0.25	**8.32 ± 0.77**	1.05 ± 0.54	**3.80 ± 0.59**	0.54 ± 0.16	**5.05 ± 0.53**	0.66 ± 0.13	nd	20.77 ± 3.24
Wine 44	1.42 ± 0.25	1.00 ± 0.15	**4.83 ± 0.44**	0.80 ± 0.26	**6.28 ± 0.35**	0.60 ± 0.20	**4.20 ± 0.32**	nd	nd	9.13 ± 1.97

Data were expressed how means ± standard deviation; nd = not detectable. Bolds: biogenic amines with a high concentration in the analyzed samples.

PCA analysis on the samples was performed to view the dataset in a reduced size and to evaluate the data matrix to highlight natural sample grouping. Figure S3A shows the loading plot of the nine amines and the total concentration of BAs in the samples, while the score plot (Figure S3B) highlights the similarities and differences between the different wine samples taken into consideration. After autoscaling, two significant components were identified equal to 37.5% and 16.1% of the variance respectively for PC1 and PC2.

To better underline which biogenic amine mostly influenced the two categories of wine, the Biplot was carried out (Figure 4). It results in white wines being grouped in the negative quadrants (on the left) compared to PC1 while red wines are grouped mainly in the positive quadrants; this is explained by the fact that red wines weigh most on the presence of HIS, TYR, and PUT. This distinction that occurs in the two types of wine highlighted how red wines are the category of wine that can pose more risks to human health, as the combined presence of these BAs can lead to the risk of food poisoning. For this reason, and even though no official limit has yet been decided, some Countries, to protect the health of consumers, have established legal or recommended limits for histamine concentrations in wine [5].

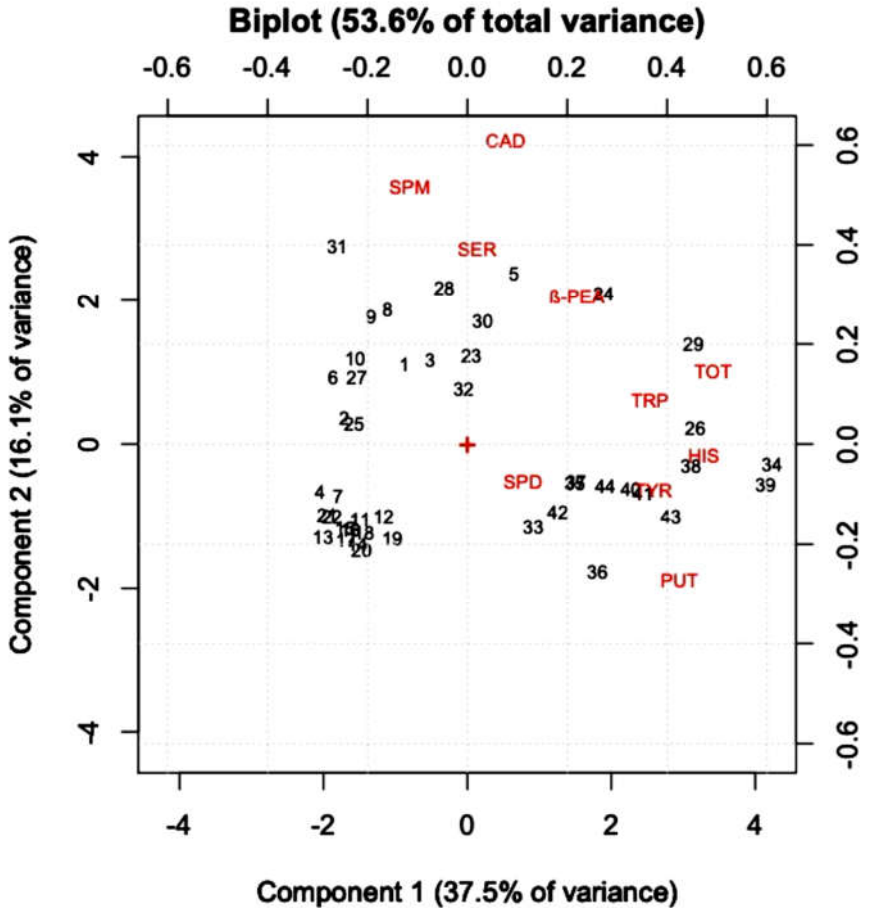

Figure 4. Biplot of white and red wines of Italian origin.

4. Conclusions

The determination of nine biogenic amines in the white and red wine samples was carried out by applying two chromatographic methods. The LC-ESI-MS analysis offers the advantage of being a fast and reliable method for the qualitative-quantitative analysis of non-derivatized BA. This allows for identifying the presence of BA in wine samples

more quickly. This is essential to quickly identify the presence of HIS and TYR in samples, since, if ingested with food, they are responsible for the main negative effects on human health (i.e., nausea, cramps, headaches, hypertension, tremors, etc.) [1]. Furthermore, the toxic effects of amines in wine can be enhanced by the synergistic effect of ethanol and acetaldehyde, which inactivates the enzymes responsible for the catabolism of BAs and increases their absorption in the gastro-intestinal wall [14]. The study highlighted the presence of all nine BA considered in the wines. Furthermore, differences in concentration were highlighted between the content of PUT, HIS, and TYR, which in red wines reached higher values, respectively of 10.52, 7.57, and 6.59 mg L^{-1}, while in white wines a lower content of 4.22 was found, 4.42 and 3.71 mg L^{-1}, respectively. This could probably be related to multiple factors: pH of wines, oenological processes, and hygienic conditions especially for fermentation processes, in relation to the microorganisms that are involved in alcoholic and malolactic fermentation [27–39]. Today, it is perhaps very difficult to obtain wines without BA, which keep all their organoleptic properties unaltered, even if one could act by controlling the critical technological factors, in particular the microorganisms involved in the fermentation processes; in this way, there would be the possibility of producing wines with low or moderate levels of BA, not dangerous for the health of consumers.

Supplementary Materials: The following are available online at www.mdpi.com/1660-4601/181/91/159/s1, Figure S1: (A) Chromatogram of BAs standard solution (*1.7 diaminoheptane (IS)) (B) Calibration curves of nine BAs. Figure S2: (A) Biogenic amines solution at four different HFBA concentrations, ranged from 1 mM to 10 mM; (B) Optimization of the volume of samples injected. Figure S3: (A) Loadings Plots of nine biogenic amines (B) Scree Plot of 44 wine samples. Table S1: Performance characteristics of HPLC-UV/Vis method. Table S2: Performance characteristics of the LC-ESI-MS method.

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3.11 Research article no. 11

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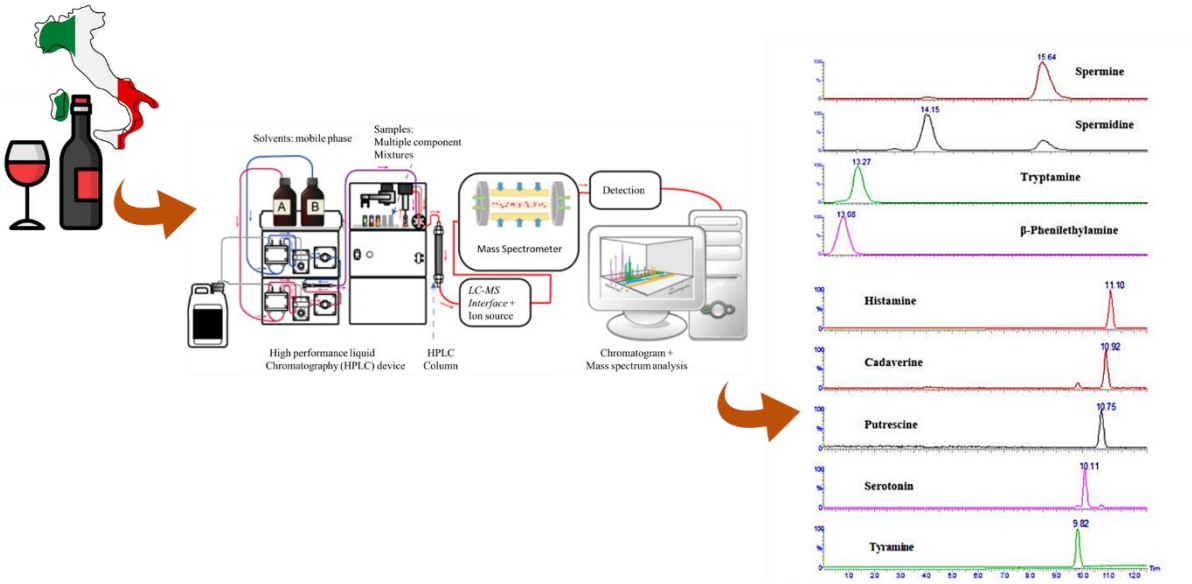

Figure 3.11. Graphical abstract of experimental study no. 11. Source: author's elaboration

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Simple, reliable determination of biogenic amines in Italian red wines. Direct analysis of underivatized biogenic amines by LC-ESI-MS

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ABSTRACT

Amines are ubiquitous compounds, and they are called “biogenic amines” (BAs) when they are synthetized by microbial decarboxylation of corresponding amino acids or “natural polyamines” when theyoriginate from endogenous metabolic pathway. BAs maybe both essential and harmful to human health. In wine, the composition of grape variety, the different types of fermentation and vinification processes and pH values are the most important contributors to BAs content. Several analytical methods have been reported for the BAs determination in wine (HPLC-UV/FLD, CE, LC-MS, etc.). As the most of them necessary require a long pre- or post-column chemical derivatization, LC coupled with MS spectrometry offers a reliable and faster determination of underivatized biogenic amines.The aim of the present work wasto investigate the content of nine BAs in 23 Italian red wines samplesusing LC-ESI-MS.

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Introduction

Aminesare ubiquitous bioactive compounds which may be both essential and harmful to human health[1]. They represent a class of organic, basic, and low-molecular weight compounds which can be natural/endogenous or exogenous in metabolism of plants, microorganisms and animals[2]. When originating from a natural metabolic pathway they are called “natural polyamines” and have physiological functions, especially for cellular metabolism as they are fundamental in membrane stabilization, protein synthesis and nucleic acid regulation[3]. Whereas, when they are synthetized through a microbial decarboxylation of the corresponding amino acids, they are defined Biogenic Amines (BAs), and are usually implicated into toxicological reactions[4]. BAs contamination in food involves symptoms that are similar to those of food poisoning: migraine headaches, nausea, gastric disorders, cardiac palpitations, respiratory suffering and psychoactive effects[5,6].Biogenic aminescan be synthesized both in fresh and perishable food(e.g. meat, fish, fruit, vegetables, etc.),which are directly exposed to decarboxylase-positive microorganisms[7];as well as fermented and/or processed food (e.g. wine, beer, cheese, coffee, chocolate, etc.) as a direct consequence of their transformation process (e.g. alcoholic and lactic fermentation)[8,9].BAs concentration mainly depends onthe protein composition of food matrix and the content of free amino acids, and it is also influenced by the presence of contaminating micro-organismsthat can be naturally present or added as starter cultureduring the transformation process[10]. However,BAs amount can be also linked tofood storage and contamination, caused by non-adequate hygienic conditions[11].

The occurrence of biogenic amines in wine depends on several factorsthat are mainly related to grape features, vinification or fermentation processes and pH values[12-14]. For the first case, different authorsreported that pedoclimatic conditions of the wine-growing area, nutritional status of vine, degree of grape ripeness and the composition of grape cluster in amino acids and natural polyamines are the main contributors to BAs content in wine[12-13,15].It has been reported that amino acids are the primary precursors of biogenic amines in wine. During alcoholic and malolactic fermentation in wine, altering yeasts and spoilage bacteria could have decarboxylating enzymes that metabolize amino acids and other substrates (e.g. aldehydes and ketones) into biogenic amines[16]. Moreover, the time of contact between the must, the grape marc and lactic acid bacteria (LAB), and the type of containers (stainless steel or oak barrels) used during vinification techniques could be significant for the synthesis of BAs in wine[17,18].pH is considered one of the most important factors influencing BAs content, as it controls the decarboxylating activity of microorganisms[10]. It is well known that high pH can positively affect the bacterial overgrowth and it consequentlypromotes the synthesis of biogenic amines in wine.Thus,explaining why higher pH values in red wines (pH: 3.4-3.5) are relevant for a higher BAs concentration compared to white wines (pH: 3.0-3.3)[18].

The main biogenic amines found and in wine are tyramine, histamine, putrescine and 2-phenylethylamine.Different authorshave shown that red wines contain more BAs than white and rosé wines, also quantifying tyramine and histamine as the most relevant BA reaching values above 8 mg/L[19-21].The European Union has not set BAs limits for the wine industry, but some countries have adopted their own regulations. Germany, Belgium and France have set a maximum histamine level of 2, 6 and 8 mg/L, respectively[21-22].

The biogenic amines determination is not simple because of their structure.

The most common approach to analyzeBAs in wine includes high performance liquid chromatography (HPLC) coupled with ultraviolet detector (UV)[23,24] fluorescence detector (FLD)[18,25], mass spectrometry (MS)[26], and capillary electrophoresis (CE)[27]. As primary or secondary amines structure do not absorb in the visible and ultraviolet range nor do they show fluorescence, pre- or post-column chemical derivatization is a necessary analytical step required for the detection[25]. The derivatization step improves the sensitivity of the analytical method; nevertheless, it has some drawbacks such as analyte loss, side reaction amines compound and longer time for the analysis. This could result into a poor resolution of the chromatographic method. However, LC coupled with MS spectrometry represents a valid and rapid ifenate technique for the detection of raw amines, as it does not require the derivatization step. Table1 shows the chemical characteristics of the nine biogenic amines analyzed in Italian red wine samples.

The aim of the present work was to determine nine BAs (tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine, serotonin, tyramine, spermidine and spermine) in 23 Italian red wines using LC-ESI-MS.

Material and methods

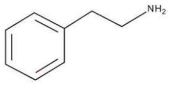
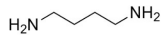
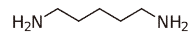
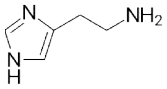
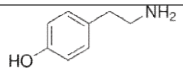
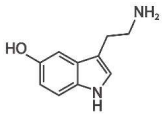
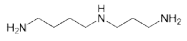
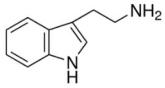
Sampling

The 23 red wine samples were purchased from local wine shop in different Italian regions: Piedmont, Tuscany, Veneto, Puglia and Sicily; for each region, red wines were respectively, for Piedmont: Barbera (Wine 1 and Wine 2), Dolcetto (Wine 3), Nebbiolo (Wine 4 and Wine 5); for Tuscany: Chianti (Wine 6 and Wine 7), Vernaccia (Wine 8), Montepulciano (Wine 9 and Wine 10); for Veneto: Pinot Nero (Wine 11), Merlot (Wine 12 and Wine 13), Cabernet (Wine 14 and Wine 15); for Apulia: Primitivo (Wine 16 and Wine 17), Negroamaro (Wine 18), Aglianico (Wine 19) and for Sicily: Nero d'Avola (Wine 20), Etna Rosso (Wine 21), Syrah (Wine 22 and Wine 23).

Chemicals

The nine biogenic amines studied were: Tryptamine (TRP), β-phenylethylamine (β-PEA), putrescine (PUT), cadaverine (CAD), histamine (HIS),serotonin (SER), tyramine (TYR), spermidine (SPD), spermine (SPM), all of which were supplied by Sigma Aldrich (St. Louis, USA), as well as heptafluorobutyric acid (HFBA) and 1,7-diaminoheptane (Internal Standard, IS).

Table 1. Chemical characteristics of the nine BA analyzed in this study

IUPAC nomenclature	*Molecular formula*	*Skeletal formula*	*Molar mass (g·mol^{-1})*
1-Phenyl-2-aminoethane (PHENYETHYLAMINE)	$C_8H_{11}N$		121.18
Butane-1,4-diamine (PUTRESCINE)	$C_4H_{12}N_2$		88.15
Pentane-1,5-diamine (CADAVERINE)	$C_5H_{14}N_2$		102.18
2-(1*H*-Imidazol-4-yl) ethanamine (HISTAMINE)	$C_5H_9N_3$		111.15
4-(2-Aminoethyl) phenol (TYRAMINE)	$C_8H_{11}NO$		137.18
3-(2-Aminoethyl)indol-5-ol (SEROTONIN)	$C_{10}H_{12}N_2O$		176.22
N'-(3-aminopropyl)butane-1,4-diamine (SPERMIDINE)	$C_7H_{19}N_3$		145.25
***N,N'*-bis(3-aminopropyl)butane-1,4-diamine (SPERMINE)**	$C_{10}H_{26}N_4$		202.35
2-(1*H*-Indol-3-yl)ethanamine (TRYPTAMINE)	$C_{10}H_{12}N_2$		160.22

Methanol of chromatographic grade was obtained from Carlo Erba (Milan, Italy) and distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

For the preparation of the amine standard solutions, an individual standard solution of 1.0mg $l^{-1}$of each amine were prepared in purified water and stored in darkness at 4±1 °C, while a standard solution containing all the amines (Mix 8) was obtained with 1 ml of each water solution diluted to 25 ml with purified water. Different aliquots of the standard solution were used to obtain the concentrations to construct calibration curves for BAs and to perform recovery experiments.

For LC-ESI-MS analysis the amine standard solutions were acidified with HFBA to obtain a final acid concentration of 5 mM and ranged from 0.1 to 16 mg $l^{-1}$with 0.8 mg l^{-1} of IS.As for the wine sample preparation, wine samples were initially filtered through a 0.20 µm membrane Millipore filter. For LC-ESI-MS analysis 25 ml of the filtered wine samples were added with HFBA to obtain a final acid concentration of 10 mM. After a second filtration, a volume aliquot of 50 µl was injected in the chromatographic column.

All measurements in the LC-ESI-MS analysis were achieved using a Thermoquest (Manchester, UK) model P2000 with an Alltima (Alltech, IL, USA) C18 reverse-phase column (250 x 4.6 mm i.d., particle size 5 µm). Mass spectrometric analysis was carried out on a Finnigan AQA benchtop single-quadrupole mass spectrometer (Thermoquest). The ESI unit operated at 4.0 kV, the capillary was heated at 200°C and nitrogen was used as desolvation and nebulizer gas at a flow rate 300 and 50 L/hour, respectively. The ESI-MS system operated in the positive ionization mode (PI). Diagnostic fragment ions were obtained by in-source collisop-induced dissociation (CID) of the protonated molecule $[M+H]^+$ after optimization of the voltage of the skimmer cone. Selected ion monitoring (SIM) was applied for the time-scheduled recording of the analytes. Data acquisition parameters are reported in Table2. Instrument control, data acquisition and processing were carried out with Mass Lab (version 2.22) from ThermoquestFinnigan (Manchester, UK).

For the LC-ESI-MS analysis, themobile phase solvents A and B were methanol (10 mMheptafluorobutiric acid) and water (10 mMheptafluorobutiric acid)respectively, at a flow rate of 1 mL/min. The column was maintained at room temperature and analytes were eluted using aninitial linear gradient program from 10% of solvent A to 85% in15 min, then passing from 85% of solvent A to 100% in 1 min,followed by an isocratic elution of 100% of A for 3 min. An additional 10 min was added to reach the initial conditions.The injected volume was 50 µl.

Table 2. Data acquisition parameters used in LC-ESI-MS for the detection of biogenic amines (SIM conditions)[29].

Biogenic amines	MW	Channel, *m/z* (relative abundance)	Cone voltage (V)	Retention window (min)
Tyramine	137.2	121.2(30), 138.3 (100)	30	0-12.85
β-phenylethylamine	121.2	105.1 (10), 122.3 (100)	30	12.85-16.00
Putrescine	88.2	89.3 (100)	40	0-12.85
Cadaverine	102.2	86.2 (10), 103.3 (100)	30	0-12.85
Histamine	111.1	95.2 (30), 112.1 (100)	40	0-12.85
Serotonin	176.2	160.3 (10), 177.2 (100)	40	0-12.85
Tryptamine	160.2	144.3 (40), 161.2 (100)	30	12.85-16.00
Spermidine	145.2	112.3 (10), 129.2 (10), 146.3 (100)	40	12.85-16.00
Spermine	202.3	129.2 (20), 112.3 (10), 203.4 (100)	40	12.85-16.00

Resultsand Discussion

Optimization of the LC-ESI-MS conditions

In order to investigate the separation of the nine BAs based on their mass/charge ratio, single amine standard solutions were injected without any column and analyzed in the full scan mode. These analytes have low relative molecular mass resulting in very small number of fragments. The MS conditions optimized to obtain maximum fragments are summarized in Tab. 3. For quantitative determination in select ion monitoring, the quasi molecular ion $[M+H]^+$ was selected for all compounds. Nevertheless, the detection of two or three confirming ions was carried out. In particular, the quasi molecular ion which has lost a NH_3 group and for spermidine and spermine the quasi molecular ion without two NH_3 molecules were selected. In the case of putrescine its low molecular weight allowed the monitoring of the quasi molecular ion only. Successively a reverse phase C18 column was installed to achieve amine separation. Biogenic amines are organic bases without any large hydrophobic side-chains; as a consequence, reverse phase chromatography is ineffective, eluting them with the dead volume. To overcome this problem, underivatized amines can be separated by ion-pair reversed phase liquid chromatography. The choice of the ion-pairing reagent has to fit two conditions: the first is to permit sufficient retention for good chromatographic separation and the second, most important, is that this reagent has to be volatile with minimum signal suppression. The additive has to allow at the same time optimum separation and recovery of amines and optimum detection by LC-ESI-MS. The addition of an acid to the mobile phase increases the retention times of the different analytes. This effect was due to the interactions between the negative charges on the inner column surface provided by the acid and the positive charges of the amines. Among the acid ion-pairing agents HFBA has demonstrated to work well in LC-ESI-MS. Moreover,a lower pH ($2<pH<3$) improves the analyte ionization in efficiency and analytical sensitivity due to the capacityof HFBA to facilitate nebulization and desolvation in the electrospray ionization source. Therefore, the use of HFBA allowed to obtain a longer total run time for best amine separation and the elution of other components present in the matrix that could co-elute with the analytes. The concentration of the ion-pairing reagent HFBA was studied as it is recommended to use a concentration as low as possible to avoid any signal suppression of the analytes. Some standard amine solutions were studied at four different HFBA concentrations (in the range from 1mM to 10 mM) of the mobile phase. Fig. 1 shows that increasing amounts of HFBA up to 5 mM result in an increase of the signal/noise ratios but at 10 mM the signal is strongly suppressed. For this reason, 5 mM was chosen as the optimal HFBA concentration for further experiments. The volume of sample injected in the column was also optimized. Fig. 2 shows the relative signal response and signal/noise ratio obtained for various injected volumes of a standard solution of tyramine in HFBA 5 mM. The best signal/noise ratio was obtained with a volume injected of 50 mL and this volume waschosen for further experiments.

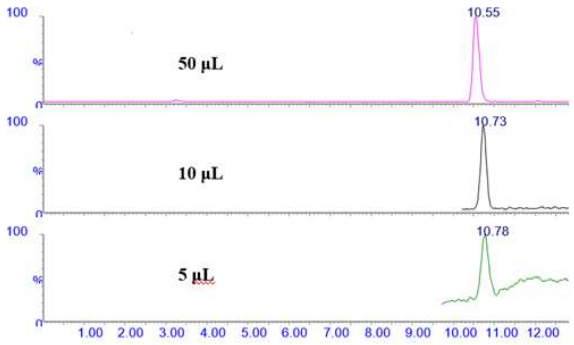


Figure 1. The increasing amounts of HFBA[29]

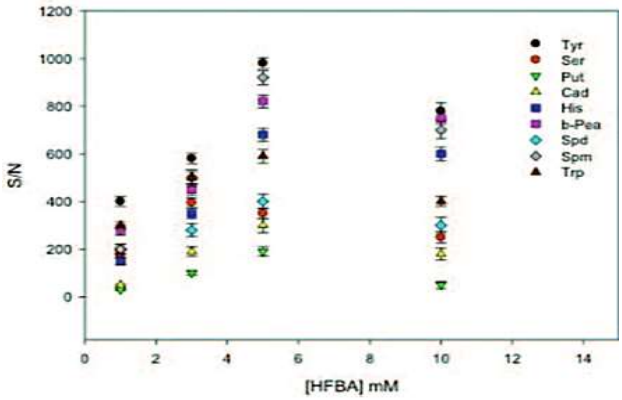


Figure 2. Signal response and signal/noise ratio obtained for various tyramine standard solution[29].

Performance characteristics of the LC-ESI-MS method

Linearity was tested using standard solutions of amines in acidified water (5 mM HFBA). Tab. 3 summarizes the results obtained. The response was linear in the range 0.1-16 µg L^{-1} and the correlation coefficients (R_2) were above 0.98, with the only exception of putrescine. The linearity "on line" (LIN) and the analytical sensitivity (AS) were calculated as reported above. The limits of detection were calculated according to the criterion of S/N=3, resulting in the range between 6.2 µg l^{-1} for tryptamine and 105.5 µg l^{-1} for putrescine.

Table 3. LC-MS method performances[29].

Biogenic Amine	R_t (min)	Conc. Range (mg l^{-1})	R^2	LIN %	AS (µg l^{-1})	LOD (µg l^{-1})
Tryptamine	15.6	0.1 - 16	0.999	98.99	23.1	42.8
β-Phenyethylamine	16.1	0.1 - 16	0.998	99.20	37.3	64.2
Putrescine	17.6	0.1 - 16	1.000	99.70	3.3	8.0
Cadaverine	18.8	0.1 - 16	0.999	99.25	8.2	17.1
Histamine	19.2	0.1 - 16	0.999	99.85	27.1	50.4
Serotonin	22.0	0.1 - 16	0.990	98.75	37.7	66.9
Tryramine	23.9	0.1 - 16	0.999	99.10	30.3	61.2
Spermidine	24.9	0.1 - 16	1.000	99.97	11.1	20.4
Sperimine	29.2	0.1 - 16	0.999	98.96	14.7	27.0

R^2: square of regression coefficient; LIN: linearity on-line; AS: analytical sensitivity; LOD: detection limit.

Determination of biogenic amines in wine samples

23 wine samples were analyzed using LC-ESI-MS method under the selected experimental conditions. Four replicates for each determination were performed. Fig. 3 shows the MS spectra for each of the nine amines studied. The complete results obtained with LC-ESI-MS for all wine samples studied are reported in Tab. 4. Each column refers to a specific biogenic amine. In the last column is reported the total amine amount, calculated for each wine sample.

By comparing the values reported in the last columns of Tab. 4 it can be also easily noted that the total amine concentrations are much higher in red wine samples 4, 8, 9, 12, 18, 22. The significant differences observed in the values reported for the wine samples can be explained bythe fact that the biogenic amine amount in wines is strongly dependent on different variables such as pH, wine aging and wine-growing area.Moreover, pH is the most important factor determining not only the biological activity of bacterial cluster in wine but also their variety and cultivar, as reported above. As for wine aging and production area, the literature reports that old wines contain significantly higher amounts of biogenic amines than young wines[12] and that in some producing areas biogenic amines are found in higher levels than in others[18]. However, as it is known in literature, red wines are generally less acidic than white ones andtherefore, biogenic amines are produced in high amounts[10]. The higher the pH, the more complex the bacterial clusters. An easier total growth and a greater bacterial diversity is observed in red wines which, therefore, show a composition of grape variety rich in amino acids and polyamines.

This is related in part to the type of winemaking and whether it involves the type of fermentation orvinification processes[17]. The most abundant amines determined with the LC-ESI-MS method resulted to be putrescine, histamine and tyramine (Tab. 5). In particular putrescine was found to be the highest value in wine samples (7.59 mg l^{-1}), followed by tyramine (6.75 mg l^{-1}) and histamine (6.01 mg l^{-1}). The correlation between putrescine, histamine and tyramine has already been noted by Martuscelliet al.[21], especially in red wines where these amines are present in greater quantities. This fact could be a consequence of malolactic fermentation which is required after alcoholic fermentation for nearly all red wines. The concentration of these amines is low after alcoholic fermentation and increases in most wines during malolactic fermentation to a very variable extent[17]. Spermine and spermidine were found in the lowest amount in red wine samples, 1.43 mg l^{-1} and 0.65 mg $l^{-1,}$ which is in accordance with the study of Liu et al.[28]. The accuracy of LC-ESI-MS methods was calculated by means of a spiking and recovery study onall red wines samples. The recovery was calculated as mean spiked concentration minus the mean original sample concentration divided by the spiked concentration. The spiked levels were 0.2mg l^{-1} and 1.0 mg l^{-1}. The LC-ESI-MS method resulted to be almost accurate with the following recovery values at 0.2 mg l^{-1}: 95.6% for tyramine; 104.2% for β-phenylethylamine; 99.9% for cadaverine; 103.3% for histamine; 96.0% for serotonin; 99.3% for tyramine; 101.0% for spermidine and 98.0% for spermine.

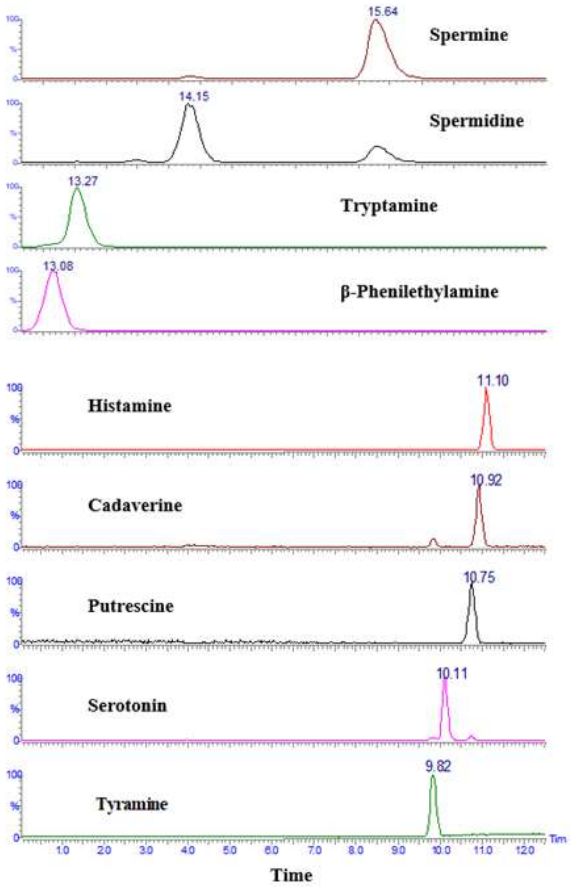


Figure 3. MS spectra of the nine biogenic amines

Table 5. Concentration range (mg l^{-1}) of the detected Biogenic Amines in wine samples, by LC-ESI-MS[29]

Biogenic Amine	Concentration Range (mg l^{-1})
Tryptamine	0.05 - 1.95
β-Phenylethylamine	0.15 - 4.05
Putrescine	0.26 – 7.59
Cadaverine	0.15 - 4.40
Histamine	0.44 - 6.01
Serotonin	0.25 - 3.55
Tyramine	0.15 - 6.75
Spermidine	0.18 - 0.65
Spermine	0.14 - 1.43

Whereas, the recovery values at 1.0 mg l^{-1} were:103.7% for tryptamine; 98.6% for β-phenylethylamine; 103.3% for cadaverine; 96.8% for histamine; 99.9 for serotonin; 97.6% for tyramine; 98.4% for spermidine and 103.1% for spermine, with theexception of putrescine whichshows lower recovery values rangingbetween 73.4%and 79.1%, respectively[29].

Table 4. LC-ESI-MS concentration (mg l^{-1}) and relative deviation standard (±RSD) of the nine biogenic amines in 23 Italian Red wines (Nd: not detectable)

Sample	*TRP*	*B-PEA*	*PUT*	*CAD*	*HIS*	*SER*	*TYR*	*SPD*	*SPM*	*Total Amines*
	Mean (±RSD)	*Mean(±RSD)*	*Mean(±RSD)*	*Mean(±RSD)*	*Mean (±RSD)*	*Mean(±RSD)*	*Mean(±RSD)*	*Mean(±RSD)*	*Mean(±RSD)*	
Wine 1	1.29±2.5	1.41±1.8	0.74±1.9	0.16±2.7	1.88±2.4	2.53±1.9	1.44±1.2	0.04±4.0	0.64±3.4	10,13
Wine 2	ND	0.31±1.8	ND	3.19±3.0	ND	0.96±3.0	2.01±2.2	0.38±2.4	1.53±1.8	8.22
Wine 3	1.31±2.4	3.05±1.8	ND	1.75±1.8	1.10±3.1	2.02±2.7	3.60±1.5	0.22±4.0	0.22±4.2	13,27
Wine 4	0.59±1.5	3.22±1.8	1.01±2.0	ND	6.21±1.4	1.02±1.8	6.85±1.4	0.75±1.9	0.33±2.9	19.28
Wine 5	0.99±2.0	0.23±2.6	0.38±3.0	3.88±1.5	3.55±2.6	0.26±1.4	0.95±2.1	ND	0.20±4.0	10,44
Wine 6	ND	ND	0.36±3.1	2.11±2.0	0.41±2.8	1.55±2.7	0.51±2.0	0.18±3.9	ND	5,12
Wine 7	0.07±2.5	0.23±2.3	ND	1.72±1.8	2.32±2.1	2.42±1.4	0.18±2.6	0.03±3.9	ND	6,97
Wine 8	2.05± 2.5	ND	0.29±3.5	2.23±1.9	1.82±3.0	2.49±2.5	1.33±1.3	0.70±2.3	0.28±1.5	11.17
Wine 9	2.04± 2.2	2.55±2.0	1.00±2.8	2.00±1.7	1.22±1.5	0.98±2.5	1.22±1.8	ND	0.33±3.1	11,34
Wine 10	ND	ND	0.33±3.2	2.15±1.9	0.45±2.7	1.71±2.5	0.42±2.1	0.25±1.6	ND	5.11
Wine 11	0.09± 3.0	1.68±1.9	1.61±2.0	ND	ND	1.89±1.9	2.1±1.5	0.50±2.7	ND	7,87
Wine 12	0.78± 2.7	2.80±2.3	0.57±2.0	4.41±3.7	2.94±2.8	ND	4.05±1.4	0.21±4.2	0.15±5.3	15.85
Wine 13	ND	ND	0.66±3.4	0.88±1.9	1.20±2.9	ND	1.98±1.8	ND	0.16±4.2	4,88
Wine 14	0.60±2.5	ND	0.77±2.9	3.22±2.4	0.66±3.1	1.56±1.7	0.99±2.0	0.33±2.5	0.16±3.8	8,29
Wine 15	0.06±2.5	0.22±2.9	ND	1.45±1.9	1.18±3.3	2.42±1.5	0.19±2.6	ND	0.22±2.9	5.22
Wine 16	0.55±2.7	2.00±1.8	1.45±1.9	1.65±2.0	1.00±2.4	1.68±1.9	0.99±2.3	0.33±2.3	0.44±2.6	10,09
Wine 17	ND	ND	0.66±2.3	1.66±2.1	ND	2.57±1.7	0.39±2.9	0.09±3.9	0.12±3.8	5,49
Wine 18	1.15±1.8	0.24±2.5	0.48±3.1	1.67±1.6	3.97±2.5	0.27±1.3	0.93±2.2	ND	ND	19.63
Wine 19	2.40±2.4	3.99±1.5	1.85±2.1	1.68±1.8	0.55±2.8	3.74±1.2	2.00±2.0	0.41±3.0	0.36±3.3	16,98
Wine 20	0.28±4.2	0.18±2.4	1.30±2.4	1.69±1.5	ND	3.25±3.7	2.93±3.3	0.31±2.2	0.36±2.3	10.97
Wine 21	0.34±3.0	ND	1.05±2.8	1.70±1.4	0.75±3.1	ND	2.05±2.1	ND	0.57±2.8	6,46
Wine 22	1.25±3.0	4.05±1.9	2.57±2.5	1.71±2.2	2.95±1.9	ND	1.88±2.4	0.05±3.1	ND	15.60
Wine 23	ND	2.73±2.0	0.87±2.9	1.72±2.1	3.66±1.6	1.53±1.5	3.84±1.5	0.27±3.0	0.68±2.9	15,3

Conclusion

The concentrations of nine biogenic amines found in red wines from different regions of Italywas were investigated using LC-ESI-MS. The method enables qualitative and quantitative detection phases to be carried out simultaneously with good performances. This approach offers a reliable and faster determination of underivatized biogenic amines,when compared with other chromatographic techniques (e.g. HPLC-UV/FID), that require a long pre- or post- column chemical derivatization.All the 23 red wine samples originated from different regions of Italy show the presence of biogenic amines, ranged between 4.88 and 19.63 mg l^{-1} and, in accordance with the concentration range (mg l^{-1}) of the detected Biogenic Amines in wine samples by LC-ESI-MS,putrescine, histamine and tyraminewere the most abundant BAs in red wine samples. This could be probably related to multiple factors such as non-adequate hygienic conditions during winemaking practices or storage, fermentation processes, wine ageing, and the oenological procedure. In addition, the presence of ethyl alcohol in wine could have a negative synergistic effect on the increasing amount of biogenic amines. Since there is no international legislation establishing maximum tolerability limits for the biogenic amines in wine, it is important to notice that a high content of BAs could be harmful for consumers' health. Therefore, a fast determination with reliable analytical methods, such as LC-ESI-MS, could be a useful tool to monitor food quality and safety parameters in wine.

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Conflicts of interest

The authors declare no conflict of interest.

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CONCLUSIONS

In modern food consumption patterns, where the demand for healthier and more sustainable products is ever-increasing, the importance of sustainable technologies in the analysis of food bioactive compounds emerges as a driving focus of innovation. Given the intersection of environmental protection and food nutritional quality, the convergence of pioneering technologies with a main focus on sustainability issues promises a new era in the analysis and research of food bioactive complexities. Such intersection not only paves the way for deep scientific advances but also underlines the need to harmonize human nourishment with the preservation of the Planet's ecosystems, as well as an essential step towards a more sustainable and health-conscious future.

In these regards, the Ph.D. project focused on the possibility of valorising main Italian market representative agri-food products and related processing by-products (i.e., durum wheat chain, wines, psychoactive foods and beverages such as tea, chocolate, and coffee, etc.) through an integrated approach for a multi-dimensional assessment of sustainability. In particular, the research project focused on the recovery of bioactive compounds from food matrices, through the development and application of emerging and sustainable technologies, such as Deep Eutectic Solvents (DESs), ultrasound-assisted extraction (UAE), etc., which enable the rapid extraction of target analytes (i.e., polyphenols, antioxidants, biogenic amines, essential fatty acids, etc.) from agri-food products, that are of particular interest for both human nutrition and in the Italian Market. In this framework, the main results of the proposed research were the object of no. 11 publications in scientific International Journals. In general, it should be emphasized that the use of sustainable technologies for the extraction and analysis of bioactive compounds in food represents a significant environmental benefit for the quality, safety, and sustainability of food. In detail, taking into account the macro-area of **research food quality and safety (FQ), (FS)**, it is worth noting that the application of green solvents (DES) coupled with UAE of polyphenols from dark chocolate, highlighted dual advantages in terms of both extraction yield and environmental assessment. Based on the results obtained, both DES pairs of natural origin (Choline Chloride:Fructose, and Betaine:Fructose) exhibited 35% higher extraction yields than conventional solvents (MeOH, and MeOH: H_2O), taking

into account different operating conditions in terms of time, temperature, and water content. The comparative Life Cycle Assessment (LCA) study highlighted the conventional solvent as 60% quantitatively more impactful than DES on the 18 damage impact categories considered, especially in terms of mineral and fossil resources availability (Vinci et al., 2023). Due to their plant-based nature, these green compounds could be directly used in the formulation of foods, and additives as well as in cosmetics and pharmaceutical preparations.

Taking into account the bioactive complexities of food products, it was possible to demonstrate that industrial as well as household preparation processes for food consumption can lead to qualitative and quantitative changes in the bioactive components as well as antioxidant properties, thus influencing their potential biological activities, as in the case of tea and coffee preparations. In particular, different operation conditions, in terms of water types (tap, distilled, and natural mineral water), as well as different infusion times (3, 5, 10 min) and temperatures (80 °C and 100 °C) were tested for the polyphenol extraction from in green and black tea matrices. The results revealed that the optimal infusion variables were obtained by extracting phenolic compounds at T = 100 °C for 10 min, both for samples of green tea (916.12–1,169.81 mg GAE/g) and black tea (932.03–1,126.62 mg GAE/g) in natural mineral water as natural extractant (Vinci et al., 2022). In these regards, the optimal infusion variables could be considered for the preparation of domestic and industrial teas, as well as those indicated on the label, to obtain infusions with higher polyphenol content and antioxidant activity and achieve greater benefit from the health-promoting effects.

Sustainable extraction methods were also investigated in the preparation of the coffee beverage by analysing and evaluating quality and sustainability of the main coffee brewing methods adopted by consumers, including professional espresso machines, Italian Moka, pods and capsules (Gobbi et al., 2023). In addition, the possibility of assessing the content of specific bioactive compounds such as total polyphenols, antioxidant activity and biogenic amines (BAs) both in raw material (coffee powders) as well as in the resulting coffee extracts, further highlighted the considerable influence of the preparation process on the bioactive content of coffee. In particular, analyses of coffee powders showed total BAs concentration ranging from 67.01 μg/g to 96.83, thus highlighting a decrease of 39% in coffee beverages (16.02–53.92 μg/g). Among all BAs, Serotonin was the prevailing amine in both ground coffee samples (62.13–84.24 μg/g)

and coffee beverage samples (12.75–33.46 μg/g), thus denoting potential health-promoting effects. Considering total polyphenol content, it was observed higher content in the starting ground coffee powders (22.96–29.61 mg GAE/g) and decreased significantly in coffee beverages, between 80% and 90%, depending on the different beverage preparation methods. The overall reduction of bioactive compounds in coffee beverages could probably be due to the high brewing temperatures and pressures, as well as the water/coffee contact surface and the particle size of the coffee powder may affect the extractant capacity of biogenic amines and phenolic compounds. Furthermore, the application of LCA methodology allowed the sustainability assessment of coffee brewing methods, thus highlighting lower environmental impact for the industrial coffee machine compared to the others, probably due to less time spent on coffee preparation and reduced packaging of raw material.

Considering food by-products, a multimethodological approach evaluating quality (antioxidant and anti-inflammatory activity), safety (BAs) parameters as well as environmental sustainability was proposed for the valorization of the "ancient Senatore Cappelli" wheat milling husks (Vinci et al., 2023). By means of HPLC-FD analyses, wheat husk samples revealed a higher content of SER amounting to 35% of the total BAs and were confirmed to occur at BAQI values <10 mg/100 g, thus denoting no loss in quality of analysed samples. While, based on in vitro assays on murine BV-2 cells, the analysed "Senatore Cappelli" husks samples resulted as non-cytotoxic, and they stimulated mRNA expression of anti-inflammatory markers (ARG-1, CD206, and Chil3), as well as the expression of genes involved in the antioxidant system.

Moreover, through the application of LCA methodology, it was possible to highlight that the impacts associated with the disposal of wheat milling by-products account for approximately 9% of total wheat production. Nevertheless, the extraction of high-value-added compounds from the wheat husk can mitigate environmental and health impacts, thereby inducing 0.41% CO_2 savings per year (12,160 kg CO_2 eq.) compared to the overall wheat-production chain. Therefore, considering the growing interest in the renewability of food resources, this multi-methodological study builds on the potential neuroprotective role in terms of reduction of neuroinflammation and oxidative stress, as well as environmental performances of waste products from sustainable agricultural supply chains.

The LCA methodology has proven to be a useful tool not only for assessing the environmental performance associated with chemical processes for food matrix extraction, but also an essential tool for quantitative environmental assessment for optimising food production processes.

Taking into account **the research macro-area Sustainability (S)**, the main research studies highlighted the potential of integrating the use of LCA in industrial processes, both for identifying hotspots and high-impact stages in the food production cycle, as well as for supporting the design of new products and/or services, thus indicating possible strategies for environmental improvement at upstream and downstream levels. In particular, LCA was applied in the wine sector considering a *cradle-to-gate* approach for the production of a 0.75L wine bottle (Vinci et al., 2022). Results highlighted the wine bottling stage as the main contributor (ranging from 39% to 69%) to the total environmental impacts, mainly due to the glass production process, as well as the use of fertilizers and pest management that were found to be the second most impactful inputs in the vineyard phase. Based on the hotspot analysis, several options were identified to reduce the environmental impact of the wine industry, among which the use of a 10% lighter glass bottle could be beneficial to the environment, thus, saving at least 0.43 kg CO_2 eq per wine bottle. Furthermore, the reuse of biomass and processing by-products (i.e., grape stalks, lees, and seeds) represented a potential alternative for soil fertilization with organic and natural nutrients.

Furthermore, the possibility of applying LCA to compare two different methods (two- and three- phases decanter) for the extraction of extra virgin olive oil considering a *gate-to-gate* approach allowed the identification improving strategies in the elaio-technical sector. In particular, the recorded results show that the two-phases system resulted to be more sustainable than three-phase extractions, thus inducing 46% CO_2 savings (equal to 212 kg CO_2 eq.) compared to the three-phase process, and changes in the range of 5–26% in the other impact categories. This finding appears mainly related to the extra addition of water and energy, as well as major waste biomass, usually required in the three-phase system. Starting from waste mass of olive oil extraction processes (i.e., olive pomace, OP) some End-of-Life (EoL) scenarios were proposed to introduce innovative technologies to improve energy efficiency, and valorise olive oil by-products. In particular, bio-gasification and composting technologies were evaluated as possible useful scenarios for the reuse of OP. Specifically, the first one appears the best available

technology for bioenergy production, since it induces greater benefits in terms of CO_2 savings. For example, bio-gasification of OP induces savings of −1756 kg CO_2 eq. in the two-step compared to the same EoL in the three-step process (−205 kg CO_2 eq.) and, in turn, greater savings than composting (−140 kg CO_2 eq. for the two-step vs. −1199 kg CO_2 for the three-step).

Therefore, based on the results obtained, it is desirable to rationalize and sustainably manage resources in agri-food chain, acting both on the upstream and downstream levels. The former should provide resource efficiency, and increased productivity in the production process, thus promoting the use of renewable resources, and orienting production toward "*zero* waste".

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