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Recovery and valorization of by-products of the agri-food chain with supercritical fluid technology

Doctoral dissertation
by

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ABSTRACT

This doctoral research investigates the potential of supercritical fluid extraction (SFE) as an enabling technology for the valorisation of lemon peels, a major agro-industrial by-product from Citrus processing, with the aim of recovering high value bioactive compounds for advanced cosmetic applications. A systematic experimental campaign was carried out by varying temperature, pressure and CO_2 flow rate to identify the most efficient extraction conditions. The explored operative window yielded extracts ranging from 1.11% to 10.18% (w/w), with the highest yield (10.18%) obtained at 50 °C, 300 bar and a CO_2 flow rate of 10 $kg \cdot h^{-1}$, thus confirming the strong influence of process parameters on extraction performance.

The chemical profile of the obtained extracts was elucidated by Gas Chromatography–Mass Spectrometry (GC–MS), revealing a complex mixture dominated by mono- and sesquiterpenes, together with discrete amounts of oxygenated terpenes such as linalool, geraniol and nerol, and additional aldehydes and esters known as key plant secondary metabolites. This composition is consistent with a multifunctional bioactive system potentially endowed with antioxidant, soothing and protective properties of interest for skin care.

To preliminarily assess safety and applicability in dermo-cosmetic products, in vitro bioscreening was performed on human skin cell models. The results demonstrated the biocompatibility of the lemon peel extracts within the tested concentration range, indicating an absence of significant cytotoxic effects and supporting their suitability as functional ingredients for personal care formulations.

Furthermore, a Life Cycle Assessment (LCA) of the valorisation process was conducted to evaluate the environmental impacts and identify the main critical stages in terms of energy

use and emissions. The LCA results highlighted that the use of SFE, compared to conventional extraction techniques, allows a significant reduction of the overall environmental impact, reinforcing the sustainable nature of the process.

Overall, the findings of this work demonstrate that an optimized SFE process can be effectively employed to recover structurally diverse and biologically relevant compounds from lemon peels, converting a low-value waste stream into a high-added-value resource. This contributes to the development of sustainable and circular strategies for the management of agricultural by-products, fully aligned with the principles of green chemistry and the bio-based economy, and lays the groundwork for further formulation, stability and efficacy studies in cosmetic and related sectors.

CHAPTER 1
INTRODUCTION

1.1 Beckground: Supercritical Fluid Technology

Supercritical carbon dioxide (CO_2) extraction is a separation technique that exploits the unique physicochemical properties of CO_2 when it is brought above its critical temperature

(31.1 °C) and critical pressure (73.8 bar). In this supercritical state, CO_2 exhibits intermediate behavior between a gas and a liquid: it combines gas-like diffusivity and low viscosity with liquid-like density, resulting in enhanced mass transfer and good solvating power toward many non-polar and moderately polar solutes. These features make supercritical CO_2 ($scCO_2$) an attractive "tunable" solvent for both analytical and industrial-scale extracton processes. (McHugh and Krukonis, 1994; Parhi, 2013).

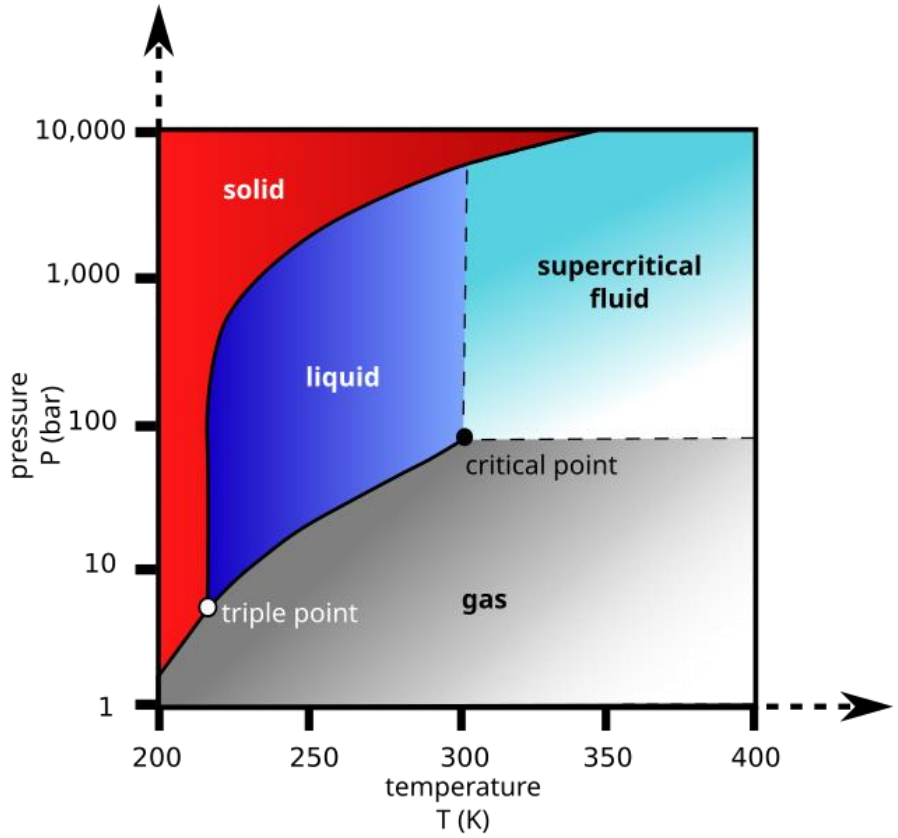


Figure 1- Phase diagram of CO2

From a process standpoint, supercritical CO_2 extraction typically involves bringing CO_2 to supercritical conditions via compression and temperature control, contacting it with a solid or liquid feed matrix in a high-pressure vessel, and subsequently depressurizing the solvent stream in one or more separators to precipitate the solubilized compounds. By adjusting

operating variables such as pressure, temperature, solvent flow rate and residence time, as well as by adding small amounts of co-solvents (e.g. ethanol), it is possible to finely modulate solvent density and selectivity, thereby targeting specific classes of molecules (e.g. lipids, essential oils, pigments, or other bioactives). (McHugh and Krukonis, 1994; Mendiola et al., 2013; Uwineza and Waśkiewicz, 2020).

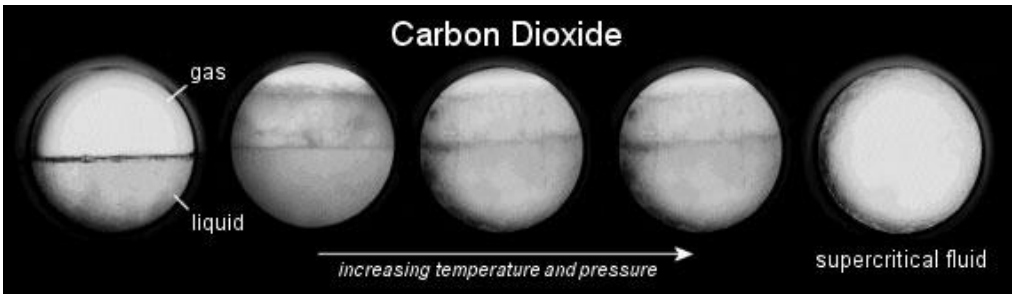


Figure 2 - CO_2 molecule under electron microscope

Compared with conventional liquid–liquid or solid–liquid extraction using organic solvents, $scCO_2$ extraction offers several advantages. CO_2 is non-toxic, non-flammable, inexpensive, readily available and classified as a "green" solvent, which allows the development of cleaner processes with minimal use of hazardous chemicals. Furthermore, the relatively mild operating temperatures, often close to ambient, limit thermal degradation and preserve the functional properties of thermo-labile compounds. The solvent can be easily removed from the final extract simply by pressure reduction, leaving negligible residues and simplifying downstream purification. These attributes have positioned $scCO_2$ as a key technology for high-value products in the food, cosmetic, nutraceutical and pharmaceutical sectors, including decaffeination of coffee and tea, extraction of hops, essential oils, carotenoids, sterols and other bioactive ingredients. (Perrut, 2000; Brunner, 2010; Uwineza and Waśkiewicz, 2020; Nozari et al., 2025).

In recent years, supercritical CO_2 extraction has gained renewed attention in the context of circular bioeconomy and sustainable processing, as it enables the valorisation of agro-industrial by-products and biomass residues into high-value extracts while reducing the environmental footprint associated with conventional solvent-based technologies. Current research focuses on process intensification, advanced modelling of phase equilibria and mass transfer, and the integration of $scCO_2$ extraction with other green technologies (e.g. subcritical water, membrane separations) to further improve selectivity, energy efficiency and overall process economics. (Brunner, 2010; Geeta et al., 2020; Słota et al., 2025; "Supercritical CO_2 as a green solvent", 2025).

1.2 Supercritical CO_2 Extraction from Lemon Waste

Lemon (Citrus limon L.) processing generates large amounts of peel, which can account for roughly 30–35% of the fruit mass and is rich in essential oils (mainly D-limonene), oxygenated terpenes (e.g. citral), flavonoids (hesperidin, eriocitrin, naringin), phenolic acids and pectins. These compounds show antioxidant, antimicrobial, anti-inflammatory and potential cardiometabolic benefits, making lemon peel a valuable feedstock for high-value ingredients in food, cosmetic and pharmaceutical sectors.

Supercritical CO_2 is particularly suitable for valorising citrus peels because it is non-toxic, non-flammable and easily removed from the extract, and its solvating power towards non-polar and moderately polar compounds can be tuned by pressure, temperature and co-solvents.

Early work on lemon systems focused on the treatment of cold-pressed peel oils rather than the direct extraction from solid peels, but these studies are fundamental to understand how $scCO_2$ fractionates and enriches bioactive terpenoid fractions.

Barth et al. (1994) studied the desorption of lemon peel oil by $scCO_2$ with the dual goal of deterpenation and removal of phototoxic psoralens. Operating in the range of ca. 7–12 MPa and moderate temperatures, they showed that $scCO_2$ could selectively remove the monoterpene hydrocarbons (mainly limonene) while lowering the psoralen content of the raffinate, resulting in safer and more aroma-intense oils.

Gironi and Maschietti (2005) further investigated supercritical fractionation of cold-pressed lemon oil in a batch apparatus equipped with an external reflux column. At 315–333 K and 8.4–10.5 MPa, they demonstrated that appropriate choice of temperature, pressure and reflux ratio allows the production of "folded" (terpene-reduced) lemon oils, enriching oxygenated aromatic components at acceptable solvent-to-feed ratios.

Díaz et al. (2005) extended this type of process modelling to citrus peel oils more generally, showing how phase equilibrium and relative volatility constraints govern the achievable separation between limonene and oxygenated aroma compounds in $scCO_2$ fractionation.

More recently, research has moved from pre-extracted oils to direct supercritical extraction of essential oil from citrus peels, including lemon. Lopresto et al. (2019) developed a process-intensified strategy for the valorisation of waste lemon peels from a Protected Geographical Indication cultivar. Dehydrated and milled peels were treated by supercritical fluid extraction (SFE) with CO_2 to recover D-limonene-rich essential oil. The study compared SFE with conventional hydrodistillation and highlighted that $scCO_2$ offers shorter extraction times, lower thermal stress on bioactives and easier solvent removal, while enabling process intensification and energy integration for improved environmental performance.

Complementary work by Toker et al. (2025) quantified limonene distribution in different citrus fractions and then optimised supercritical extraction from lemon peel specifically. Dried, ground lemon peels exhibited the highest limonene content (~58,000 ppm). Using $scCO_2$ in the range 35–50 °C and 12.5–20 MPa, response surface methodology indicated an optimal region around 40 °C and 15 MPa for maximising D-limonene yield, with matrix pre-treatment (drying and grinding) playing a crucial role in mass transfer and overall recovery.

At a broader scale, Costa et al. (2025) evaluated supercritical CO_2 extraction of limonene from orange, lemon, lime and tangerine peels at 150 bar (15 MPa) and 35 °C for 30 minutes. Besides reporting significant limonene recoveries from lemon waste, they coupled the experimental SFE data with an economic assessment, estimating manufacturing costs in the range of 50–530 USD/kg extract and showing that energy recovery in the CO_2 cycle improves economic feasibility.

Taken together, these studies indicate that $scCO_2$ in the range 10–20 MPa and 35–60 °C efficiently recovers lemon essential oil rich in limonene and other terpenoids, while allowing selective modification of composition (e.g. deterring highly volatile terpenes or removing psoralens) via pressure, temperature and process configuration (simple batch vs. rectification column).

1.3 Recovery of phenolic compounds and flavonoids

While $scCO_2$ is an excellent solvent for non-polar terpenoids, its low polarity limits the extraction of phenolic compounds and flavonoids from citrus peels unless a polar co-solvent

is added. This issue is clearly highlighted in the review by M'hiri et al. (2014), which compares various extraction methods for citrus peel phenolics and notes that supercritical CO_2 alone is generally unsuitable for efficiently recovering polar phenolics.

A key lemon-inclusive study is the work of Romano et al. (2022), who extracted bioactive compounds from orange, tangerine and lemon peels using both liquid and supercritical CO_2, with and without ethanol as co-solvent. They systematically evaluated extraction yield, total polyphenol content, individual polyphenolic profile, antiradical activity and volatile organic compounds. The highest yields were obtained when 20% ethanol was employed as co-solvent in both liquid CO_2 (20 MPa, 20 °C) and supercritical CO_2 (30 MPa, 60 °C). For lemon peels, liquid CO_2 + 20% ethanol provided extracts particularly rich in naringin (≈19.9 mg g^{-1} dry extract) and limonene, with high antiradical capacity (up to ~60 µmol Trolox equivalents g^{-1} by ABTS and DPPH assays).

These findings confirm that adding a modest fraction of ethanol markedly enhances the recovery of flavonoids and phenolic acids from lemon peel while preserving a substantial terpenoid fraction, thus yielding multifunctional extracts with both antioxidant polyphenols and volatile aroma compounds.

A more recent contribution by Domínguez-Rodríguez et al. (2025) proposed a sustainable sequential extraction of citrus peels (grapefruit, lime and lemon). In their scheme, $scCO_2$ is first used to extract volatile terpenoids; a subsequent pressurised liquid extraction step (ethanol/water) recovers phenolic compounds from the deoiled peels. This "biorefinery" approach maximises overall utilisation of the peel matrix while reducing solvent consumption and enabling fractionation into an essential-oil-rich and a phenolic-rich fraction.

At a more general level, Tyśkiewicz et al. (2018) reviewed supercritical fluid extraction of phenolic compounds and concluded that $scCO_2$ combined with polar modifiers (typically 5–20% ethanol or methanol) can successfully extract a variety of phenolic acids and flavonoids from plant matrices, with improved antioxidant activity of the resulting extracts compared to conventional methods.

This supports the strategy adopted by Romano and co-workers for citrus peels and suggests that further optimisation of co-solvent type and percentage could enhance phenolic recovery from lemon peel specifically.

1.4 Biological activity and potential applications of lemon peel $scCO_2$ extracts

The bioactivity of lemon peel CO_2 extracts reflects their combined terpenoid and phenolic composition. Supercritical and liquid CO_2 extracts from citrus peels (including lemon) are typically dominated by monoterpenes such as D-limonene, with additional oxygenated terpenes and a flavanone-rich phenolic fraction (e.g. naringin, hesperidin) (Romano et al., 2022; Xu et al., 2025). This dual composition underpins both strong antimicrobial/antifungal effects—primarily associated with the essential-oil fraction—and antioxidant, anti-inflammatory and metabolic effects linked to flavonoids and other phenolics (Alam et al., 2014; Salehi et al., 2019).

Limonene-rich essential oils from lemon peels exhibit marked antioxidant and antimicrobial properties. Several studies on citrus peel essential oils, where limonene is the major component, report significant antibacterial and antioxidant activity against a range of foodborne and environmental pathogens (Meryem et al., 2023; Dias et al., 2019). In particular, Ammad et al. (2018) showed that Citrus limon essential oil effectively controls fungal diseases of grapevine wood, demonstrating fungistatic and fungitoxic effects against

pathogens such as Eutypa spp., Botryosphaeria dothidea and Fomitiporia mediterranea. Similar antifungal properties have been reported for citrus essential oils against Candida albicans and other phytopathogenic fungi, with D-limonene identified as a key contributor to the antifungal effect and possible synergistic interactions with minor components (Radithia et al., 2022; Ajayi-Moses et al., 2019; Salvatore et al., 2022). These data support the use of lemon peel $scCO_2$ extracts—particularly their essential-oil fractions—as natural preservatives and antifungal agents in agro-food and post-harvest applications.

Phenolic-enriched fractions obtained from citrus peels by CO_2-based processes (often using ethanol as a co-solvent) show high total polyphenol content, strong antiradical activity and significant levels of flavanones such as naringin. Romano et al. (2022) demonstrated that liquid and supercritical CO_2 extractions from orange, tangerine and lemon peels with 20% ethanol yield extracts with elevated naringin content and high ABTS/DPPH antiradical capacity compared with conventional solvent extraction. Beyond extraction data, a large body of pharmacological literature links naringin and related citrus flavonoids to beneficial effects on metabolic syndrome, dyslipidaemia, hypertension and obesity, mediated by antioxidant and anti-inflammatory mechanisms (Alam et al., 2014; Raja Kumar et al., 2019; Salehi et al., 2019). Naringenin and other citrus flavonoids have also been shown to modulate oxidative-stress pathways and inflammatory mediators (Xu et al., 2025), reinforcing the rationale for exploiting lemon peel $scCO_2$ extracts as sources of bioactives for functional foods and nutraceutical formulations.

From a process and application standpoint, several reviews on citrus food-waste valorisation emphasise that integrating green extraction technologies—such as supercritical CO_2, pressurised liquid extraction, ultrasound- or microwave-assisted extraction—enables the production of high-value ingredients (essential oils, flavonoids, pectins) for the food,

nutraceutical, cosmetic and packaging sectors while reducing the environmental burden of citrus processing residues (Anoopkumar et al., 2023; Suri et al., 2021; Anticona et al., 2020; Ammar et al., 2025; Selvaprasad et al., 2025). At a broader scale, Costa et al. (2025) evaluated supercritical CO_2 extraction of limonene from different citrus peels (including lemon) and coupled the experimental data with an economic assessment, showing that energy recovery and process integration can make citrus-waste valorisation via $scCO_2$ economically attractive. In line with this, Ray et al. (2023) highlighted supercritical extraction of bioactives from fruit waste as a green and sustainable approach, particularly when embedded in biorefinery schemes.

In this broader biorefinery context, lemon peel $scCO_2$ extracts can be positioned as multifunctional ingredients: limonene-rich fractions for antimicrobial/antifungal and aromatic functions (e.g. natural preservatives, coatings, active packaging), and phenolic-enriched fractions for antioxidant and anti-inflammatory functionalities in functional foods, nutraceuticals and dermo-cosmetic products. Overall, the available evidence indicates that lemon peel $scCO_2$ extracts combine technological advantages (solvent-free, tunable composition, mild temperatures) with a robust bioactivity profile, making them promising candidates for sustainable, high-value applications across food, cosmetic and pharmaceutical formulations.

1.5 In vitro bioscreens for cellular response analysis of citrus extracts

In vitro bioscreens based on mammalian cell models are widely used to characterise the biological activity and safety of citrus extracts before moving to in vivo studies. These screening platforms typically combine viability and cytotoxicity tests (e.g. MTT, neutral red,

AlamarBlue), oxidative-stress markers (reactive oxygen species, antioxidant defence), inflammatory mediators (NO, cytokines, COX-2/iNOS expression) and functional read-outs such as cell migration, differentiation or barrier integrity. Recent reviews on citrus bioactives emphasise that the majority of mechanistic data for citrus flavanones and peel extracts derives from such in vitro cellular models, particularly in skin, intestinal and immune cells (Sorrenti et al., 2023; Denaro et al., 2021; Silla et al., 2025).

A large group of in vitro bioscreens explores the effect of citrus extracts on skin cell homeostasis, photoprotection and wound healing using human keratinocytes (HaCaT, primary NHEK) and dermal fibroblasts. Pérez-Sánchez et al. (2014) developed a combined preclinical–clinical model to evaluate a nutraceutical mix of citrus bioflavonoids and rosemary extract (Nutroxsun®). In HaCaT keratinocytes exposed to UVB, they assessed cell viability (MTT), intracellular ROS (H_2DCFDA), DNA damage (comet assay) and inflammatory markers, demonstrating that the citrus–rosemary formulation significantly preserved viability, reduced oxidative damage and improved genoprotection; the same formulation reduced minimal erythema dose in human volunteers, supporting the translational value of the bioscreen.

Building on this approach, Sánchez-Marzo et al. (2020) tested two formulations composed of flavanone-enriched citrus extract plus olive (± rosemary) in UVB-irradiated HaCaT cells. Their in vitro bioscreen combined antioxidant assays with flow-cytometric analysis of γH2AX activation as a marker of DNA damage, showing that the formulation enriched in citrus flavanone aglycones and rosemary diterpenes offered superior protection against UVB-induced genotoxicity.

Tomasello et al. (2022) evaluated a phytocomplex from red orange (Citrus sinensis) on UVA/B-induced photoaging in skin cells, using viability assays, ROS measurements and markers of senescence and extracellular matrix degradation, and reported significant photoprotective and anti-ageing effects in vitro.

Citrus peel extracts from other species have been screened for more specific cellular responses. Abe et al. (2020) showed that an aqueous peel extract from Citrus sudachi suppresses proliferation of HaCaT and normal human epidermal keratinocytes while promoting their differentiation. Their bioscreen measured proliferation, phosphorylation of EGFR and ERK1/2, and expression of differentiation markers, demonstrating that the extract inhibits EGFR–ERK signalling and enhances calcium-induced differentiation, with potential applications in hyperproliferative skin disorders.

More recently, Citrus hystrix extracts have been evaluated in wound-healing bioscreens. Ratanachamnong and co-workers reported that water and peel extracts rich in flavonoids and phenolic acids show strong antioxidant activity and promote keratinocyte and fibroblast migration in a scratch assay, while protecting cells from oxidative injury (H_2O_2 challenge) by improving viability and reducing intracellular ROS (Ratanachamnong et al., 2023; Purba et al., 2024).

Taken together, these skin-cell bioscreens illustrate how citrus extracts are interrogated for photoprotective, antioxidant, pro-differentiating and wound-healing activities using coherent panels of in vitro endpoints.

A second cluster of in vitro bioscreens focuses on intestinal and immune cells to evaluate anti-inflammatory and barrier-protective effects of citrus extracts. Denaro et al. (2021) first performed a cell-free antioxidant/anti-inflammatory screen on nine representative citrus

flavanones, then formulated a flavanone mix (neoeriocitrin, eriocitrin, hesperidin, neohesperidin, diosmin) and tested it on an IL-1β-stimulated Caco-2 intestinal cell model. Their bioscreen combined measurements of intracellular ROS, NF-κB activation and IL-8 release, showing that the flavanone mix had stronger antioxidant and anti-inflammatory effects than individual compounds, highlighting synergy among citrus flavanones.

Fernández-Fernández et al. (2021) studied citrus pomace and orange pomace biscuit extracts through an in vitro digestion model followed by Caco-2 cell assays. They evaluated the bioaccessibility of phenolics and then used the bioaccessible fractions in intestinal cells subjected to oxidative stress, assessing cell viability and oxidative markers; the results indicated that digested citrus fractions retained significant antioxidant capacity and offered protection against H_2O_2-induced damage.

Immune-cell bioscreens often employ RAW 264.7 macrophages to probe anti-inflammatory mechanisms of citrus extracts. Karthikeyan et al. (2021) examined flavonoids from Citrus unshiu peel in LPS-stimulated RAW 264.7 cells, using viability (MTT), nitrite production (Griess assay), cytokine levels and expression of iNOS and COX-2; they showed that the flavonoid fraction significantly decreased NO and pro-inflammatory cytokines while downregulating iNOS/COX-2, confirming a potent anti-inflammatory profile.

Li et al. (2025) applied a similar macrophage-based bioscreen to a macroporous resin-purified extract from thinned young citrus fruits, combining classical inflammatory markers with metabolomics to map how the extract reshapes cellular metabolic pathways during the inflammatory response.

Beyond classical ethanolic extracts, biotransformed citrus extracts have been screened in co-culture systems of macrophages and adipocytes. One study on biotransformed citrus extract

reported reduced inflammatory mediators and improved adipokine profiles in LPS-stimulated RAW 264.7 cells and 3T3-L1 adipocytes, evaluated via NO, cytokines and gene-expression assays, supporting potential applications in obesity-associated low-grade inflammation (Medrano-Fernandez et al., 2017, cited in Fernández-Fernández et al., 2021).

1.6 Life cycle assessment (LCA) and lemon-waste valorisation via supercritical CO_2

Lemon processing (juice, essential oils, candied peel) generates large amounts of peel and pulp, which are often under-utilised or disposed of with significant environmental burdens. Recent LCA studies on citrus chains show that both cultivation (fertiliser use, energy for irrigation) and waste management are major contributors to global warming potential, eutrophication and ecotoxicity (Machin-Ferrero et al., 2022; Chen et al., 2021; Teigiserova et al., 2022).

In particular, Machin-Ferrero et al. (2022) quantified the environmental profile of Argentine lemon and its derivatives, demonstrating that processing stages and residue handling strongly influence the overall footprint, while Chen et al. (2021) highlighted that conventional disposal routes for citrus residues in China are often environmentally sub-optimal and that valorisation scenarios can substantially improve performance.

Within this context, LCA is increasingly applied to evaluate citrus-waste biorefineries and cascading valorisation concepts where supercritical CO_2 ($scCO_2$) extraction of limonene is a key first step (Joglekar et al., 2019; Teigiserova et al., 2022; Medina-Herrera et al., 2024).

Supercritical CO_2 extraction has long been investigated for citrus peel essential oils as a cleaner alternative to steam distillation and hexane extraction. Early studies by Mira et al. (1999) and Berna et al. (2000) optimised $scCO_2$ extraction of orange peel essential oil,

showing high limonene yields at relatively mild temperatures and highlighting the potential for solvent-free flavour production.

More recent work has extended these concepts to mixed citrus wastes and to lemon peel specifically. Romano et al. (2022) used liquid and supercritical CO_2 (with ethanol as co-solvent) to extract essential oils and phenolic fractions from orange, tangerine and lemon peels, demonstrating that CO_2-based processes can recover limonene-rich oils and flavanone-rich extracts with competitive or superior yields compared with conventional solvent extraction.

For lemon peel, Toker et al. (2025) optimised supercritical fluid extraction from dehydrated waste lemon peels, exploring 35–50 °C and 12.5–20 MPa and identifying process windows that maximise limonene content in the extract.

Mai et al. (2022) developed a two-stage $scCO_2$ process for waste Citrus grandis peel, obtaining both a limonene-rich essential-oil fraction and a naringin-rich solid fraction, and explicitly motivated their work by the need to reduce organic-solvent use in citrus-waste processing.

Together with broader reviews positioning $scCO_2$ as a green solvent with low toxicity and high recyclability (Sajal, 2025), these studies support the idea that lemon peel waste can be upgraded into high-value ingredients (limonene, flavonoids) using CO_2-based technologies as the front end of a biorefinery.

Costa et al. (2025) took a step further by explicitly addressing the techno-economic performance of $scCO_2$ limonene extraction from orange, lemon, lime and tangerine peels. Using dried citrus wastes and operating at 150 bar and 35 °C for 30 min, they estimated

manufacturing costs for limonene between 50 and 530 US$/kg extract, depending on the citrus species and process assumptions, and showed that implementing energy recovery in the CO_2 cycle could reduce production costs by about

While their study does not present a full ISO 14040/44-compliant LCA, the detailed mass- and energy-balance data provide a useful basis for future cradle-to-gate environmental assessments of lemon-waste valorisation via $scCO_2$.

Several LCA studies have analysed citrus-waste biorefineries in which essential-oil removal (often limonene) is a key stage, even if the extraction is not always performed with $scCO_2$. Joglekar et al. (2019) modelled a fruit-peel biorefinery for citrus waste and found that unit operations such as hydrolysis and flashing were dominant contributors to global warming potential, underlining the importance of energy integration and waste-heat recovery.

Teigiserova et al. (2022) performed an LCA of a scaled-up cascading biorefinery for orange peel waste producing limonene, citric acid and animal feed. They showed that valorisation pathways outperform simple disposal options in most impact categories, but also that electricity mix and process energy demand critically shape the environmental profile.

Medina-Herrera et al. (2024) reviewed citrus-waste biorefineries and summarised several LCA case studies. They reported that processing orange and lemon peels in integrated biorefineries with anaerobic digestion is environmentally preferable to land spreading or landfill, and that prior removal of essential oils (to mitigate limonene inhibition in anaerobic digestion) can further improve performance if the extraction step is energy-efficient.

Ortiz-Sanchez and Cardona Alzate (2021) compared different productive chains for orange peel waste and concluded that scenarios incorporating by-product recovery and energy co-generation reduce overall impacts compared with current low-value uses.

For whole lemons and derived products, Machin-Ferrero et al. (2022) performed an LCA in a circular-economy context and identified agricultural inputs and energy use in industrial processing as main hotspots, but also showed that valorising residues into added-value products is a promising improvement strategy.

Although most of these citrus-focused LCAs model essential-oil removal via steam distillation or conventional techniques, their system boundaries and inventory data can be adapted to alternative front-end technologies such as $scCO_2$. In particular, they provide guidance on how to allocate environmental burdens between lemon juice, lemon essential oil, limonene, animal feed and energy recovery, which is critical when assessing lemon-waste valorisation chains that start with supercritical extraction.

The environmental behaviour of $scCO_2$ extraction has been investigated more explicitly in LCAs and techno-economic-environmental assessments of other feedstocks. Moncada et al. (2014), for example, compared several extraction technologies for citronella and lemongrass essential oils in a Colombian case study, combining process simulation with economic and environmental indicators and showing that cleaner extraction routes can be competitive when energy integration is optimised.

Khalati et al. (2023) evaluated techno-economic performance, safety and life-cycle impacts for supercritical CO_2 extraction of volatile oil from Aquilaria sinensis, illustrating typical trade-offs: reduced solvent toxicity and emissions versus higher electricity demand for CO_2 compression and circulation.

Becerra et al. (2022) performed a comparative LCA of alternative processes to obtain verbenone and carvone from essential oils, including routes based on purified and raw essential oils, and underlined that solvent use, energy intensity and by-product credits are key determinants of environmental performance.

Outside the citrus sector, Candela et al. (2025) carried out an LCA for rice-bran lipid valorisation using $scCO_2$ and green solvents, combining process simulation and life-cycle modelling according to ISO 14040–44; they concluded that the most sustainable configuration balances wax yield, energy use and solvent consumption rather than simply maximising extraction yield.

Taken together with the $scCO_2$ review by Sajal (2025), which synthesises environmental and performance data from 163 case studies, these works suggest a general pattern that is highly relevant for lemon peel: $scCO_2$-based valorisation typically reduces impacts related to volatile organic solvents (human toxicity, photochemical ozone formation) and can enable higher-value product portfolios, but may increase impacts in energy-related categories (climate change, resource depletion) if electricity and heat are not optimally managed.

Despite the growing literature on citrus-waste valorisation, there is still no fully developed cradle-to-grave LCA focused specifically on lemon peel valorisation via supercritical CO_2 extraction of limonene and co-products. Existing studies provide complementary pieces: (i) process-scale data and cost estimates for mixed citrus (including lemon) $scCO_2$ extraction (Costa et al., 2025); (ii) detailed LCAs of lemon and citrus chains, where residues are handled via conventional routes or generic biorefineries (Machin-Ferrero et al., 2022; Chen et al., 2021; Joglekar et al., 2019; Teigiserova et al., 2022); and (iii) methodological

examples of LCA for $scCO_2$ extraction in other sectors (Moncada et al., 2014; Khalati et al., 2023; Candela et al., 2025).

Future LCAs on lemon-waste valorisation with $scCO_2$ will therefore need to integrate these strands: using experimental data from lemon-peel extraction studies (Toker et al., 2025; Romano et al., 2022) for inventory building, adopting citrus-specific system boundaries and allocation rules from existing LCAs, and explicitly modelling energy-integration options (heat recovery, CO_2 recirculation) in line with current industrial $scCO_2$ practice. On this basis, LCA can identify under which conditions lemon peel $scCO_2$ biorefineries genuinely improve the environmental profile of lemon-processing chains and how they should be designed to support circular-economy objectives.

CHAPTER 2 SFE EXTRACTION

2.1 Introduction

The sustainable valorisation of agro-industrial by-products is gaining increasing relevance within both the scientific community and industrial sectors, in line with the principles of the circular economy and the need to reduce the environmental impact of production chains. Among the residues generated in large quantities, lemon peels represent a particularly valuable biomatrix due to their industrial-scale availability and their high content of

bioactive compounds of nutritional, cosmetic, and pharmaceutical interest, including terpenes, flavonoids, limonoids, and other secondary metabolites. Nevertheless, the efficient recovery of these compounds requires the adoption of green extraction technologies that are capable of ensuring high selectivity while preserving molecular integrity, avoiding the use of petrochemical solvents, and reducing downstream purification steps.

In this context, supercritical CO_2 extraction (SC-CO_2) emerges as an enabling and environmentally sustainable technology. The particular physico-chemical properties of CO_2 in supercritical conditions—namely, gas-like diffusivity and liquid-like solvating power allow for an adjustable extraction selectivity controlled by pressure and temperature. Additional advantages include moderate operating conditions, low toxicity, and the absence of solvent residues in the final extracts, which is of specific relevance for applications in cosmetics, nutraceuticals, and food-grade formulations.

However, the overall efficiency of the extraction process is not determined by operating conditions alone. It is strongly influenced by the structural characteristics and moisture content of the raw biomass, which in turn affect mass transfer phenomena and the accessibility of solutes to the supercritical fluid. The establishment of optimized pre-treatment protocols (e.g., forced-air drying, lyophilization, controlled grinding, granulometric selection) is therefore a crucial step to maximize yield reproducibility and to avoid degradation or loss of volatile fractions. Pre-treatment also enables the standardization of biomass feeding strategies into the extraction vessel, improving packing density and internal flow distribution during supercritical operation.

Within this framework, the activities conducted first focused on the systematic definition and comparison of pre-treatment protocols for two categories of lemon peels: (i) fresh peels,

directly obtained from citrus-processing facilities, and (ii) ethanol-exhausted peels, derived from industrial infusion processes. The experimental plan examined the influence of lyophilization parameters, dehydration kinetics, and particle size reduction on moisture removal efficiency and on the preservation of volatile and semi-volatile target components. Subsequently, pilot-scale extraction trials were performed using a 7 L supercritical fluid extraction unit, in which extraction parameters—pressure, temperature, and CO_2 mass flow—were varied in a controlled manner following a design of experiments (DoE) approach. The objective was to determine the conditions that simultaneously optimize extraction efficiency, compositional selectivity, and process scalability.

The combined evaluation of pre-treatment effects and extraction conditions provides a framework for establishing standardized process parameters for the valorisation of citrus by-products. These results contribute to a broader strategy aimed at transforming processing residues into value-added bioactive ingredients, thus supporting the development of integrated biorefinery models and advancing the transition toward sustainable and circular production systems.

2.2 Materials and Methods

2.2.1 Matrix sampling

The matrix used for the supercritical CO_2 extraction experiments consisted of lemon peels (Citrus limon Burm.f.) sourced from the production chain of Nastro d'Oro Distillery (Sorrento, Italy), where they are generated as a by-product of limoncello manufacturing. The peels were supplied already separated from the whole fruit by the agricultural provider, ensuring traceable origin, varietal uniformity, and seasonal consistency of the raw material. This approach guarantees a matrix with reproducible chemical composition, minimizing

variability associated with manual peeling processes and reducing oxidative degradation during handling.

After acquisition, the peels underwent a 48-hour infusion process in food-grade ethanol, during which ethanol selectively extracts the volatile and aromatic fraction rich in monoterpenes—mainly limonene, β-pinene, myrcene, and related compounds. At the end of this step, the peels are depleted in essential oils, yet they retain substantial levels of phenolic metabolites, flavonoids, and structural polysaccharides, predominantly located in the albedo layer and cell wall matrix. These characteristics make the material particularly suitable for extraction strategies targeting polar and semi-polar bioactive compounds for potential cosmetic and nutraceutical applications.

Figure 3 - Lemon peels in infusion

For the extraction trials, the matrix was used in two distinct conditions:

- Lyophilized and micronized exhausted peels, obtained by freeze-drying and controlled size reduction to increase surface area and enhance CO_2 diffusion within the solid matrix.
- Exhausted peels used in their native form, without thermal or mechanical pre-treatment, to evaluate the influence of residual moisture and native tissue structure on extraction kinetics and efficiency.

2.2.2 Freeze-drying

Freeze-drying was carried out using a BÜCHI Lyovapor L-300 system (BÜCHI Labortechnik AG, Flawil, Switzerland), equipped with a chemically resistant diaphragm vacuum pump and an integrated condenser unit with an ice capacity of ≥ 8 kg. The instrument operates with automatic pressure control (P-Controller) and dynamic shelf temperature regulation, enabling stable thermodynamic gradients required for sublimation and ensuring high process reproducibility.

Prior to freeze-drying, the exhausted lemon peels were pre-frozen at –20 °C for 12 h, arranged in a uniform layer (<1 cm) on stainless steel trays to ensure homogeneous ice crystal formation and prevent tissue collapse. The freeze-drying cycle consisted of:

Controlled Freezing Phase:

- Shelf temperature: –40 °C, 2 h
- Purpose: full solidification of intracellular water.

Primary Sublimation Phase:

- Chamber pressure: 1 mbar Condenser temperature: –104 °C Shelf temperature ramp: –35 °C → –10 °C Duration: 18–24 h

Purpose: removal of unbound water through direct sublimation, monitored by pressure stabilization trends and mass-loss progression.

Secondary Drying Phase:

- Pressure: ≤ 1 mbar
- Shelf temperature ramp: up to +20 °C
- Duration: 6–8 h

Final residual moisture content was confirmed to be < 5% (w/w), ensuring microbiological stability and structural preservation.

Figure 4 - Freeze-drying of lemon peels

The obtained material exhibited a porous, low-density microstructure with enhanced specific surface area, which improved CO_2 penetration and mass transfer dynamics during the supercritical extraction stage. This treatment also prevented bed compaction and channeling within the extraction vessel, ensuring consistent extraction performance.

2.2.3 Milling of lemon peels

After freeze-drying, the matrix underwent mechanical milling to achieve controlled particle size reduction and increase the specific surface area for mass transfer during supercritical CO_2 extraction. Milling was carried out using a stainless-steel knife mill equipped with a radial rotor and ventilated cutting chamber, operating at a fixed rotational speed of 10,000 rpm. The knife mill was selected over hammer mills or cryogenic methods to minimize specific fracture energy and prevent shear-induced heat accumulation, which could degrade thermolabile phenolic compounds.

The freeze-dried material was processed in 30 g batches, with milling cycles limited to 60 seconds and intermittent cooling pauses to maintain the chamber temperature below 35 °C, thereby preventing volatilization of residual aromatic fractions. The milled product was subsequently sieved using a vibratory sieve with a 500 μm mesh, isolating the fine fraction ($d < 500$ μm) and standardizing the Particle Size Distribution (PSD). Oversized particles were reintroduced into the mill until the desired distribution was obtained.

Figure 5 - milling of lemon peels

The final PSD was evaluated using the RRSB (Rosin–Rammler–Sperling–Bennett) distribution model, yielding a median particle diameter (d_{50}) of approximately 350 µm with a narrow span, indicative of good uniformity. This particle size range:

- enhances bed permeability,
- increases solid–CO_2 interfacial surface area,
- prevents channeling and compaction in the extraction basket,
- improves reproducibility and efficiency of supercritical extraction.

The milled matrix was stored in airtight containers under dry and light-protected conditions to prevent oxidative and hygroscopic degradation prior to extraction.

2.2.3 Supercritical Fluid Extractor

Supercritical CO_2 extractions were performed using a pilot-scale supercritical fluid extraction system (SCFN-L7®, SepareCo, Turin, Italy), equipped with a 7 L high-pressure stainless-steel extraction vessel specifically designed for processing solid and semi-solid plant matrices. The system is configured with three dedicated separation stages, enabling the fractionation of the extracted compounds according to differences in volatility, polarity and molecular weight, thereby improving both extract purity and process selectivity.

The extraction unit is rated for operating pressures up to 300 bar and temperatures up to 80 °C, with a CO_2 flow capacity of 33 $kg \cdot h^{-1}$, ensuring suitable throughput for both experimental optimization studies and preliminary process scale-up. CO_2 circulation, heating, cooling and decompression are fully automated.

System operation is controlled through a PLC (Programmable Logic Controller) integrated with a SCADA (Supervisory Control and Data Acquisition) interface, enabling:

- continuous monitoring of pressure, temperature, and flow rate,
- automated feedback-based process modulation,
- data logging for traceability and reproducibility,
- alarm and safety interlock management during high-pressure operation.

Prior to extraction, the pre-treated biomass was manually loaded into the extraction vessel and compacted gently to avoid channeling while maintaining adequate permeability to the CO_2 flow. After sealing, the vessel was pressurized and heated to the selected supercritical conditions, and the extraction was allowed to proceed for the predetermined residence time.

The outgoing supercritical stream was directed through a three-stage separation train, configured as follows:

- Separator S1 (pressure let-down unit): A controlled stepwise depressurization initiated selective precipitation of less volatile constituents.
- Separator S2 (heated cyclone separator): Maintained at elevated temperature to prevent premature condensation of volatile and low-molecular-weight compounds. Under these conditions, water and light terpenoid species remain in the supercritical or gaseous phase, minimizing coalescence and phase entrapment.
- Separator S3 (cooled cyclone separator): Operated at low temperature to promote condensation and collection of volatile and semi-volatile components, including essential oil (EO) fractions.

Figure 6 - SFE Plant

The final extract was recovered at ambient pressure, minimizing structural alteration and oxidation.

The recovered EO fractions were immediately transferred into amber borosilicate vials and stored at 4 °C in darkness to prevent oxidation, photo-degradation, and thermal decomposition. Food-grade CO_2 (purity ≥ 99.9%) was supplied by Nippon Gases (Milan, Italy), ensuring compliance with standards for nutraceutical and cosmetic applications.

The extraction yield was determined gravimetrically by relating the mass of extract recovered to the mass of the dry matrix subjected to extraction. The yield was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Extract mass (g)}}{\text{Dry matrix mass (g)}} \times 100$$

where:

- Dry matrix mass (g) corresponds to the accurately weighed mass of the plant material loaded into the extraction vessel after pre-treatment and moisture normalization, ensuring that yield values are independent of residual water content.
- Extract mass (g) represents the mass of the collected fraction recovered after the separation stage, once residual CO_2 was fully released and no additional mass variation was detected upon repeated weighing.

To minimize weighing uncertainty, each sample was measured using an analytical balance (readability: 0.1 mg), and results were recorded as the mean ± standard deviation of three independent extraction replicates. Prior to weighing, extracts were stored for 24 h

in the dark at 4 °C, allowing complete depressurization and stabilization under atmospheric conditions, thus preventing artifacts associated with solvent retention or condensation.

This gravimetric approach enables direct comparability between extraction runs and provides quantitative evaluation of process efficiency as a function of biomass pre-treatment, extraction pressure, temperature, and CO_2 flow rate.

2.3 Results and Discussion

2.3.1 Matrix Pretreatment

SFE performance is highly sensitive to the physical state of the feed. Particle size, shape, specific surface area, bed porosity, bulk density, and residual moisture govern CO_2 penetration and internal diffusion, as well as external mass-transfer and pressure-drop along the packed bed (22). In practice, inadequate pretreatment can lead to channelling, early saturation of the solvent phase, and poor reproducibility between runs. For these reasons, the matrix was systematically pre-conditioned before extraction.

Lemon peels were first rinsed to remove soluble sugars and surface impurities, manually trimmed to eliminate albedo in excess where present, and cut into strips (~5–10 mm width) to improve drying kinetics. Samples were then freeze-dried (bench-scale lyophilizer) under controlled conditions: shelf temperature −45 °C during primary drying, chamber pressure 0.10–0.15 mbar, followed by a secondary-drying step at +20 °C to desorb bound water. Three lyophilization durations (12, 24, and 36 h) were screened (Table 1). End-point criteria were: (i) mass-loss rate <0.01 g h^{-1} over the last 2 h; (ii) target residual moisture <5% w/w (AOAC 925.10) or, alternatively, <0.30 water activity (aw); and (iii) preservation of a porous, non-collapsed microstructure (visual and tactile assessment). Among the tested

conditions, 24 h was identified as the minimum time ensuring the same extent of mass reduction and residual moisture achieved by longer cycles, while maintaining structural integrity comparable to the "infused peels" reference used for benchmarking. Shorter cycles (12 h) led to higher residual moisture and partial pore collapse, which are known to hinder CO_2 diffusivity and depress yield (22).

After drying, peels were cryo-assisted milled (knife mill with intermittent liquid-nitrogen dosing) to mitigate thermal rise and volatilization losses. The ground material was sieved to defined fractions (250–500 μm; 500–1000 μm; 1000–2000 μm). For SFE campaigns, the 500–1000 μm cut was selected as a trade-off between enlarged external area and acceptable bed permeability. Very fine powders (<250 μm) increased pressure drop and favored channeling, whereas coarse particles (>1 mm) reduced interfacial area and prolonged internal diffusion paths. Prior to loading, the lot was gently mixed to homogenize particle-size distribution and equilibrated at room conditions in sealed containers with desiccant. Bulk density (ρb) and bed porosity (ε) were determined gravimetrically in the extraction basket (typical values: ρb ≈ 0.32–0.38 g cm^{-3}; ε ≈ 0.40–0.48), and moisture was confirmed by oven method (105 °C) and spot-checked by Karl Fischer titration on representative samples.

The pretreated matrix was packed without over-tamping to avoid density gradients, and stainless-steel frits (10 μm) plus a thin inert spacer were used to prevent fines entrainment. This standardized pretreatment protocol—freeze-drying for 24 h to low aw, followed by controlled grinding/sieving—yielded stable bed hydrodynamics and enhanced mass-transfer, improving extraction efficiency and run-to-run reproducibility.

Matrix	Duration (h)	Reduction %
Exhausted peel	65	87%
Exhausted peel	36	87%
Exhausted peel	24	87%

Table 1 - Different freeze-drying time and water reduction %

2.3.2 Optimization of SFE

Optimization of SFE requires the simultaneous consideration of thermodynamic and kinetic factors, as extraction efficiency depends on both the solubility of target solutes in the supercritical phase and the rate at which these solutes are transported from the solid matrix into the bulk fluid. In supercritical CO_2 systems, solvent density, solute vapor pressure, diffusivity, and mass-transfer resistance are interdependent variables shaped primarily by pressure, temperature, flow rate, and extraction time. Therefore, establishing optimal operating conditions involves identifying the regime where solvent power and mass-transport rates are maximized without compromising the stability of heat-sensitive compounds.

Several studies report that, at constant temperature, increasing pressure improves extraction yields by increasing the density of CO_2 and thus enhancing its ability to solvate non-polar and moderately polar constituents . However, this effect is not linear. Beyond certain thresholds (generally 250–300 bar for citrus matrices), diffusivity decreases due to increased fluid viscosity and reduced molecular mobility, resulting in slower penetration of CO_2 into plant microstructures. Therefore, pressure selection must balance the competing effects of density-driven solubility and transport-related diffusional limitations.

Temperature similarly influences extraction through a dual mechanism. As temperature increases, the vapor pressure of solutes increases, improving their thermodynamic

propensity to partition into the CO_2 phase . In contrast, increases in temperature at constant pressure reduce CO_2 density, which tends to diminish its solvent strength. This trade-off generates an “optimal temperature window” characteristic of supercritical systems, often between 40 and 55 °C for matrices rich in monoterpenes and phenolic derivatives. Importantly, temperatures exceeding 55–60 °C risk thermally induced rearrangements, oxidation, or hydrolysis of sensitive compounds, including limonoids and oxygenated monoterpenes. This aspect is particularly relevant in citrus peel extracts, where antioxidant and antimicrobial activities depend on preserving native structural integrity.

The influence of CO_2 flow rate is primarily kinetic. Machmudah et al. demonstrated that, under high pressure and moderate temperature, increasing flow rate enhances mass transfer through both internal diffusion and external film transport, ultimately improving solute desorption and convective removal. Nonetheless, excessively high flow rates may shorten the residence time of the supercritical phase, preventing the system from reaching local equilibrium. Therefore, flow rate must be sufficiently high to maintain concentration gradients without hindering solute dissolution dynamics.

Extraction time (or total CO_2 throughput) dictates the extent of solute depletion from the matrix. During the initial extraction period, easily accessible compounds (surface-located and weakly bound) are rapidly solubilized, resulting in a sharp increase in yield (so-called “constant-rate” phase). Subsequently, as extraction progresses into the “falling-rate” phase, transport becomes diffusion-limited, and additional yield increases require disproportionately longer extraction times. Thus, prolonged extraction improves recovery but must be evaluated in the context of overall process efficiency, CO_2 consumption, and potential changes in selectivity.

Based on these considerations, optimization of SFE in this study followed a single-parameter variation strategy, modifying pressure, temperature, and flow rate individually while maintaining all other variables constant. Each parameter set was applied in triplicate to ensure statistical reliability, and extraction yields are reported as mean ± standard deviation.

Table 2 summarizes the extraction conditions investigated and their corresponding yields. The observed extraction efficiencies ranged from 1.37% to 10.18%. Notably, the highest yield (10.18%) was obtained at 50 °C, 300 bar, and a CO_2 flow rate of 10 $kg \cdot h^{-1}$. These conditions reflect an optimal thermodynamic–kinetic balance in which:

- High pressure (300 bar) ensures elevated CO_2 density and solvation power.
- Moderate temperature (50 °C) increases solute vapor pressure without causing degradation. Sufficient flow rate (10 $kg \cdot h^{-1}$) maintains strong concentration gradients and efficient mass transfer.

Extraction time allows transition through both constant-rate and diffusion-limited stages while remaining industrially feasible.

n.	**Matrix**	**Treatment**	**Quantity (g)**	**Pressure (bar)**	**Flux (Kg h^{-1})**	**Temperature (°C)**	**Kg CO_2**	**Yield %**
1	Exhausted peel	NL	600	300	20	50	80	9.45 ± 1.9
2	Exhausted peel	L	300	300	20	50	80	8.2 ± 0.5
3	Exhausted peel	L	300	300	10	50	80	10.18 ± 1.0
4	Exhausted peel	L	300	300	20	60	80	2.21 ± 0.3
5	Exhausted peel	L	300	200	20	50	80	1.37 ± 0.5
6	Exhausted peel	L	300	200	20	60	80	2.22 ± 0.3
7	Exhausted peel	L	300	200	10	60	80	2.61 ± 1.4
8	Exhausted peel	L	300	200	10	50	80	2.26 ± 1.2

9	Exhausted peel	L	300	300	10	60	80	3.70 ± 0.4

Table 2- Extraction parameters for lemon peels. L: Lyophilization; NL: not lyophilization; Yield: mean three replicates ± SD of three replicates

Accordingly, 50 °C, 300 bar, and 10 $kg \cdot h^{-1}$ were selected as the standardized operational conditions for subsequent extractions and characterization assays, ensuring both high yield and preservation of the functional integrity of bioactive compounds.

CHAPTER 3 CHEMICAL ANALISYS

3.1 Introduction

Gas chromatography (GC) is an analytical separation technique widely employed for the qualitative and quantitative characterization of volatile and semi-volatile organic compounds. The underlying principle of GC is based on the differential distribution of analytes between a mobile gas phase and a stationary phase, according to their physicochemical properties, such as volatility, polarity, and intermolecular interactions. The chromatographic system consists of a thermostatically controlled oven housing the analytical column, typically a fused-silica capillary several meters in length, coated internally with a thin film of stationary phase. The structural and chemical nature of this stationary phase critically influences analyte–phase interactions, and therefore governs retention behavior and separation efficiency.

Sample introduction is generally achieved via an autosampler coupled to a heated injector, where the analyte is volatilized and carried into the column by an inert carrier gas (commonly helium, hydrogen, or nitrogen). As analytes migrate through the column, they partition continuously between the gaseous mobile phase and the stationary phase coating the inner column wall. Compounds with lower affinity for the stationary phase or higher vapor pressure elute more rapidly, while those exhibiting stronger interactions require longer transit times. At the column outlet, the separated analytes are detected by a suitable detector, capable of converting their presence into a measurable electrical signal and transmitting it to a data processing system for peak integration and identification.

In advanced analytical workflows, gas chromatography is frequently hyphenated with mass spectrometry (GC-MS), wherein the effluent from the chromatographic column is introduced directly into a mass spectrometer. Mass spectrometry provides structural information based

on analyte ionization and fragmentation patterns, which are highly characteristic for individual chemical species. The combined GC-MS technique therefore enables compound identification through both retention time and mass spectral matching against established databases, offering superior selectivity and analytical confidence. For this reason, GC-MS is considered a gold-standard methodology for the characterization of complex organic mixtures.

In the present study, GC-MS analysis was employed to characterize the chemical composition of an essential oil obtained through supercritical CO_2 extraction. Essential oils are natural products derived from aromatic plants and are generally characterized by a complex and heterogeneous chemical profile. They consist predominantly of volatile and thermolabile constituents, including monoterpenes, sesquiterpenes, and their oxygenated derivatives. The relative abundance and structural diversity of these compounds are responsible for the biological, sensory, and functional properties of essential oils, such as antimicrobial, antioxidant, and aromatic activities. However, this chemical complexity also necessitates the use of analytical techniques capable of resolving structurally similar compounds, isomers, and trace constituents with high sensitivity and precision.

Supercritical CO_2 extraction provides a gentle and selective means of obtaining essential oils, allowing the recovery of volatile compounds under moderate temperatures and without the use of organic solvents. This is particularly advantageous for preserving thermosensitive and aroma-defining molecules. Consequently, GC-MS serves as an indispensable tool in quantifying and elucidating the compositional profile of supercritical CO_2 extracts, enabling detailed assessment of extraction efficiency, quality attributes, and potential bioactivity.

3.2. Materials and Methods

3.2.1 Plant Material and Sample Preparation

Fresh lemon peels (Citrus limon (L.) Burm. f.) were sourced from certified organic processing batches to ensure reproducibility and to avoid contamination from pesticides or post-harvest treatments. Peels were manually separated from the pulp, rinsed with distilled water to remove surface sugars and impurities, and immediately frozen at −20 °C to limit enzymatic degradation. Freeze-drying was carried out using a bench-top lyophilizer under controlled pressure (0.10–0.15 mbar) and shelf temperature (−45 °C), followed by secondary drying at +20 °C to reduce bound moisture. The dried material was cryo-milled and sieved to obtain a particle-size fraction of 500–1000 µm, selected to optimize mass transfer and bed permeability in supercritical extraction.

3.2.2 Supercritical CO_2 Extraction Procedure

Extraction was performed using a pilot-scale supercritical fluid extraction unit equipped with a 7 L stainless-steel extraction vessel. Operational parameters were optimized based on a single-variable approach and finalized at 50 °C, 300 bar, and CO_2 flow rate of 10 $kg \cdot h^{-1}$. Each extraction run was carried out for a fixed duration of 120 minutes after reaching steady-state pressure and temperature. Separator vessels were operated in staged pressure reduction to prevent co-precipitation of waxes and heavy terpenoids. Extracts were collected in amber, gas-tight vials with PTFE-lined caps, stored at 4 °C in complete absence of light, and analyzed within 7 days to prevent oxidative shifts in volatile composition.

3.3.3 Sample Dilution and Pre-Analytical Handling

Prior to GC-MS analysis, essential oil samples were equilibrated to room temperature and diluted 1:50 (v/v) in HPLC-grade n-hexane. To minimize analyte loss or discrimination:

- All glassware was silanized. Samples were handled using gas-tight syringes. Solutions were filtered through 0.22 µm PTFE syringe filters to remove particulates that could damage the chromatographic column. No heating steps were applied to avoid thermal degradation of monoterpene and oxygenated fractions. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Analyses were performed on a GC-MS system equipped with an autosampler and a single-quadrupole mass spectrometer. Chromatographic separation employed a (5%-phenyl)-methylpolysiloxane capillary column (e.g., 30 m × 0.25 mm i.d., 0.25 µm film thickness) suited for terpene profiling. To ensure analytical repeatability, each sample was injected in triplicate, and retention-time alignment was monitored using internal QC standards.

3.3.4 Compound Identification, Quantification, and Data Processing

Peak integration was performed using the manufacturer's chromatographic software with adaptive baseline correction. Compounds were identified based on:

- Retention time (tR) relative to chromatographic standards;
- Mass spectral matching against the NIST/EPA/NIH Mass Spectral Library (v. 2.3) with similarity index ≥ 85%;
- Linear Retention Index (LRI) calculation using n-alkane standards (C8–C20) analyzed under identical conditions, with acceptance tolerance ±10 LRI units.

Relative composition (%) was determined by area normalization without response factor correction, as commonly adopted for essential oil profiling. To verify stability, the same sample was re-analyzed after 5 and 10 days of refrigerated storage, confirming absence of significant compositional drift (variability <3%).

3.3. Results and Discussion

The samples provided by MATER consisted of lemon peel matrices. A total of 30 samples were analyzed, subdivided into three categories based on their physical and processing state (Table x):

- Exhausted freeze-dried matrix,
- Exhausted non-freeze-dried matrix,
- Fresh matrix.

The term exhausted matrix refers to lemon peels after infusion in ethanol, i.e., the residual solid material obtained following the solvent extraction step.

The analyses were conducted using an untargeted GC-MS profiling approach at the Instrumental Analysis Laboratory of the Department of Pharmacy. The objective was to determine the qualitative and semi-quantitative composition of the essential-oil-based extracts. GC-MS was selected due to its capability to resolve the high number of volatile and semi-volatile components typically present in essential oils.

The analyses were carried out using an Agilent 6850 Series II gas chromatograph coupled to a single-quadrupole Agilent 5973 Network mass spectrometer equipped with an electron impact (EI) ionization source, operated at 70 eV, with a scan range from m/z 40 to 350. Chromatographic separation was performed using a DB-5ms capillary column (poly-5% diphenyl-95% dimethylpolysiloxane, 30 m × 0.32 mm i.d., 0.25 µm film thickness). Helium (He) was used as the carrier gas at a constant flow rate of 1.0 $mL \cdot min^{-1}$.

The injector temperature was set to 280 °C, operating in split mode (split ratio 9:1). A volume of 5 µL of each essential oil sample was injected using an Agilent 7683 automatic liquid

sampler. The oven temperature program was set from 50 °C to 300 °C at a heating rate of 10 °C·min^{-1}, for a total runtime of 40 minutes.

Compound identification was performed by comparing the mass fragmentation patterns with those contained in the NIST.11 mass spectral library. Only compounds showing a match score ≥ 60% were considered, and only peaks with a relative abundance ≥ 0.30% were reported.

Samples collected from separator S3, considered to be pure oily extracts, were injected without any pretreatment. In contrast, extracts collected from separator S1 were obtained in solid or powder form, and were therefore solubilized in an ethyl acetate:n-hexane solvent mixture prior to analysis. All samples were stored at 4 °C in the absence of light until analysis. The absence of interfering peaks was confirmed by injection of pure solvent controls (ethanol, ≥99.0%).

Matrix	Extractor	Injection Date	Pre-Injection Treatment
Extract from exhausted freeze-dried peels (27.05.2024)	S3	30-May-2024	None
Extract from exhausted non-freeze-dried peels (28.05.2024)	S3	30-May-2024	None
Extract from non-freeze-dried peels (28.05.2024)	S1 + S2	Not injectable	Centrifuged 20 min; filtered with 0.45 µm and 0.22 µm syringe filters
Extract from exhausted non-freeze-dried peels (29.05.2024)	S3	30-May-2024	None
Extract from exhausted non-freeze-dried peels (29.05.2024)	S2	11-Jun-2024	None
Filtered residue post vacuum extraction (29.05.2024)	S1	30-May-2024	Whatman paper filtration

Extract from fresh peels (04.06.2024)	S3	11-Jun-2024	Syringe filtration
Extract from fresh peels (05.06.2024)	S3	11-Jun-2024	Syringe filtration
Extract from fresh peels (06.06.2024)	S3	11-Jun-2024	Syringe filtration
Extract from exhausted freeze-dried peels (11.06.2024)	S2	20-Jun-2024	None
Extract from exhausted freeze-dried peels (11.06.2024)	S3	20-Jun-2024	None
Extract from exhausted freeze-dried peels (14.06.2024)	S3	20-Jun-2024	None
Extract from exhausted freeze-dried peels (17.06.2024)	S3	25-Jun-2024	None
Extract from exhausted freeze-dried peels (18.06.2024)	S3	25-Jun-2024	None
Extract from exhausted freeze-dried peels (19.06.2024)	S3	28-Jun-2024	None
Extract from exhausted freeze-dried peels (19.06.2024)	S1	02-Jul-2024	Solubilized in 50:50 ethyl acetate:n-hexane
Extract from exhausted freeze-dried peels (20.06.2024)	S3	28-Jun-2024	None
Extract from exhausted freeze-dried peels (26.06.2024)	S3	28-Jun-2024	None
Extract from exhausted freeze-dried peels (27.06.2024)	S3	28-Jun-2024	None
Extract from exhausted freeze-dried peels (28.06.2024)	S3	08-Jul-2024	None
Extract from exhausted freeze-dried peels (01.07.2024)	S3	08-Jul-2024	None
Extract from exhausted freeze-dried peels (02.07.2024)	S3	08-Jul-2024	None
Extract from exhausted freeze-dried peels (03.07.2024)	S3	08-Jul-2024	None
Extract from exhausted freeze-dried peels (10.07.2024)	S3	17-Jul-2024	None

Extract from exhausted freeze-dried peels (15.07.2024)	S3	17-Jul-2024	None
Extract from exhausted freeze-dried peels (09.07.2024)	S3	23-Jul-2024	None
Extract from exhausted freeze-dried peels (16.07.2024)	S3	23-Jul-2024	None
Extract from exhausted freeze-dried peels (17.07.2024)	S3	23-Jul-2024	None
Extract from exhausted freeze-dried peels (18.07.2024)	S3	23-Jul-2024	None
Extract from fresh peels, Sorrento (22.07.2024)	S3	26-Jul-2024	None
Extract from fresh peels, Sorrento (25.07.2024)	S3	26-Jul-2024	None

Table 3- Overview of lemon peels extrats samples

A total of 38 essential oil extracts were analyzed, and approximately 100 compounds were identified overall. In nearly all samples, monoterpene hydrocarbons (such as limonene, γ-terpinene, and α-pinene) represented the predominant constituents. Although the composition of essential oils is highly complex, the detected compounds may be grouped into two main classes based on their biosynthetic origin. The first and major class consists of terpenes and terpenoids, while the second comprises aromatic and aliphatic compounds.

- Hydrocarbons (molecules composed exclusively of C and H): Monoterpene hydrocarbons (C_{10}), aliphatic or aromatic, unsaturated mono- or bicyclic compounds, e.g., *myrcene, limonene, pinene, phellandrene*. Sesquiterpene hydrocarbons (C_{15}), e.g., *caryophyllene, bisabolene*.
- Compounds containing C, H, and O: Alcohols (–OH functional group), e.g., *linalool, geraniol, nerol, borneol*. Aldehydes (–CHO functional group), e.g., *citral (geranial + neral), vanillin*. Phenols (aromatic –OH), e.g., *thymol*. Ethers (C–O–C functional

group), e.g., *eucalyptol*. Esters (O–C=O functional group), e.g., *linalyl formate, geranyl acetate, bornyl acetate, benzyl benzoate, terpinyl acetate*.

Based on extraction performance (yield and qualitative profile), six extracts were selected for subsequent biological evaluation and were labeled A–F.

Table x summarizes the extraction parameters for each selected sample. The following tables (Table 4 through Table 9) report the chemical composition of each extract, together with the corresponding mass spectra.

These selected extracts will be subjected to in vitro cytotoxicity testing on specific cell lines in order to preliminarily assess their potential toxicity. Furthermore, their biofunctional properties will be evaluated through targeted biochemical assays, with the aim of determining their suitability for cosmetic formulation applications.

Extract	**Pressure (bar)**	**Temperature (°C)**	**CO_2 Flow ($kg \cdot h^{-1}$)**	**Yield (%)**	**Matrix**
A	300	50	20	8.6	BLL 27.05.2024
B	300	50	10	18.76	BLL 10.06.2024
C	200	60	20	2.47	BLL 10.07.2024
D	200	50	10	3.12	BLL 15.07.2024
E	300	60	10	4.00	BLL 16.07.2024
F	200	60	10	3.60	BLL 18.07.2024

Table 4- Parameters of the selected extracts; BLL: exhausted and freeze-dried lemon peels.

No.	Identified Compound	Relative Abundance (%)
1	β-Pinene	1.00
2	β-Myrcene	0.35
3	β-Phellandrene	0.37
4	D-Limonene	18.98
5	Eucalyptol (1,8-Cineole)	0.53
6	γ-Terpinene	7.85
7	trans-β-Terpineol	1.09
8	α-Terpinolene	0.49
9	(+)-Linalool	2.04
10	Nonanal	0.98
11	(R)-(+)-Citronellal	0.58
12	1-Nonanol (Nonyl alcohol)	0.34
13	α-Terpineol	1.84
14	Nerol (cis-Geraniol)	1.99
15	β-Citral	1.60
16	Geraniol	3.17
17	Citral (Geranial + Neral)	2.39
18	Nerol acetate	2.75
19	Caryophyllene	1.83
20	trans-α-Bergamotene	1.00
21	(Z,E)-α-Farnesene	2.57
22	(E)-β-Farnesene	0.36
23	β-Bisabolene	4.59
24	1,5-Heptadiene, 2,5-dimethyl-3-methylene-	2.05
25	1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene	0.43
26	Ethyl palmitate	0.37
27	5,7-Dimethoxycoumarin (Citropten)	0.61
28	Bis(2-ethylhexyl) hexanedioate*	0.51

Table 5 - Composition of extract A

No.	Identified Compound	Relative Abundance (%)
1	α-Pinene	0.70
2	β-Pinene	2.69
3	(–)-β-Pinene	2.01
4	D-Limonene	31.35
5	γ-Terpinene	6.51
6	trans-β-Terpineol	0.46
7	(+)-Linalool	0.84
8	Nonanal	0.54
9	(R)-(+)-Citronellal	0.35
10	α-Terpineol	1.68
11	Nerol (cis-Geraniol)	1.53
12	β-Citral (Neral)	1.60
13	Geraniol	1.89
14	Citral (Geranial + Neral)	2.35
15	α-Citral (Geranial)	2.64
16	Nerol acetate	3.21
17	Geranyl acetate	1.34
18	3,5-Heptadienal, 2-ethylidene-6-methyl-	0.76
20	Caryophyllene	1.87
21	trans-α-Bergamotene	2.04
22	Alloaromadendrene	0.48
23	(+)-Valencene	2.46
24	1,5-Heptadiene, 2,5-dimethyl-3-methylene-	1.39
25	β-Bisabolene	3.63
26	Ethyl palmitate	0.38
27	5,7-Dimethoxycoumarin (Citropten)	0.65
28	Ethyl linoleate	0.32
29	Ethyl linolenate	0.35
30*	Bis(2-ethylhexyl) hexanedioate*	7.86

Table 6 - Composition of Extract B

No.	Identified Compound	Relative Abundance (%)
1	Trimethylphosphine	2.85
2	Eucalyptol (1,8-Cineole)	0.39
3	1-Octanol	0.56
4	Linalool	1.93
5	(–)-4-Terpineol	2.74
6	L-α-Terpineol	9.43
7	Nerol (cis-Geraniol)	8.97
8	β-Citral (Neral)	4.21
9	Geraniol	9.96
10	Citral (Geranial + Neral)	6.22
11	8-Hydroxymenthol	1.46
12	Nerol acetate	1.75
13	Geranyl acetate	0.63
14	Vanillin	0.78
15	Ethyl vanillin	0.52
16	Syringaldehyde	0.55
17*	N-Butylbenzenesulfonamide*	6.06
18	Palmitic acid	0.92
20	Scoparone	1.26
21	5,7-Dimethoxycoumarin (Citropten)	9.56
22	Oleic acid	3.14
23	Stearic acid	0.49
24	11,13-Dimethyl-12-tetradecen-1-ol acetate	0.91

Table 7- Composition of Extract C

No.	Identified Compound	Relative Abundance (%)
1	Linalool	1.44
2	(–)-4-Terpineol	5.29
3	L-α-Terpineol	10.02
4	Nerol (cis-Geraniol)	8.32
5	β-Citral (Neral)	3.75
6	Geraniol	11.18
7	Citral (Geranial + Neral)	4.54
8	Vanillin	1.31
9	N-Butylbenzenesulfonamide*	7.41
10	Tridecanoic acid	1.79
11	Scoparone	1.35
12	5,7-Dimethoxycoumarin (Citropten)	8.70
13	Petroselinic acid	5.31
14	2-Methyl-(Z,Z)-3,13-octadecadien-1-ol	1.51

Table 8 - Composition of Extract D

No.	Identified Compound	Relative Abundance (%)
1	6-Methyl-3,5-heptadien-2-one	0.71
2	Cyclopentanol, 1-(methylenecyclopropyl)-	1.37
3	Linalool	2.84
4	(–)-4-Terpineol	3.04
5	L-α-Terpineol	14.02
6	Nerol (cis-Geraniol)	9.48
7	β-Citral (Neral)	1.40
8	Geraniol	10.14
9	Citral (Geranial + Neral)	1.95
10	Nerol acetate	0.52
11	N-Butylbenzenesulfonamide*	3.64
12	Palmitic acid	1.94
13	Scoparone	0.76
14	5,7-Dimethoxycoumarin (Citropten)	3.55
15	Linoleic acid (9,12-octadecadienoic acid)	10.75

Table 9 - Composition of Extract E

Detected Compounds	Relative Abundance
1. 2,3-Dehydro-1,8-cineole	1.70
2. Linalool	2.28
3. (-)-4-Terpineol	3.58
4. L-α-Terpineol	12.27
5. Nerol (cis-geraniol)	8.32
6. β-Citral	2.41
7. Geraniol	8.26
8. Citral	2.78
9. Nerol acetate	0.53
10. Vanillin	0.63
11. Benzenesulfonamide, N-butyl-	3.26
12. Palmitic acid	2.10
13. Scoparone	0.64
14. 5,7-Dimethoxycoumarin (Citropten)	3.54
15. 9,12-Linoleic acid	14.63
16. Butyl linoleate	5.14
17. Linoleic acid, 10,13-methyl ester	0.40
18. Glycerol-2-linoleate	1.35
19. 1H-Indene, 2-butyl-5-hexyl-octahydro-	0.39
20. Methyl 9,12-heptadecadienoate	0.75
21. n-Propyl linoleate	7.94

Table 10 - Composition of Extract F

CHAPTER 4
BIOCOMPATIBILITY ANALYSIS OF EXTRACTS

4.1 Introduction

The biocompatibility assessment of extracts A, C, and E (for composition details, see the previous chapter/report) was performed using immortalized human keratinocyte cell lines as an in vitro model of skin tissue. This experimental choice is motivated by the physiological relevance of keratinocytes in the epidermal barrier and by safety requirements for ingredients intended for potential dermo-cosmetic applications or skin-contact devices. The primary objective of this chapter is to quantify any dose-dependent cytotoxicity of the extracts and to define a concentration range devoid of measurable adverse effects (in vitro NOAEL), thereby laying the groundwork for subsequent functional studies.

The primary endpoint was cell viability, determined by the colorimetric MTT assay, which estimates mitochondrial metabolic activity in viable cells. To minimize analytical bias, the study included negative controls (vehicle), cytotoxic positive controls (e.g., a standard surfactant/oxidant), optical interference controls (extract blanks at the same concentrations without cells), and checks for signal linearity. The extracts were tested across a graded concentration range and at multiple exposure times, with technical and biological replicates ($n \geq 3$), and statistical analysis by ANOVA with multiple comparisons versus control (e.g., Dunnett's test). In line with common in vitro cytotoxicity practices, a reduction in viability below widely accepted thresholds (≈70% relative to control) was considered indicative of a relevant cytotoxic effect.

4.2 Materials and Methods

4.2.1 Cell cultures

Two independent experiments were performed, each including five replicates for every extract under investigation. Human keratinocytes (HaCaT line), employed as an epidermal in vitro model, were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded into 96-well plates at approximately 6,000 cells in 190 µL per well. After a 24-hour attachment period under standard culture conditions (37 °C, humidified 5% CO_2), cells were exposed for 24 hours to five concentrations of each extract spanning 50 µg/mL to 2.5 mg/mL. The specific test concentrations are reported in the accompanying table.

To ensure data quality and interpretability, each plate included vehicle (negative) controls, a cytotoxic positive control, and extract-only blanks (medium plus extract without cells) to monitor potential optical interference in the colorimetric readout. Plate maps were arranged to minimize edge effects and to randomize treatment positions across technical replicates. Following exposure, cell viability was quantified as described in the MTT assay section, and results from the two independent runs were combined after confirming reproducibility across experiments.

Solutions	[ ] cells
1	50 ug/mL
5	0,250 mg/mL
10	0.5 mg/mL
25	1.25 mg/mL
50	2.5 mg/mL

The extracts, previously lyophilized, were first solubilized in 100% DMSO and then diluted to prepare stock solutions at 50 mg/mL in 90% H_2O / 10% DMSO (v/v). The solvent mixture (90% H_2O / 10% DMSO) served as the negative (vehicle) control, while 100% DMSO was used as the cytotoxic positive control. The final DMSO concentration in all treatment conditions was 0.5% (v/v).

4.2.2 MTT Assay

The assay relies on the ability of mitochondrial dehydrogenases in viable cells to reduce MTT (blue tetrazolium bromide) to insoluble purple formazan crystals. After the treatment period, the culture medium was removed. Each well was then incubated with 200 µL of MTT working solution (5 mg/mL) for 2 hours and 15 minutes at 37 °C in a humidified 5% CO_2 atmosphere. The MTT solution was subsequently aspirated, and 200 µL of DMSO were added to each well to solubilize the formazan. Plates were gently agitated to ensure complete dissolution, and absorbance was measured at 550 nm using a microplate spectrophotometer.

To account for potential optical interference by the extracts, extract-only blanks (medium + extract + MTT without cells) were included and used for background correction. Vehicle control wells (solvent only) were set to 100% viability. Cell viability was calculated as:

$$\text{Viability (\%)} = \frac{A_{550,\,sample} - A_{550,\,blank}}{A_{550,\,vehicle} - A_{550,\,blank}} \times 100.$$

4.2.3 Antimicrobical activity

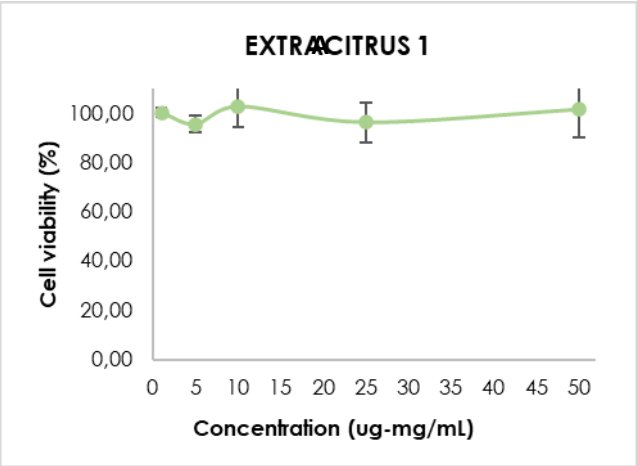
Antimicrobial activity was evaluated by an agar diffusion assay (Kirby–Bauer well/disk variant) using representative bacterial and fungal strains. Lyophilized extracts were solubilized in 100% DMSO and diluted to the desired test concentrations; unless otherwise

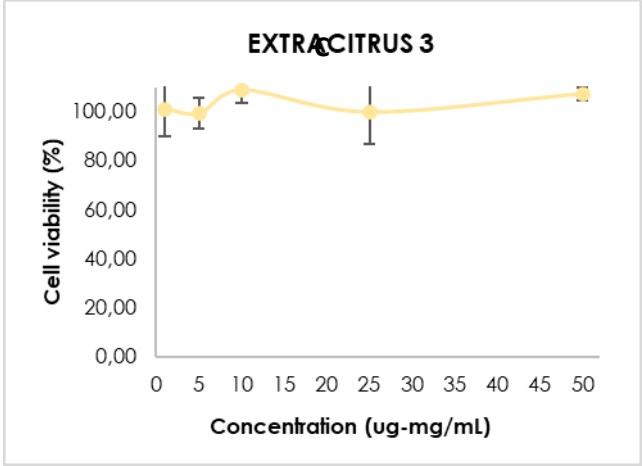
stated, the vehicle was adjusted so that the final DMSO content applied to plates matched that of the negative control. Test organisms included Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, and the yeast Candida albicans. Bacterial assays were performed on Mueller–Hinton agar, while Sabouraud dextrose agar was used for C. albicans. Fresh cultures were standardized to a 0.5 McFarland turbidity (approximately 1–2 × 10^8 CFU/mL for bacteria and ~10^6 CFU/mL for yeast), then lawn-inoculated across the agar surface with sterile swabs. Sterile 6 mm wells (or, alternatively, paper disks) were loaded with defined volumes of the extract solutions prepared at multiple concentrations established during preliminary trials; plates received in parallel a solvent-only negative control (100% DMSO applied in the same volume regime) and a reference positive control chosen from literature-supported agents effective against the target organisms (e.g., ciprofloxacin or gentamicin for bacteria; amphotericin B or fluconazole for C. albicans). Inoculated plates were incubated under standard conditions—35 ± 2 °C for 18–24 h for bacteria and 30–35 °C for 24–48 h for C. albicans—in a non-CO_2, humidified environment. After incubation, inhibition zones were measured in millimeters, including the well/disk diameter; each condition was tested in at least triplicate on independent plates, and data were summarized as mean ± standard deviation. To control for optical or chromatic interference by colored extracts, extract-only blanks were applied to uninoculated plates and used to inform plate reading. Statistical comparisons of halo diameters were conducted by one-way ANOVA with post hoc multiple comparisons versus the vehicle control (Dunnett's test), using a two-sided $\alpha = 0.05$.

4.3 Results and Discussion

4.3.1 Biocompatibility test results

Cell viability was expressed as a percentage relative to the vehicle control. As shown by the concentration–response curves (Figure 1), none of the extracts exhibited significant cytotoxicity toward HaCaT cells after 24 h of exposure within the tested concentration range. Across all conditions, mean viability remained above commonly accepted in vitro thresholds for non-cytotoxicity (≈70% of control), with no statistically significant reductions versus the vehicle by one-way ANOVA with Dunnett's post hoc test ($\alpha = 0.05$). The reference positive control produced the expected decrease in viability, confirming assay sensitivity and the absence of ceiling/floor effects. On this basis, the entire concentration window evaluated can be considered biocompatible for subsequent functional assays (in vitro NOAEL within the tested range).





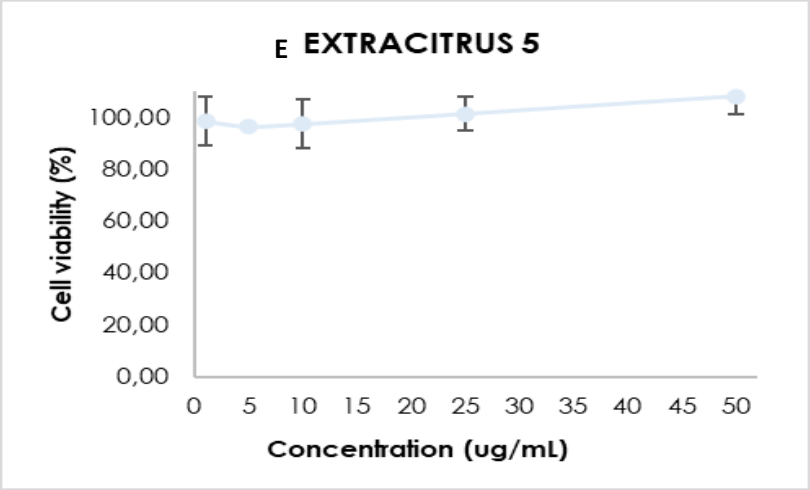


Figure 7 - Cell viability of the selected extracts on human keratinocytes.

Biocompatibility was reassessed on a new batch of immortalized human keratinocytes (HaCaT) and, in parallel, on immortalized human dermal fibroblasts (HDF). HaCaT cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. HDF cells were cultured in Fibroblast Medium (FM) supplemented with the vendor-recommended Fibroblast Growth Supplement (FGS). All cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2 and handled according to the respective supplier guidelines. Prior to experimentation, both cell lines were confirmed mycoplasma-free, used within recommended passage ranges, and seeded at sub-confluent density to ensure logarithmic growth. Unless otherwise specified, assays were performed on cells that had been equilibrated for at least 24 h under standard culture conditions.

4.3.2 In vitro bioscreen

Samples were tested across a concentration range of 5 to 1000 µg/mL for 48 h on both immortalized human keratinocytes (HaCaT) and immortalized human dermal fibroblasts (HDF). Extracts were solubilized in DMSO and diluted in culture medium; the vehicle was

matched in all conditions. To exclude solvent-related artifacts, DMSO cytotoxicity was profiled independently at graded concentrations up to 0.8% v/v (the maximal DMSO content present at the highest extract dose), showing no appreciable impact on cell survival within the working window (data not shown).

Cellular responses to treatment are reported as a **Cell Survival Index (CSI)**, a composite metric integrating metabolic viability with total cell number. Viability was quantified by the MTT assay (mitochondrial dehydrogenase activity), while total cell counts and live/dead ratios were obtained with a TC20™ automated cell counter using trypan blue exclusion in a single-pass readout. For each condition, MTT absorbance values (blank-corrected; vehicle set to 100%) and total cell counts (normalized to vehicle) were combined as:

$$\text{CSI (\%)} = \left(\frac{\text{MTT viability (\%)}}{100}\right) \times \left(\frac{\text{Total cell count (\%)}}{100}\right) \times 100.$$

This formulation penalizes treatments that reduce metabolic activity, proliferative capacity, or both, providing an aggregate measure of cell survival.

Concentration–response relationships were modeled by nonlinear regression (four-parameter logistic, variable slope) in GraphPad Prism 8.0. Curve fits were constrained to biologically plausible bounds (Bottom ≥ 0, Top ≤ 120), and doses were log-transformed prior to fitting. Data are presented as mean ± SEM with **n = 12** per concentration (pooled from three independent experiments). Unless otherwise specified, statistical significance versus the vehicle control was assessed by one-way ANOVA followed by Dunnett's post-hoc test (two-sided $\alpha = 0.05$). Between-cell-line differences in curve parameters (e.g., Top, Bottom, EC50) were explored by extra-sum-of-squares F-tests.

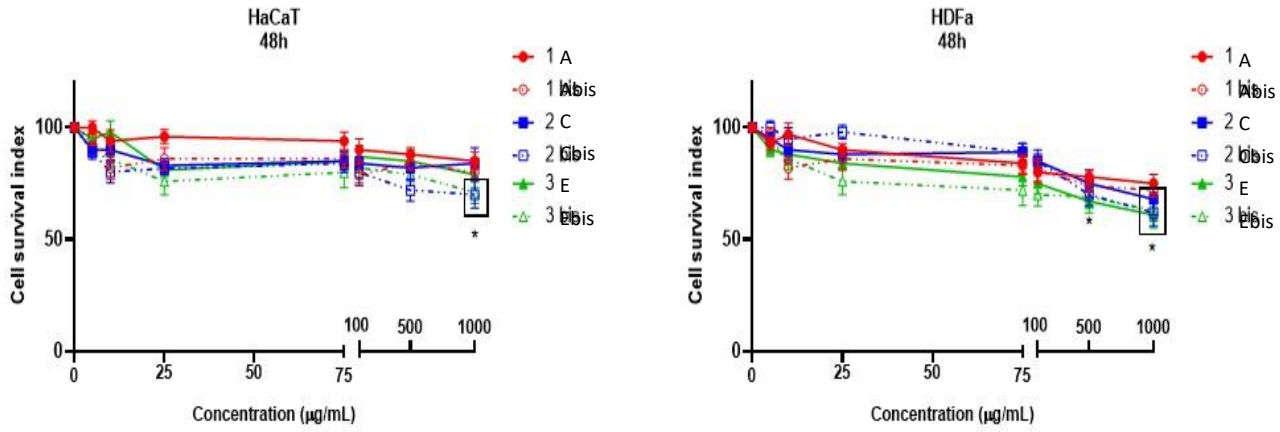


Figure 8 - Biocompatibility test results on keratinocytes (HaCaT) and fibroblasts (HDFa).

Using the agar diffusion ("halo test") method, the extracts A, C, and E were challenged against Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, and Candida albicans. Across the concentration/volume window optimized in preliminary trials, none of the extracts generated measurable inhibition zones (halo diameter = 0 mm, within reading error), while the reference antibiotic/antifungal produced clear, strain-appropriate halos and the vehicle control (100% DMSO, volume-matched) showed no inhibition. These internal controls verify inoculum quality, plate performance, and readout validity, indicating that the lack of halos is attributable to the extracts rather than assay failure.

Operationally, agar diffusion preferentially detects small, sufficiently water-mobile actives; thus, the null result does not exclude antimicrobial potential per se. The extracts are complex, likely enriched in hydrophobic and/or higher-molecular-weight constituents with limited agar permeability and local bioavailability. Additional masking factors may include: (i) solubility limits at the application site, (ii) non-specific binding of constituents to agar

components, (iii) rapid local quenching by matrix effects, and (iv) DMSO fraction constraints imposed to preserve microbial viability. Notably, P. aeruginosa presents an added barrier due to efflux and outer-membrane properties, whereas S. aureus/S. epidermidis and C. albicans can display tolerance that requires sustained exposure rather than a single bolus dose on solid media.

Given these constraints, broth-based assays are the logical next step. Minimum inhibitory/fungicidal concentration (MIC/MBC/MFC) testing by CLSI/EUCAST microdilution would address diffusion limitations and provide quantitative potency estimates. Time–kill kinetics could reveal slow-onset or concentration-dependent effects that disk/well diffusion can miss. If sub-MIC effects are suspected (e.g., anti-virulence or membrane-modulatory actions), checkerboard assays for synergy with standard antibiotics, post-antibiotic effects, or quorum-sensing/biofilm inhibition tests (crystal violet biomass, metabolic resazurin readouts) are advisable. Nanoformulation (e.g., cyclodextrin complexation, liposomes, or polymeric micelles) and pH/ionic-strength tuning may further enhance dispersion and target engagement without exceeding acceptable solvent levels.

From a safety-by-design perspective, the absence of agar-detectable antimicrobial activity is consistent with the extracts' in vitro biocompatibility profiles on HaCaT and HDF cells reported above, and reduces concern for broad, non-selective cytotoxicity at the skin interface. Overall, the halo test indicates no agar-diffusible antimicrobial effect for the extracats A, C, and E under the conditions used. However, a structured follow-up program—standardized microdilution, time–kill studies, biofilm and anti-virulence endpoints, and formulation-guided delivery—remains warranted to fully resolve antimicrobial potential and mechanism, if any.

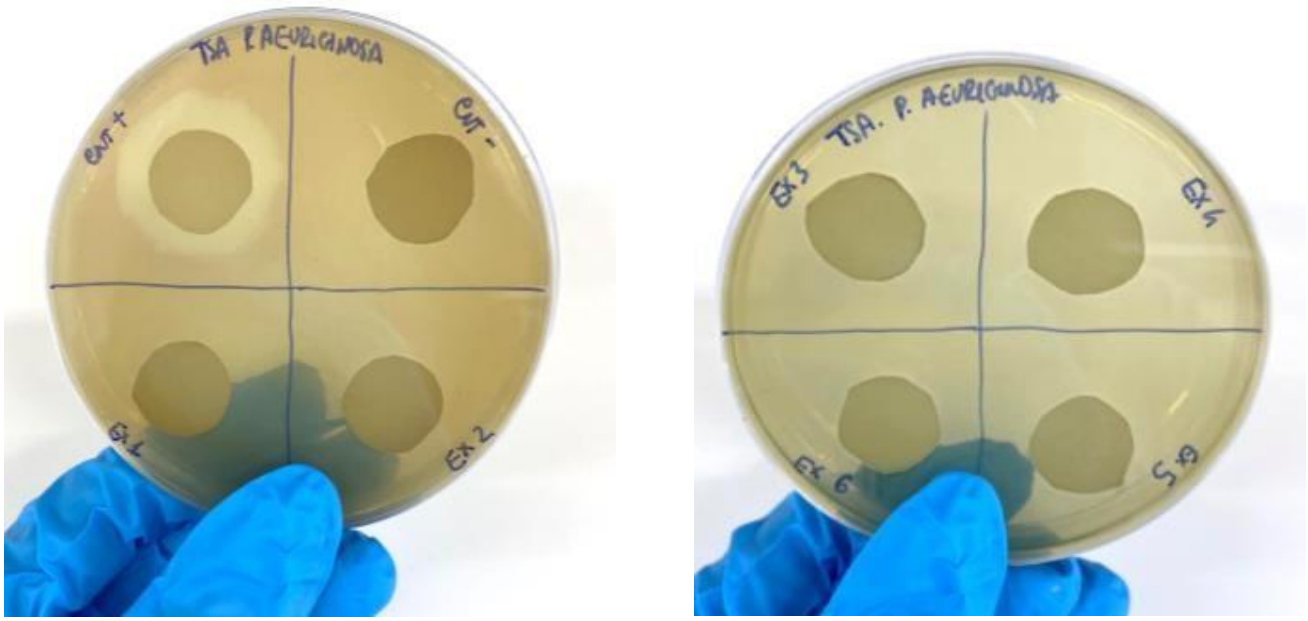


Figure 9 - Results of the HALO test on Pseudomonas aeruginosa for the extracts under investigation.

Based on the in vitro assays and the qualitative–quantitative composition of each extract, all samples proved biocompatible within the tested ranges, with Extract A showing a more favorable cell-survival profile than C and E. None of the extracts displayed agar-diffusible antimicrobial activity in the halo test against the bacterial and fungal strains considered. In terms of fragrance-determining constituents, the extracts exhibited a broadly similar bouquet of volatiles, suggesting comparable olfactory behavior.

From a compositional standpoint, the methoxycoumarins scoparone and citropten were both detected in Extract E and were absent in Extract C; scoparone was exclusive to E among the samples examined, while citropten was present in both A and E. These findings position Extract A as the leading candidate for skin-contact applications when safety and sensory attributes are weighed together. The absence of detectable halos should not be interpreted as definitive lack of antimicrobial potential, given the diffusion limits of agar assays; quantitative broth microdilution (MIC/MBC/MFC), time–kill kinetics, or formulation-

guided strategies (e.g., improved solubilization or nanoencapsulation) may still reveal activity not observable on solid media. Finally, the presence of methoxycoumarins invites routine checks on photostability and photobiology in final formulations, even though these compounds are generally less photoreactive than classic furocoumarins.

CHAPTER 5 LCA OF SUPERCRITICAL CO_2 EXTRACTION FROM LEMON PEELS

5.1 Introduction

The growing awareness of the environmental impact of industrial processes has made sustainability a central priority for many companies. In this context, assessing the ecological footprint of production activities has become essential to minimise waste and optimise the use of resources. This thesis addresses this need by providing an in-depth analysis of the environmental sustainability of the process, a project focused on the valorisation of citrus-processing by-products.

Citrus fruits, well known for their richness in bioactive compounds, have great potential not only from a nutritional perspective but also for nutraceutical applications. Among the by-products generated during limoncello production, exhausted lemon peels represent a particularly valuable resource, as they still contain flavonoids, essential oils and dietary fibres. Recent studies have highlighted the therapeutic properties of citrus-derived flavonoids, especially in the prevention of obesity and diabetes, reinforcing the idea that these by-products should be valorised rather than discarded . Overall, citrus-processing residues can account for up to 50% of the original raw material, thus offering a significant opportunity for recovery and reuse strategies.

The extraction of bioactive compounds from these matrices not only reduces environmental impacts, but also contributes to the development of high value-added functional ingredients. In this framework, innovative extraction methods such as Supercritical Fluid Extraction (SFE) have shown great potential for optimising the recovery of bioactive compounds while reducing the consumption of organic solvents and energy resources. The research project is fully aligned with the “Do No Significant Harm” principle, meaning that it is designed so as not to cause significant harm to the environment. The main objective is to valorise by-

products from the citrus supply chain within a circular-economy perspective, through the use of a low-impact technology such as SFE.

In SFE, extraction is performed using CO_2 instead of conventional organic solvents. At the end of the extraction step, the CO_2 is recovered in a storage vessel and reused in subsequent extraction cycles. This operational strategy prevents the release of significant amounts of CO_2 into the environment, thus avoiding additional contributions to greenhouse gas emissions. Moreover, the small fraction of CO_2 remaining in the extract rapidly evaporates at room temperature, allowing the production of formulations that are extremely safe for both humans and the environment.

This thesis specifically focuses on the limoncello production chain, as the industrial partner, Distillerie Nastro D'Oro, is specialised in the manufacture of limoncello. The aim is to explore the feasibility of extracting bioactive compounds from exhausted lemon peels generated during the company's production process. The process under investigation initially involves infusion of lemon peels in ethanol, followed by a decantation step and cold mixing of the semi-finished product with granulated sugar and water. The resulting liquid is then subjected to coarse filtration and finally bottled. The exhausted peels, instead of being disposed of, are freeze-dried and milled, and then treated by supercritical CO_2 extraction. This approach enables the recovery of valuable bioactive compounds, minimising waste and maximising the overall efficiency and added value of the process.

To evaluate the environmental sustainability of this system and to optimise resource use and consumption, a Life Cycle Assessment (LCA) was carried out. LCA is a standardised tool used to assess how products and services affect the environment throughout their entire life cycle, by considering specific inputs and outputs within a clearly defined set of system

boundaries. In this thesis, LCA is applied to the process in order to identify the main environmental hotspots, compare alternative process configurations and provide quantitative evidence to support decision-making towards a more sustainable, circular management of citrus by-products.

5.2 Materials and Methods

Life Cycle Assessment (LCA) was adopted as the main methodological framework to quantify the potential environmental impacts associated with the process, in accordance with the ISO 14040 and ISO 14044 standards. LCA is a systematic and quantitative method used to evaluate the potential environmental impacts related to a product, process or service over its entire life cycle, from the extraction of raw materials to end-of-life management. This life-cycle perspective includes all relevant stages: raw material extraction, manufacturing and processing, packaging, transport and distribution, use phase, potential reuse or recycling, and final disposal. In this study, LCA was applied to the limoncello production chain integrating the valorisation of exhausted lemon peels via supercritical CO_2 extraction, with the aim of identifying environmental hotspots and assessing the benefits and trade-offs of peel valorisation in a circular economy perspective.

In line with LCA principles, the analysis adopts a holistic and relative approach. All relevant life cycle stages and elementary flows are considered to the extent allowed by data availability and the defined system boundaries, focusing on environmental aspects and related potential impacts while avoiding the transfer of burdens from one life cycle stage or impact category to another. The functional unit was defined as the production of one bottle (1 L) of finished limoncello, which provides a clear reference for quantifying inputs and outputs and allows an objective comparison between alternative scenarios, namely: (i) a

conventional system in which exhausted lemon peels are treated as waste and disposed of, and (ii) an enhanced system in which peels are recovered, freeze-dried, milled and valorised through supercritical CO_2 extraction. This functional unit ensures that all results are expressed per unit of comparable service, enabling meaningful benchmarking of different process configurations.

Consistent with ISO standards, the LCA study was structured into four interconnected phases. First, in the goal and scope definition phase, the objectives of the study, intended applications of the results, target audience and comparison scenarios were specified. The product system was described, the functional unit was defined as mentioned above, and the system boundaries were established to include agricultural production of lemons (where relevant), transport to the distillery, limoncello production steps (peel infusion in ethanol, decantation, cold mixing with sugar and water, coarse filtration and bottling), management of exhausted peels, and the additional operations required for peel valorisation (freeze-drying, grinding and supercritical CO_2 extraction). Assumptions, limitations, allocation procedures for multi-functional processes and data quality requirements were also defined at this stage.

Second, a Life Cycle Inventory (LCI) analysis was carried out to collect and quantify all relevant inputs and outputs associated with the defined system. Primary data were gathered from the industrial partner (Distillerie Nastro D'Oro) for site-specific processes such as limoncello production, energy and material consumption, and on-site management of by-products. For background processes (e.g. electricity mix, upstream production of ethanol, sugar, packaging materials, transport), secondary data were obtained from established LCA databases and literature. The inventory includes flows of energy, water, raw and auxiliary

materials, as well as emissions to air, water and soil and waste streams linked to each life cycle stage. All data were normalised to the functional unit.

Third, a Life Cycle Impact Assessment (LCIA) was performed to translate the LCI results into potential environmental impacts. Inventory flows were classified into relevant impact categories (e.g. climate change, ozone depletion, acidification, eutrophication, human toxicity and ecotoxicity), and characterisation models were applied to calculate category indicators such as Global Warming Potential. The choice of impact categories and indicators was made in line with the goal and scope of the study and with commonly used LCIA methods. Where appropriate, optional LCIA steps such as normalisation and grouping were explored to facilitate interpretation and comparison between the conventional and valorisation scenarios, while avoiding subjective weighting that could bias the results.

Finally, an interpretation phase was conducted to critically analyse the outcomes of the LCI and LCIA in relation to the defined goals and scope. This phase involved identifying environmental hotspots along the limoncello value chain, assessing the relative contribution of peel valorisation steps to overall impacts, evaluating the robustness and sensitivity of the results to key assumptions and data uncertainties, and formulating recommendations for process optimisation. The interpretation focuses on providing clear and transparent insights for decision-makers, highlighting both the potential environmental benefits of integrating supercritical CO_2 extraction of lemon peel by-products and any trade-offs that may arise in specific impact categories.

The goal of this LCA is to evaluate the environmental impacts associated with the main stages of the production process, which is aimed at both limoncello manufacturing and the valorisation of exhausted lemon peels, a production residue that would otherwise be

managed as waste. The analysis makes it possible to identify critical points in terms of resource consumption, waste generation and emissions, with the ultimate aim of guiding improvement actions and sustainability strategies in a circular economy perspective.

This LCA is not designed as a public comparative assertion between alternative products or processes. Consequently, it is not subject to an external critical review by a third party as required by ISO 14044 for comparative studies. Nevertheless, the methodological approach, system definition and impact assessment procedures have been developed in accordance with the ISO 14040 and ISO 14044 standards, in order to ensure consistency, transparency and robustness of the results.

5.3 Results and Discussion

5.3.1 Process description

The process is designed to valorise exhausted lemon peels generated during limoncello production at Distillerie Nastro D'Oro. The overall system consists of a sequence of food-grade and semi-industrial operations, which together define the foreground processes modelled in the LCA. The process can be divided into two main segments: (i) limoncello production and (ii) valorisation of the exhausted lemon peel matrix.

5.3.2 Limoncello production

Distillerie Nastro D'Oro source lemon peels directly from the supplier GALANO SORRENTO AZIENDA AGRICOLA S.R.L., thus avoiding the on-site peeling step and the associated generation of fresh peel by-products. The lemon peels are immersed in food-grade ethanol for 48 hours. The standard proportion adopted is 1 L of ethanol per kg of lemons, corresponding to approximately 150 g of lemon peel. The ethanol, obtained from agricultural feedstocks (molasses), acts as a solvent for the extraction of essential oils and aromatic

compounds from the peels. At the end of the maceration period, an aromatic infusion is obtained, with an average density of about 0.840 kg/L, which represents the first intermediate product of the process and the starting point for subsequent limoncello formulation steps.

5.3.3 Decantation

The aromatic infusion is then transferred into dedicated stainless-steel tanks for gravitational decantation. This step allows the natural settling of suspended solids and impurities. During decantation, an average loss of approximately 1% of the semi-finished product is observed, mainly due to the separation of sediment and residues. The clarified liquid collected from the upper part of the tank constitutes the direct input to the following mixing step. Transfer between tanks is carried out using an electric pump with a nominal power of 0.37 kW, and electricity consumption is modelled as proportional to the volume of infusion handled.

5.3.4 Cold mixing

The decanted infusion is then blended with granulated sugar and potable water according to the company recipe: 0.375 L of infusion for every 0.5 L of water and 250 g of sugar. Mixing is performed at room temperature using an electric mixer with a power rating of 0.75 kW, typically operated for about 1 hour per batch. The output of this unit operation is a raw, unfiltered limoncello, in which the flavour profile is defined and the alcohol, sugar and water contents are adjusted to the desired final characteristics. From an LCA perspective, this step concentrates a significant portion of the material inputs (sugar and water) and associated upstream burdens.

5.3.5 Filtration

The unfiltered limoncello is subjected to mechanical filtration to remove residual peel particles, fibres and any undissolved sugar crystals. The filtration process yields, on average, 98% finished product (filtered limoncello) and 2% solid residue from the filter medium. Electricity consumption in this phase includes the operation of the filtration pump and the

transfer of the filtered product to storage or directly to the bottling line. The actual yield of the filtration step can vary depending on the clogging level and age of the filters: performance tends to be optimal with new filters and slightly lower with filters that have been in use for several consecutive days. In the LCA model, this variability is acknowledged, and average yields are adopted as representative values.

5.3.6 Bottling and labelling

The finished limoncello is packaged in 1 L glass bottles using a semi-automatic line composed of a filling unit, capping machine, labelling unit, conveyor belts and an air compressor. The line has a nominal capacity of approximately 600 bottles per hour under standard operating conditions. In addition to the direct energy consumption of the bottling line, the LCA also accounts for the environmental burdens associated with the production of the glass bottle and aluminium cap, by including two additional processes: “glass bottle production” and “aluminium cap production”. These processes capture the upstream energy and material requirements and ensure that the packaging contribution is properly reflected in the overall environmental profile of 1 L of finished limoncello.

5.3.7 Valorisation of exhausted lemon peels

Beyond the core limoncello production steps, the process integrates a dedicated valorisation pathway for the exhausted lemon peels, which are normally treated as waste. In the enhanced system, the peels are stabilised and processed to obtain a bioactive extract via supercritical CO_2 extraction.

5.3.8 Freeze-drying of exhausted lemon peels

The exhausted peels arising from limoncello production are first subjected to freeze-drying (lyophilisation) to remove residual moisture and stabilise the matrix for storage and subsequent processing. Freeze-drying reduces the mass of the peel matrix by approximately 86%, mainly due to water removal. The water released is non-contaminated and is modelled

as a neutral emission to the environment. The freeze-dried product is a dry, stable matrix with extended shelf-life, suitable for downstream size reduction and extraction. In the LCA, this unit operation is associated with considerable energy use, which is explicitly quantified and allocated per functional unit.

5.3.9 Grinding

The lyophilised lemon peel is then mechanically ground to obtain a particle size distribution suitable for efficient extraction in the SFE unit. As the material is already dry and stable, mechanical grinding is assumed to have negligible mass losses, and the output yield is considered to be 100% of the input in the form of ground matrix. Any minor losses (e.g. dust deposition) are considered negligible at the scale of the assessment. The grinding step is modelled mainly through its electricity consumption and is treated as a preparatory unit operation that enhances mass transfer during extraction.

5.3.10 Supercritical CO_2 extraction (SFE)

The ground peel matrix is finally processed by supercritical CO_2 extraction (SFE), using carbon dioxide as the extraction solvent under supercritical conditions. This technology allows the recovery of bioactive compounds from the lemon peel without the use of organic solvents, thereby contributing to a lower environmental impact and a cleaner extract. Experimental trials were conducted by varying operating parameters such as pressure, temperature and CO_2 flow rate. The extracts obtained were subsequently characterised at the Department of Pharmacy, University of Naples Federico II, to assess their chemical composition and bioactive potential.

The optimisation of extraction performance, in terms of both yield and quality of the recovered fractions, led to the selection of the following operating conditions as the reference set-up for the process: 300 bar, 60 °C and a CO_2 flow rate of 10 kg/h. Under these conditions,

the process was found to maximise the quality of the extract, enhancing the valorisation of the bioactive content of the exhausted lemon peels [15]. In the LCA model, these operating parameters are used to calculate the specific energy requirements and CO_2 circulation per functional unit, as well as to quantify the amount of bioactive extract obtained from a given mass of peel.

Overall, the integration of freeze-drying, grinding and SFE into the limoncello production chain transforms a residual stream, previously handled as waste, into a source of high-value bioactive ingredients. From a life-cycle perspective, this creates an extended product system in which waste minimisation, resource efficiency and added value are jointly considered, providing the basis for evaluating the environmental trade-offs and benefits of implementing the concept at industrial scale.

5.3.11 LCA Evaluation

The Life Cycle Assessment was carried out using a "gate-to-gate" approach, focusing on the internal stages of the production process for the valorisation of exhausted lemon peels, as implemented at the Distillerie Nastro D'Oro facility. Specifically, the analysed system starts with the reception of lemon peels at the distillery and ends with the production of bottled limoncello as the final product. Within these boundaries, both the limoncello production steps and the additional valorisation operations applied to the exhausted peels (e.g. nutraceutical extraction) are included.

The following elements are included within the system boundaries:

- all material inputs (lemon peels, ethanol, water, granulated sugar);
- electricity consumption associated with pumps, mixers, filtration units, bottling equipment and, where applicable, the units involved in peel valorisation (e.g. freeze-dryer, grinder, SFE unit) in the modelled scenario;

- material outputs, both intermediate and final (infusion, raw limoncello, filtered limoncello, freeze-dried and ground matrix, SFE extracts, solid residues from filtration);
- direct and indirect emissions generated by the process, to be quantified at the inventory modelling stage (e.g. ethanol losses, electricity use from the national grid).

The following life-cycle stages are excluded from the system boundaries:

- the agricultural phase (lemon cultivation, harvesting and transport from field to the peel supplier);
- the peeling step, as it is carried out by third parties and lies outside the direct operational control of Distillerie Nastro D'Oro;
- secondary and tertiary packaging, downstream logistics and distribution, and the final consumption phase of the product;
- end-of-life scenarios for the bottle and packaging (e.g. recycling, disposal), which are not explicitly modelled in this gate-to-gate study.

The system boundary is graphically represented in the LCI flow diagram attached to this thesis, which illustrates the foreground processes and their connections to background processes sourced from LCA databases. Adopting a gate-to-gate perspective makes it possible to isolate the environmental impacts directly attributable to the operational stages under the company's control, thereby enabling a targeted evaluation of process efficiency, improvement opportunities and the potential environmental benefits arising from the integration of exhausted peel valorisation.

The functional unit selected for this study is the processing of 500 kg of fresh lemons, from which approximately 75 kg of lemon peels are obtained and subsequently processed for

limoncello production. All input and output flows – both energy and material – were collected, estimated and normalised with respect to this functional unit, ensuring consistency and comparability of the results across all stages of the process.

This choice allows for a systematic quantification of the environmental impacts associated with each process step, enabling an assessment of the overall efficiency of the system and the specific contribution of by-product valorisation operations (in particular, the management and treatment of exhausted lemon peels). The adopted functional unit is especially effective in representing the real dynamics of the production cycle, as it reflects a typical processing batch at the industrial plant and provides a direct link between operational data and environmental performance indicators.

Moreover, a clearly defined functional unit offers a robust reference for interpreting the results, both in terms of internal optimisation (reducing energy consumption, minimising waste, increasing the efficiency of peel valorisation) and for potential benchmarking with analogous processes in the agri-food and alcoholic beverage sectors.

To represent the operational stages of the production process in a clear and systematic way, a flow diagram was developed to describe the main unit operations, highlighting for each of them the corresponding inputs, outputs and energy requirements. The diagram has a dual purpose: on the one hand, it provides a concise visual overview of the production sequence; on the other, it serves as a key operational tool for the Life Cycle Inventory (LCI) phase of the LCA, facilitating the mapping of material and energy flows and ensuring consistency between the collected data and the system model.

In this study, the analysed process was broken down into seven main stages, corresponding to the unit operations identified along the limoncello production chain and the valorisation

pathway of exhausted lemon peels. The flow diagram was constructed by combining data supplied by the industrial partners with information gathered during the characterisation of the by-products, so as to realistically reflect actual operating conditions. The data associated with each stage of the diagram were then processed and normalised for the compilation of the life cycle inventory, forming the quantitative basis for the subsequent impact assessment phase.

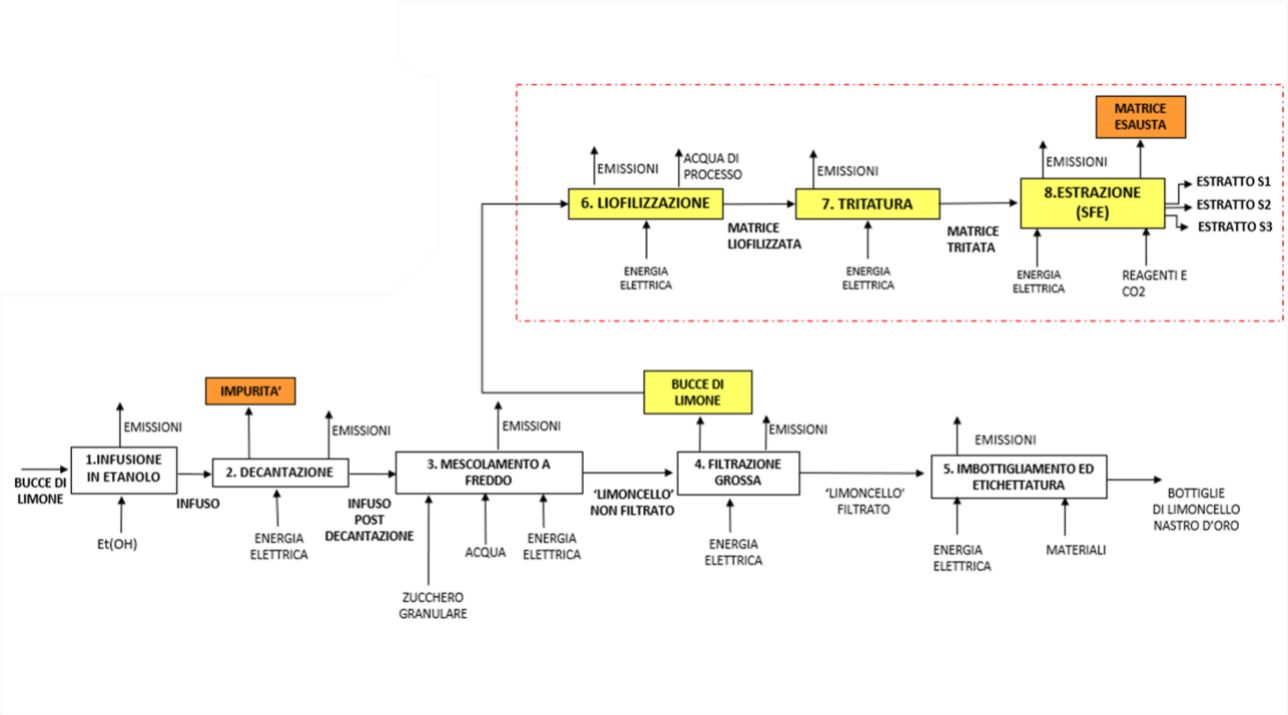


Figure 10 - Flow chart

The Life Cycle Inventory (LCI) represents the collection and quantification of the input and output flows related to the system under study, across all stages included within the system boundaries. The analysis was carried out following a “gate-to-gate” approach for the production of a lemon peel infusion, excluding harvesting and transport operations performed by third parties.

To ensure the consistency, transparency, and reproducibility of the LCA carried out on the process, it was necessary to adopt a structured set of operational hypotheses and calculation assumptions. These assumptions derive from a combination of primary sources (company data and direct measurements) and secondary sources (technical literature, LCA databases), and form the basis on which the life cycle inventory was built. The entire analysis system is normalised with respect to the functional unit of 500 kg of fresh lemons processed, from which an average yield of 15% lemon peels is obtained (corresponding to 75 kg). This choice makes it possible to realistically represent the operating conditions of the plant and provides a homogeneous basis for quantifying the inputs and outputs of the process.

a*) Density and conversions*

- The density of ethanol is assumed to be 0.789 kg/L, a value used to convert solvent volumes into mass.
- The density of the semi-finished product after decantation is assumed to be 0.840 kg/L, based on direct measurements.
- The electricity consumption for moving the semi-finished product from one stage to the next is calculated by considering an electric pump of 0.37 kW operating for 10 minutes for a flow of 500 litres.
- The electricity consumption for cold mixing is calculated by considering an ANSALDO asynchronous motor, TYPE A1C0812CA224, operating for one hour for 500 litres of product.

- The energy consumption for filtration was calculated by considering the same pump used in the decantation stage. The filtration time for 500 litres of limoncello is 40 minutes.
- The energy consumption of the freeze-drying stage was quantified directly, taking into account the equipment used at the R&D Laboratory of Mater s.r.l. The BUCHI LIOVAPOR 300 device has a power of 2.17 kW.
- The electricity consumption due to grinding was calculated considering a laboratory grinder of 1.5 kW.
- The energy consumption of the SFE stage was quantified directly on the basis of the Mater s.r.l. plant.

b) Assumptions on recipe and composition

For the material inventory of the cold mixing phase, the proportions of the industrial recipe of Distilleria Nastro d'Oro were used:

- 0.5 litres of water
- 250 g of sugar
- 375 mL of ethanol–lemon peel infusion
- Average alcoholic strength of the infusion: 84–86%.

c) Process efficiencies and yields

- The decantation stage generates a 1% loss in the form of solid impurities.
- During coarse filtration, the yield is 98%, while the remaining 2% consists of solid residues (albedo, residual fibres).
- Freeze-drying reduces the mass of the matrix by 86%, releasing water as a non-polluting output flow.

- SFE provides a yield of useful extract of about 2.34%, with the CO_2 employed being almost completely recovered and recirculated.

d) Exclusions and simplifications

- Secondary packaging materials (cardboard boxes, pallet wrap films, etc.) are not considered.
- Ethanol losses due to evaporation were assumed to be incorporated in the LCIA calculation, based on average emission factors from the literature.

The inventory was built starting from the data collected in the company and completed using recognised LCA databases (e.g. Ecoinvent) for glass, aluminium and technical CO_2 production models, as well as for energy flows related to the Italian electricity grid. Emission factors were taken from certified sources, in line with the ISO framework.

The Life Cycle Inventory (LCI) represents the core of the LCA, as it systematically collects, organises and quantifies all material and energy flows that pass through the production system. The aim of this phase is to identify and map the inputs (raw materials, energy resources, auxiliary materials) and the outputs (intermediate products, final products, waste, emissions) associated with each of the stages included within the system boundaries. The process was divided into eight main stages, covering both limoncello production and the valorisation of exhausted peels for the extraction of bioactive compounds. The inventory was built on the basis of data collected in the company, supplemented by secondary sources and supporting modelling.

The following tables summarise for each stage:

- Process inputs (raw materials, energy, auxiliary materials);

- Useful outputs (intermediate or final products);
- Waste and losses;
- Estimated emissions, where available, referring to the LCIA chapter for the detailed analysis of environmental impacts.

All values were expressed in units of mass (kg) or volume (litres), where appropriate, with an indication of the electricity consumption in kWh for each operating stage. The flows relating to primary packaging materials (bottles, caps, labels) were integrated as separate sub-stages.

The first five stages of the process represent the limoncello production cycle, from infusion in ethanol to the packaging of the final product. The subsequent three stages describe the transformation pathway of the exhausted peels into high value-added extracts. The reported data highlight the significant energy intensity of freeze-drying and supercritical CO_2 extraction. The inventory also takes into account the impacts associated with the production of primary packaging materials.

Phase	***Input***	***Value***	***Unit***	***Output***	***Value***	***Unit***
0. Collection and transport	Lemons	500	kg	–	–	–
	Peel yield	15	%	–	–	–
	Transport	–	ton·km	–	–	–
1. Infusion in ethanol	Lemon peels (FU)	75	Kg	Infusion	475	kg
	Ethanol	500	Lt	–	–	–
	–	400	kg	Emissions	accounted in LCIA impact assessment	–
2. Decantation	Infusion	475	kg	Infusion after decantation	470.25	kg
	Total electricity consumption	0.13	kWh	Impurities	4.75	kg

	–	–	–	Emissions	accounted in LCIA impact assessment	–
3. Cold mixing	Infusion after decantation	470.25	kg	Unfiltered limoncello	1589.85	kg
	Water	746.4	Lt =kg	–	–	–
	Granulated sugar	373.2	kg	–	–	–
	Total electricity consumption	0.94	kWh	Emissions	accounted in LCIA impact assessment	–
4. Coarse filtration	Unfiltered limoncello	1589.85	kg	Filtered limoncello	1558.053	kg
	Total electricity consumption	0.93	kWh	Albedo and lemon peels	31.8	kg
	–	–	–	Emissions	accounted in LCIA impact assessment	–
5. Bottling and labelling	Final limoncello	1558	lt	Limoncello bottles	1558	units
	Total electricity consumption	22.07	kWh	–	–	–
	Materials 1 = 1 L glass bottles	1558	units	–	–	–
	Materials 2 = caps	1558	units	–	–	–
	Materials 3 = labels	1558	units	Emissions	accounted in LCIA impact assessment	–

Table 11 - Life Cycle Inventory (LCI) of the process (Phases 1–5)

Phase	**Input**	**Value**	**Unit**	**Output**	**Value**	**Unit**
6. Lyophilisation	Matrix	31.8	kg	Lyophilised matrix	4.45	kg
	Total electricity consumption	72	kWh	Non-contaminated water	27.35	kg
	–	–	–	Emissions	calculated in LCIA impact assessment	–

7. Grinding	Lyophilised matrix	4.45	kg	Ground matrix	4.45	kg
	Total electricity consumption	0.12	kWh	Emissions	calculated in LCIA impact assessment	–
8. SFE extraction	Ground matrix	4.45	kg	Extract S1	0.1	kg
	–	–	–	Extract S2	0	kg
	Total electricity consumption	258	kWh	Extract S3	0	kg
	CO_2 used and recirculated	1260	kg	Exhausted matrix	4.35	kg
	–	–	–	Emissions	calculated in LCIA impact assessment	–

Table 12- ife Cycle Inventory (LCI) of the process (Phases 6–8)

Production of glass bottle

Material/Energy flow	**Direction**	**Quantity**	**Unit**
White glass for packaging	Input	0.60	kg
Electricity for the forming process	Input	0.05	kWh
Natural gas used to heat the glass during forming	Input	0.02	Nm^3
Water for cooling and cleaning	Input	0.20	L
Result: one glass bottle	Output	1	Unit

Table 13- Life Cycle Inventory (LCI) of the "Glass Bottle Production" process

Production of aluminium cap

Material/Energy flow	**Direction**	**Quantity**	**Unit**
Primary aluminium for the production of the aluminium cap	Input	0.001	kg
Electricity for pressing and forming	Input	0.003	kWh
Water for washing and cooling	Input	0.001	L
Result: one aluminium cap	Output	1	Unit

Table 14- Life Cycle Inventory (LCI) of the "Aluminium Cap Production" process

The assessment of environmental impacts (Life Cycle Impact Assessment – LCIA) was carried out in accordance with international standards, using the ReCiPe 2016 method in both its Midpoint (H) and Endpoint (H) versions. This methodology was selected because it is one of the most up-to-date and robust models currently available for quantifying environmental burdens and it covers a wide spectrum of impact categories that are particularly relevant for agro-food systems and nutraceutical extraction processes.

In practical terms, the LCIA followed the usual sequence of phases. First, during the classification step, all the elementary flows identified in the Life Cycle Inventory – that is, the inputs of resources and the outputs of emissions and wastes – were assigned to the corresponding impact categories defined by ReCiPe 2016. Subsequently, in the characterisation phase, each flow was multiplied by the appropriate characterisation factor, thereby quantifying the potential contribution of that flow to the different environmental impacts. This allowed the transformation of a heterogeneous set of inventory data into comparable impact indicators. A further normalisation step was then applied, in which the obtained results were related to reference values representative of global conditions; this procedure facilitates the interpretation of the magnitude of the impacts by placing them in a broader context. For the Endpoint analysis only, an additional aggregation phase was performed in order to obtain a single score for each damage area, thus simplifying the comparison between scenarios and helping to identify the processes that contribute most to the overall impact. All calculations were based on a combination of primary inventory data, collected directly from the process under study, and secondary data from the Ecoinvent 3 database, as described in the LCI section.

With regard to the impact categories, the ReCiPe 2016 Midpoint (H) framework made it possible to evaluate a series of environmental issues that are particularly significant for the

system. Among these, climate change (GWP 100) is of primary importance, as it reflects the contribution of greenhouse gas emissions to global warming over a 100-year time horizon. The analysis also considered stratospheric ozone depletion, which captures the potential effect of specific substances on the thinning of the ozone layer, and ionising radiation, which accounts for potential human exposure to radioactive emissions. Further attention was devoted to the formation of photochemical oxidants, relevant both for human health and terrestrial ecosystems, and to fine particulate matter formation, which is closely linked to respiratory and cardiovascular risks.

The study also examined terrestrial acidification and freshwater and marine eutrophication, indicators that describe the potential for soil acidification and for nutrient enrichment of aquatic environments, respectively, with possible consequences such as biodiversity loss and algal blooms. Ecotoxicity was evaluated for terrestrial, freshwater and marine ecosystems, in order to estimate the possible toxic effects of chemical substances on different environmental compartments. Human toxicity, in both its carcinogenic and non-carcinogenic components, was included to account for potential long-term health effects related to exposure to hazardous substances. In addition, the LCIA considered land use, which reflects the pressure exerted on land resources, as well as the scarcity of mineral and fossil resources, indicators that capture the depletion of non-renewable materials and energy carriers. Finally, water consumption was assessed to quantify the contribution of the system to the use of freshwater resources, a parameter of growing importance in the context of climate change and water stress.

For the Endpoint analysis (ReCiPe 2016 Endpoint H), all these Midpoint indicators were further aggregated into three overarching damage categories: Human Health, Ecosystem Quality and Resource Availability. Impacts contributing to Human Health summarise the

potential effects of emissions and resource use on morbidity and mortality; those related to Ecosystem Integrity describe the consequences on biodiversity and ecosystem functioning; and the category of Resource Availability expresses the long-term implications of resource extraction in terms of future scarcity and increased extraction costs. This integrated perspective provides a clearer and more intuitive understanding of how the process affects the environment along its life cycle and supports decision-making aimed at reducing its overall footprint.

The LCA results clearly show that the environmental profile of limoncello production is largely dominated by the bottling and labelling stage. This phase alone accounts for more than 60–70% of the total impacts in many of the midpoint categories considered, with particularly high contributions to Global Warming Potential, Mineral Resource Scarcity and Human Toxicity, cancer effects. Such dominance is mainly linked to the primary packaging material, i.e. the glass bottle, whose production is highly energy-intensive and requires significant amounts of mineral raw materials (silica, soda, limestone) and auxiliary inputs. In addition, the relatively high weight of glass compared with alternative packaging solutions amplifies the impacts associated with upstream transport and, to a lesser extent, end-of-life management.

The remaining process stages—infusion, decantation, cold mixing, coarse filtration, lyophilisation, grinding and supercritical extraction—make a moderate contribution to the overall impact. Their influence is more evident in categories related to toxicity and photochemical ozone formation, where electricity consumption and the use of solvents and process auxiliaries play a more prominent role. Nevertheless, even when these contributions are summed, they remain secondary compared with those associated with glass packaging,

confirming that the core environmental hotspot of the system lies in the packaging step rather than in the extraction or formulation of the product itself.

Overall, the production and packaging system for limoncello can therefore be described as impact-driven by the primary glass packaging. This finding suggests that any strategy aimed at improving the environmental performance of limoncello should prioritise interventions on the bottling stage. Examples include the use of lightweight bottles, higher shares of recycled glass, alternative packaging formats, or reuse and refill schemes. While process optimisation in the earlier stages remains relevant, especially for reducing specific toxicological impacts, the LCIA clearly indicates that packaging design and material choice are the key levers for substantially lowering the life-cycle footprint of the product.

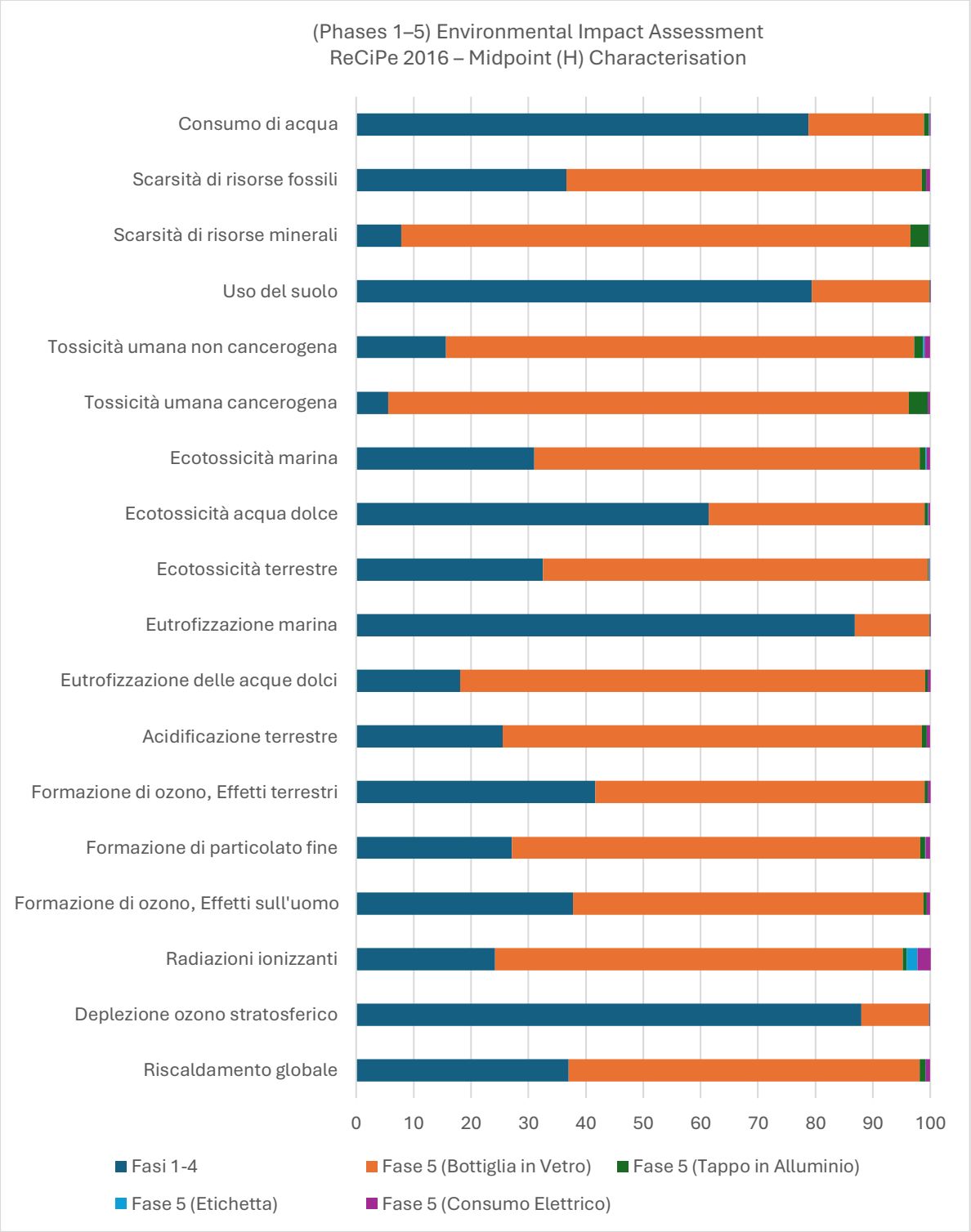


Figure 11- Environmental Impact Analysis (LCIA) – Midpoint approach for the process (Phases 1–5)

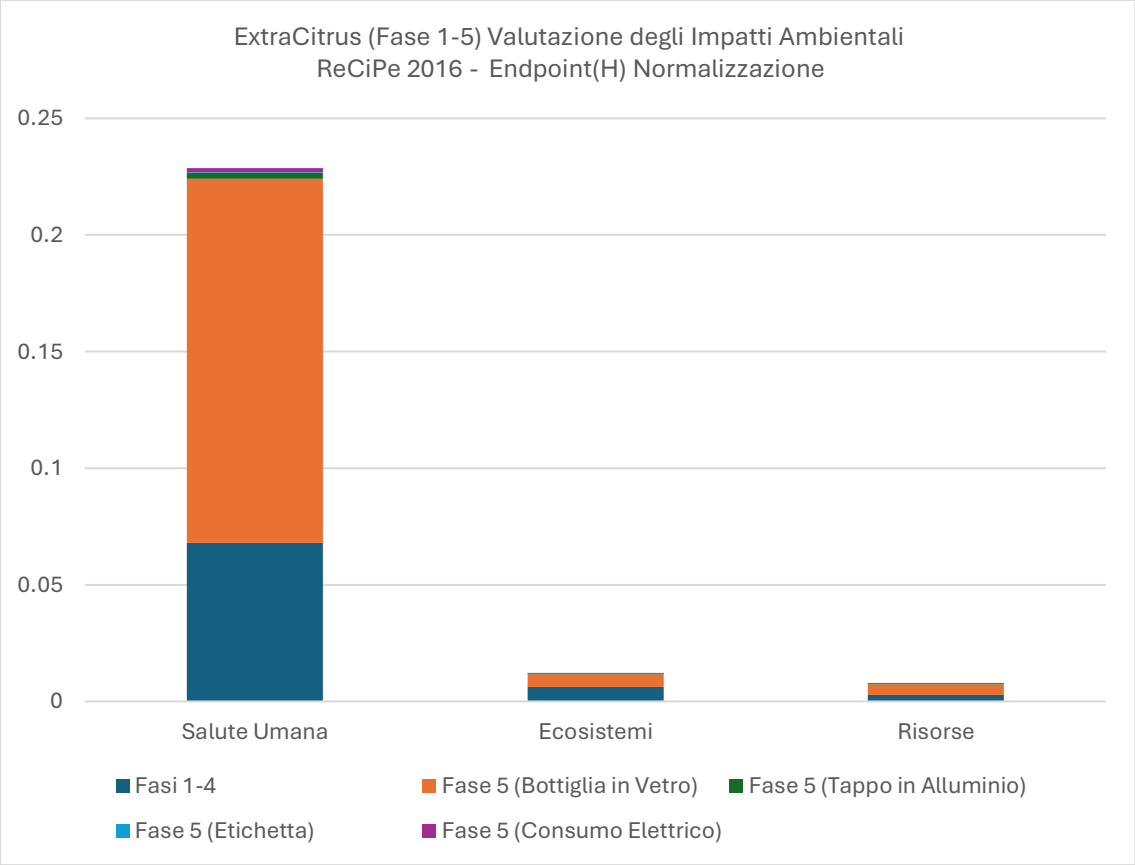


Figure 12 - Environmental Impact Analysis (LCIA) – Endpoint approach for the process (Phases 1–5)

For the production of extracts from the exhausted lemon peels, the Life Cycle Impact Assessment shows a clear differentiation between the various process stages. The supercritical CO_2 extraction (SFE) step emerges as particularly energy-intensive, mainly due to the high electricity demand associated with the compressor, the chiller (cooling unit) and the boiler (heating unit). Maintaining high pressures and controlled temperatures over prolonged operating times requires a continuous energy input, which translates into a non-negligible contribution to several impact categories, especially those linked to climate change, resource depletion and certain toxicity indicators.

Similarly, the lyophilisation phase represents a highly impactful step in terms of energy consumption. Freeze-drying involves repeated cooling, freezing and sublimation cycles under vacuum, which demand substantial amounts of electricity to operate refrigeration

systems and vacuum pumps. As a result, lyophilisation contributes significantly to the overall environmental footprint of the valorisation chain, often ranking just behind SFE in the analysed categories.

By contrast, the grinding phase shows negligible environmental impacts, both in terms of electricity use and associated emissions. The energy required to mechanically grind the lyophilised matrix is relatively low compared with the previous thermal and high-pressure operations, and its contribution to the total LCIA results remains marginal in all examined categories.

Overall, the valorisation of exhausted peels leads to a marked increase in cumulative energy demand when compared with a scenario in which the peels are not further processed. However, this additional burden is offset by the recovery of high value-added compounds that can be used in nutraceutical, cosmetic or functional ingredient applications. In this perspective, the process contributes to reducing the amount of waste sent to disposal, improving the circularity of the system and potentially displacing the production of conventional ingredients with a higher environmental footprint. From a life cycle standpoint, the trade-off between increased energy use and waste reduction, coupled with the generation of value-added products, supports the rationale for the valorisation strategy, especially if future improvements in energy efficiency and the use of low-carbon electricity sources are implemented.

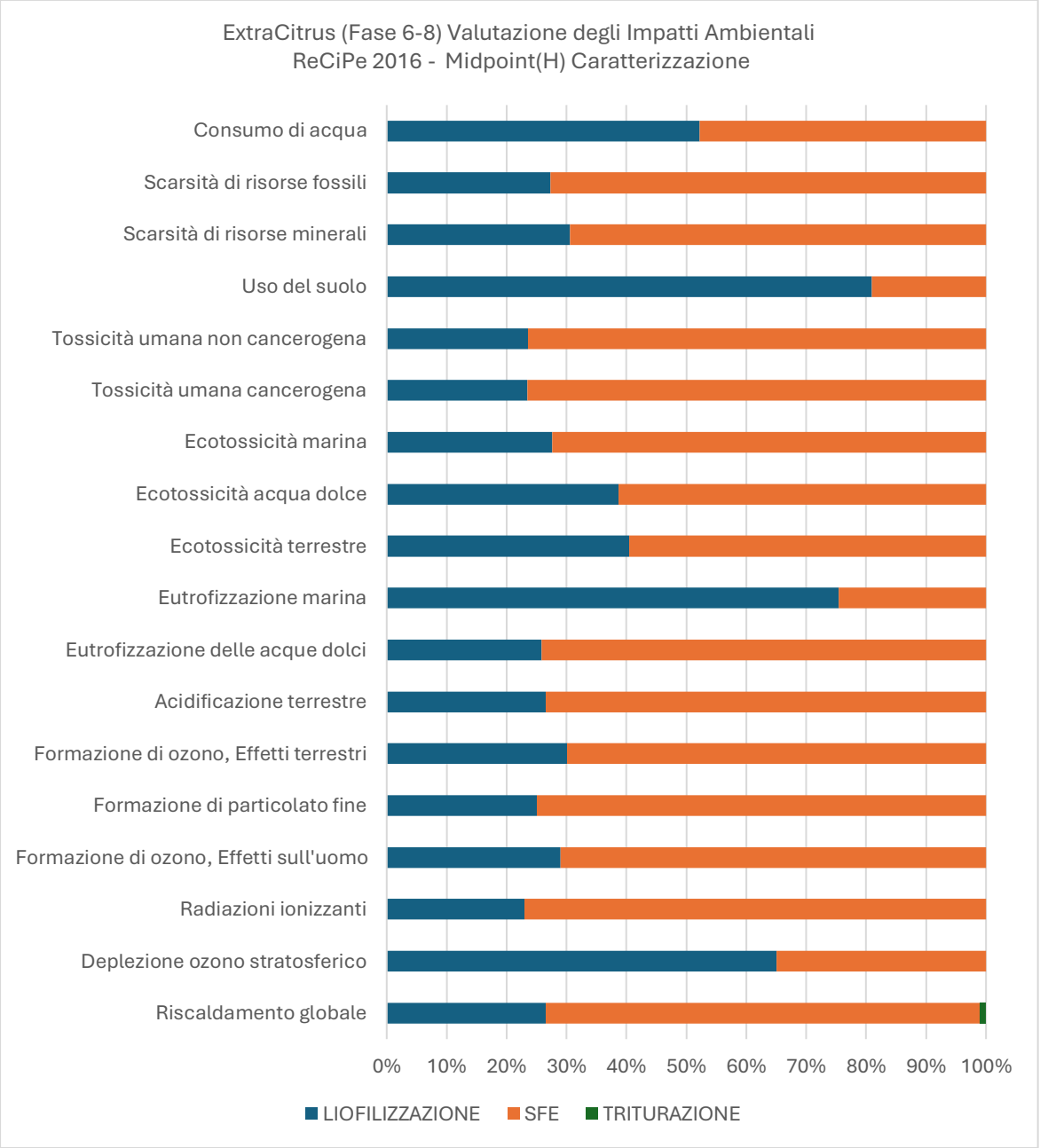


Figure 13 - Environmental Impact Analysis (LCIA) – Midpoint approach for the process (Phases 6–8)

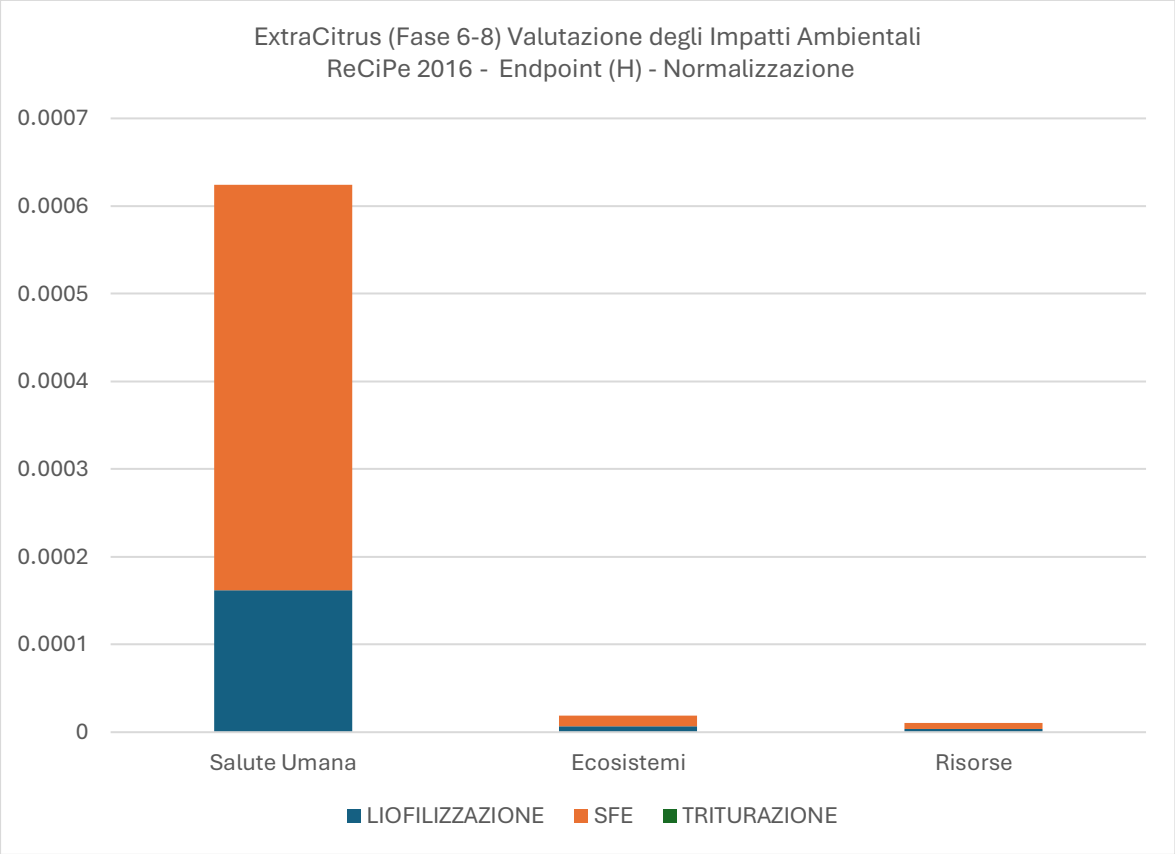


Figure 14 - Environmental Impact Analysis (LCIA) – Endpoint approach for the process (Phases 6–8)

In order to identify additional opportunities to improve the sustainability performance of the process, specific comparative environmental assessments were carried out on two critical elements of the production chain. The first concerns the type of bottle used for packaging, comparing a baseline scenario with virgin glass bottles against an alternative scenario employing bottles made from recycled glass. The second focuses on the source of energy supply used throughout the process, contrasting conventional grid electricity with electricity derived entirely from renewable sources.

These scenario analyses make it possible to quantify the potential environmental benefits associated with the adoption of more sustainable alternatives and to understand how design choices at the packaging and energy-supply level influence the overall life cycle impacts. In

this way, the LCIA does not only describe the current footprint of the system, but also provides practical guidance for directing future optimisation strategies and investment decisions, for example by prioritising the use of recycled materials or low-carbon energy mixes.

Figures 15 and 16 compare the environmental impacts associated with the use of virgin-glass bottles and recycled-glass bottles, according to the ReCiPe 2016 Midpoint (H) and Endpoint (H) approaches, respectively. These graphical comparisons highlight the extent to which changing the packaging material can contribute to reducing impacts in key categories and to lowering the aggregated damage scores for Human Health, Ecosystem Quality and Resource Availability.

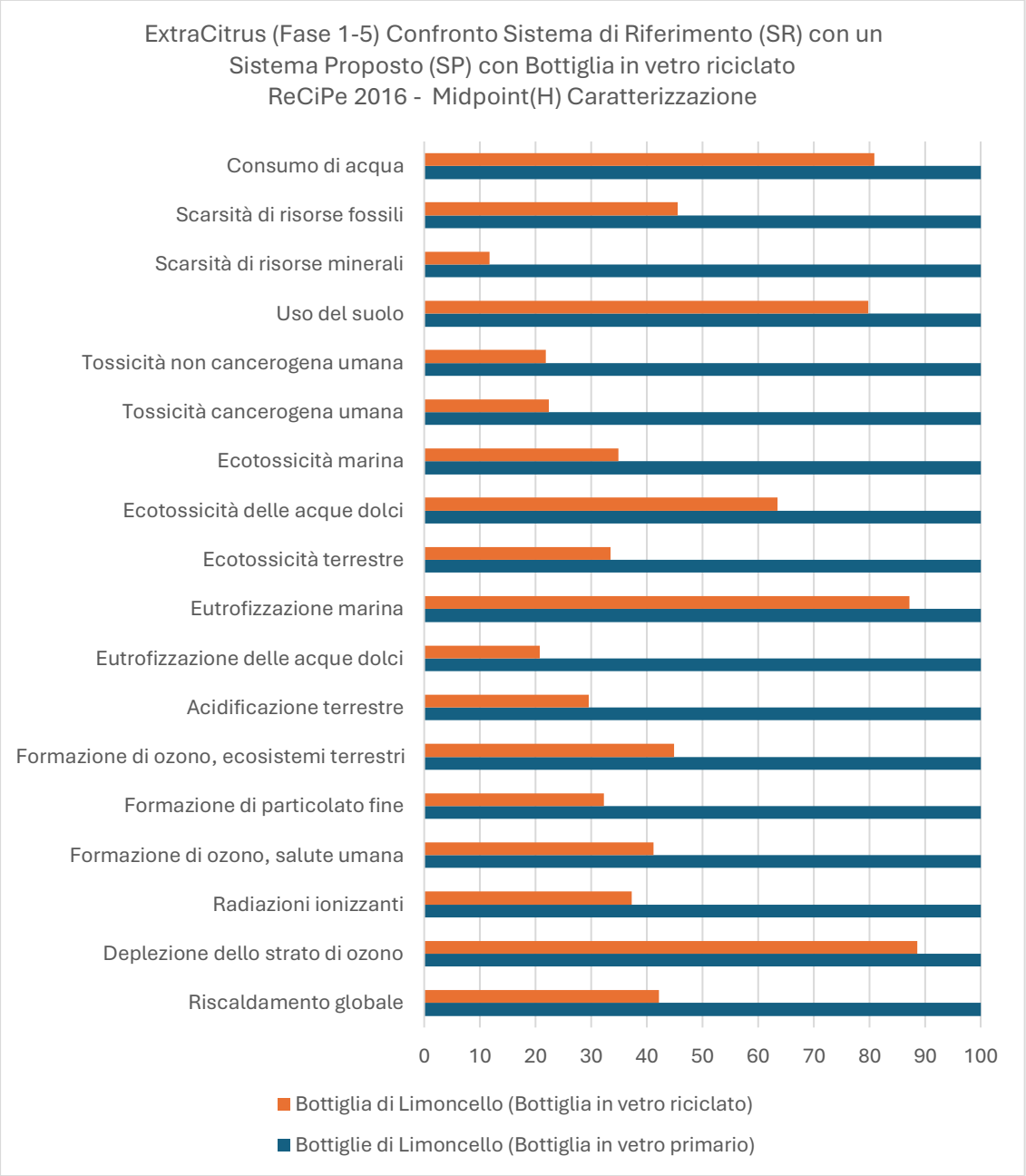


Figure 15 – – Confronto tra Scenario di Riferimento (bottiglia in vetro primario) e Scenario Proposto (bottiglia in vetro riciclato) – Midpoint

In Figure 15, a marked reduction in impacts can be observed in almost all environmental categories when recycled glass bottles are used instead of virgin glass. The most evident improvement concerns the Global Warming Potential (GWP) category, where greenhouse gas emissions are significantly reduced thanks to the lower energy demand and the higher

efficiency associated with recycled glass production. At the same time, substantial benefits emerge in categories related to human toxicity and mineral resource use, as the decreased need for primary raw materials and the partial elimination of highly energy- and resource-intensive industrial processes lead to a lower overall environmental burden. Taken together, these results clearly indicate that shifting from virgin to recycled glass packaging represents an effective strategy for mitigating the life cycle impacts of the limoncello system.

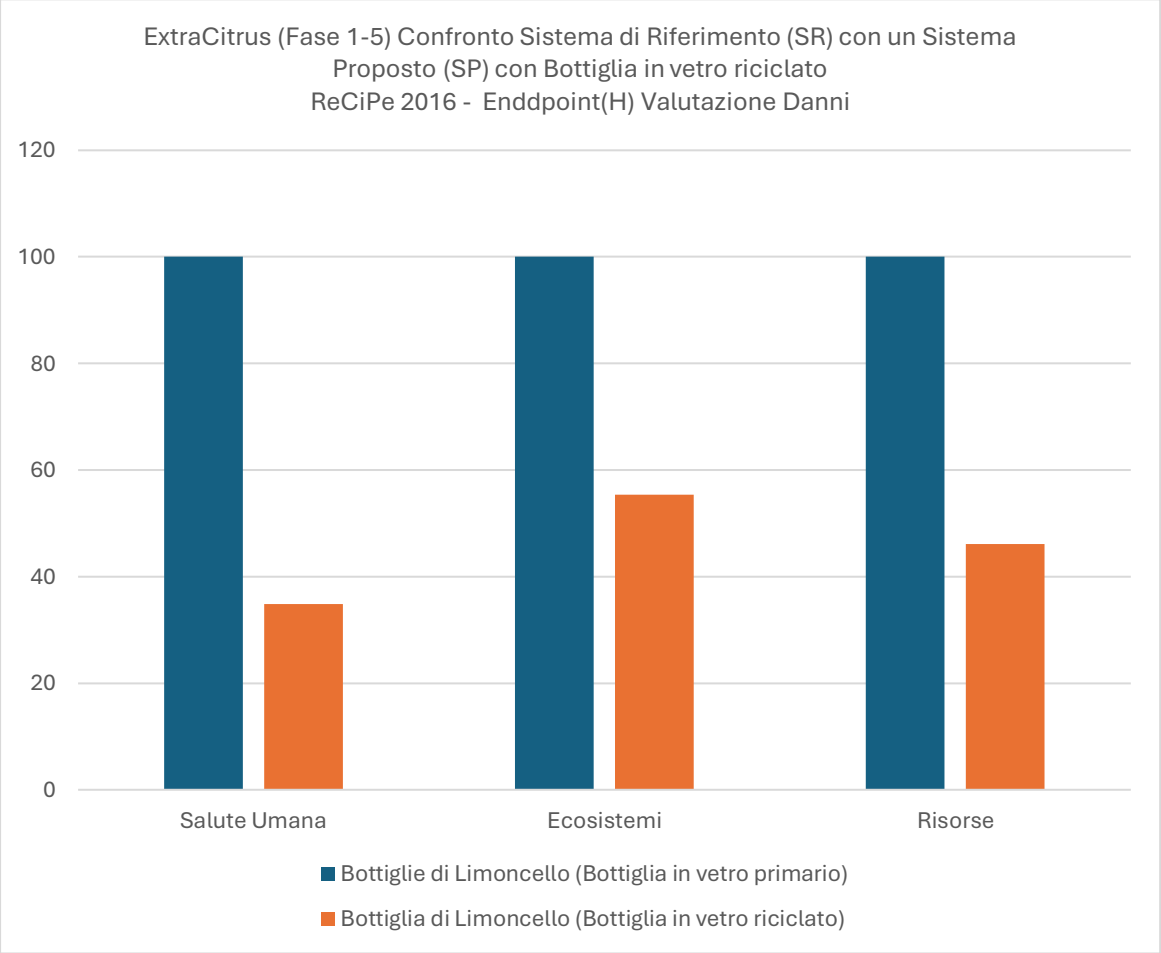


Figure 16 - Confronto tra Scenario di Riferimento (bottiglia in vetro primario) e Scenario Proposto (bottiglia in vetro riciclato) – Endpoint

Figure 16 (Endpoint), which aggregates the midpoint results into damage categories, visually confirms the environmental benefits of using recycled glass, especially in terms of reducing potential damage to human health and improving the long-term availability of resources. By

condensing multiple impact pathways into single scores, the Endpoint analysis makes it even clearer that the choice of packaging – and in particular the production of the glass bottle – is one of the main drivers of the overall environmental footprint. This comparison reinforces the idea that packaging design and material selection are strategic levers for significantly reducing the life cycle impacts of the product system.

Figures 17 and 18 further explore the improvement potential associated with energy supply by focusing on the three key valorisation stages for the exhausted lemon peels: lyophilisation, grinding and supercritical CO_2 extraction. For these phases, the LCIA compares the current scenario, in which electricity is supplied from the conventional Italian grid mix, with an alternative scenario based on electricity generated entirely from renewable sources. This comparative assessment makes it possible to quantify how much of the environmental burden associated with energy-intensive operations can be mitigated simply by switching to a low-carbon energy mix. In the case of lyophilisation and SFE, where electricity use is particularly high, the adoption of renewable energy leads to notable reductions in climate change impacts and improvements in several toxicity- and resource-related categories, thereby enhancing the overall sustainability profile of the valorisation chain.

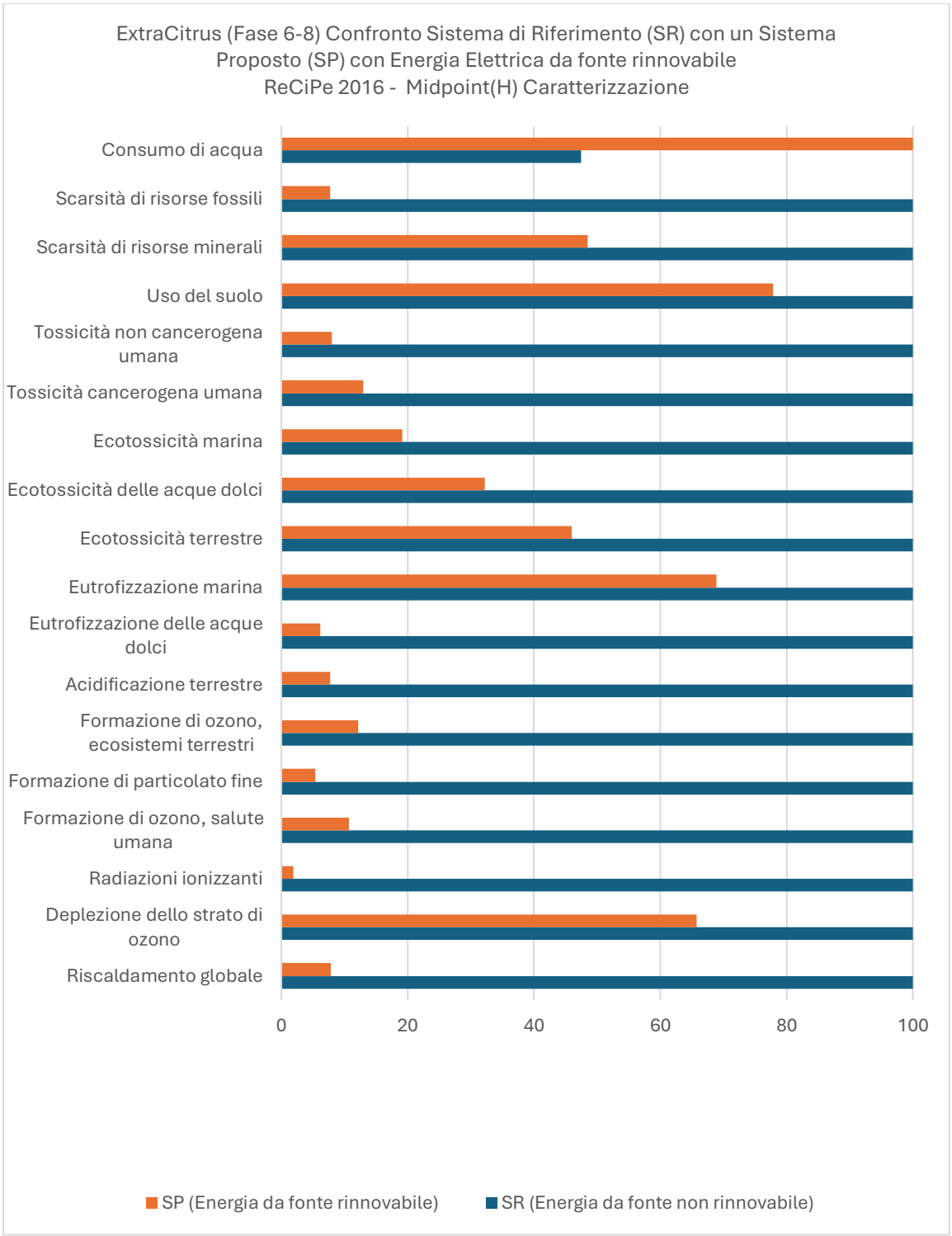


Figure 17 - Confronto Sistema di Riferimento (SR) con un Sistema Proposto (SP) con Energia Elettrica da fonte rinnovabile – Midpoint

In Figure 17, a drastic reduction in Global Warming–related impacts can be observed when the system switches from conventional grid electricity to renewable energy sources. The adoption of a renewable energy supply leads to a decrease of up to 80% in CO_2 emissions and in the consumption of fossil resources, highlighting how strongly the environmental profile of the valorisation phases depends on the carbon intensity of the electricity mix.

In addition to climate change and fossil resource depletion, several other impact categories also show marked improvements. Categories associated with fine particulate matter formation, which are closely linked to atmospheric emissions from fossil-based power generation, exhibit a clear reduction, indicating a lower contribution to air pollution and related health risks. Likewise, indicators of human toxicity benefit from the use of renewables, as the reduction in upstream emissions and pollutants associated with fossil fuel extraction, processing and combustion translates into a more favourable overall toxicological profile. Altogether, the results illustrated in Figure 8 confirm that decarbonising the energy supply is a highly effective strategy for mitigating the environmental impacts of the energy-intensive stages of the process, amplifying the benefits achieved through the circular valorisation of exhausted lemon peels.

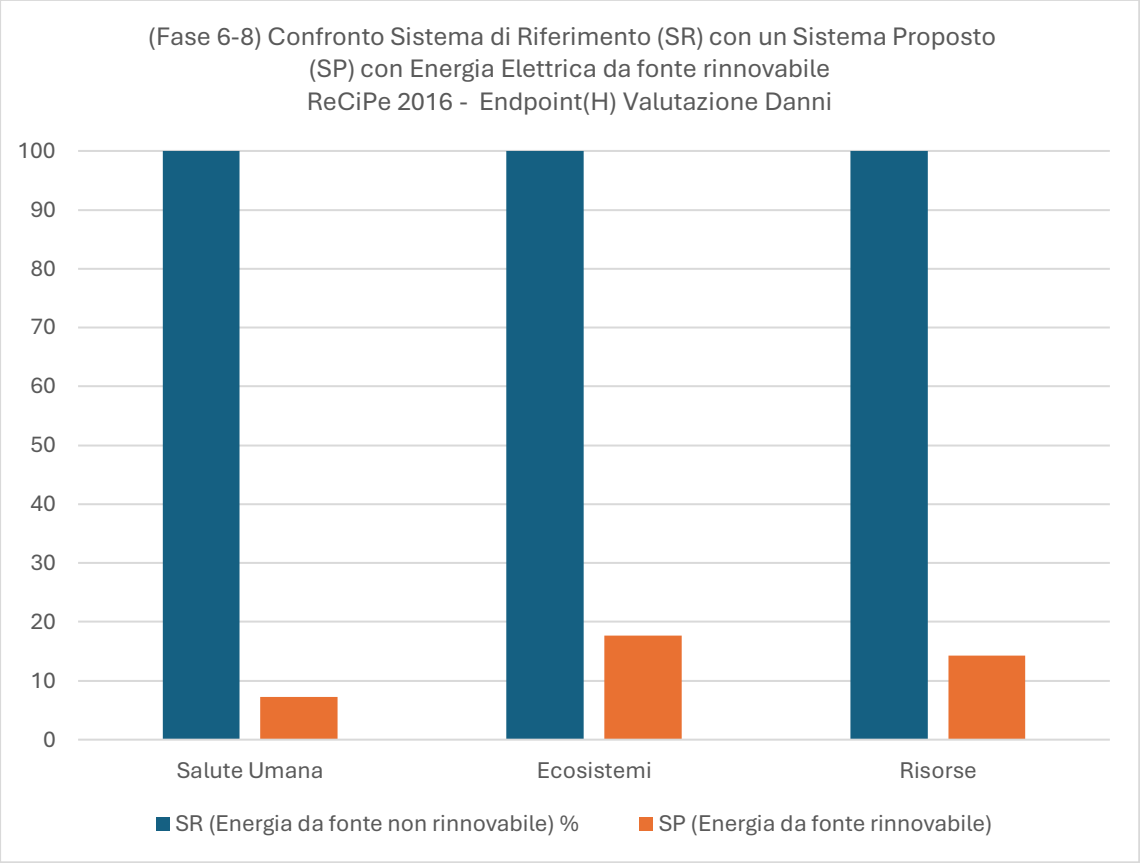


Figure 18 - Confronto Sistema di Riferimento (SR) con un Sistema Proposto (SP) con Energia Elettrica da fonte rinnovabile – Endpoint

Figure 18 (Endpoint) further reinforces this message, highlighting a substantial reduction in the potential damage to human health and ecosystems achieved through the decarbonisation of the electricity used in the most energy-intensive stages of the process (freeze-drying and SFE extraction). By comparing the different scenarios, the analysis clearly shows that targeted interventions on both materials and energy sources do not produce marginal effects, but translate into concrete and quantifiable environmental benefits.

In particular:

- The use of recycled glass significantly lowers the demand for virgin raw materials and reduces the overall impact of the bottling phase, since less energy is required for glass production and fewer emissions are associated with the extraction and

processing of primary resources. This choice therefore contributes to mitigating resource depletion and climate change impacts linked to packaging.

- The shift towards renewable energy substantially improves the environmental footprint of the transformation stages, especially those with high energy consumption, by reducing greenhouse gas emissions and other pollutants. In this way, the adoption of low-carbon electricity mixes actively supports the transition towards climate neutrality and a more sustainable production model.

Taken together, these findings confirm the robustness of the recommendations put forward in the report and indicate clear priority directions for the future optimisation of the process, namely increasing the share of recycled materials in packaging and maximising the use of renewable energy in all energy-intensive operations.

The analysis showed that:

The bottling and labelling stages account for the largest share of impacts in limoncello production (stages 1–5), particularly for Global Warming Potential (GWP), fossil resource use and human toxicity (carcinogenic). Within this block, the production of the glass bottle emerges as the main contributor to the overall impact of the system, both because of the high energy demand of melting and forming processes and the use of virgin raw materials. This result confirms that packaging is not a neutral element, but rather one of the real environmental hotspots across the entire product life cycle.

With regard to the valorisation stages of exhausted peels, in particular freeze-drying and supercritical CO_2 extraction, these are characterised by high energy consumption. This leads to significant impacts especially in terms of fossil resource demand and greenhouse gas emissions, especially when the energy used is supplied by conventional electricity mixes.

Although these stages add an “energy burden” to the system, they are closely linked to the transformation of the peels into high value-added products (nutraceutical or functional extracts).

Despite the increase in energy consumption associated with the valorisation of exhausted peels, this activity contributes positively to the overall sustainability of the system. In particular, it allows:

- A reduction in the amount of organic waste destined for disposal, thereby limiting the impacts associated with waste treatment (for example emissions from composting or landfilling);
- A reduction in the demand for new resources for the production of bioactive ingredients, partially replacing virgin raw materials with compounds extracted from by-products;
- A reduction in the potential environmental impact associated with the management of agro-industrial waste along the citrus supply chain.

The recovery of peels, through their transformation into nutraceutical extracts, therefore represents a clear example of the application of circular economy principles, in which a process residue is reintroduced into the production cycle as a resource.

The environmental comparisons carried out further showed that:

- The use of recycled glass bottles makes it possible to measurably reduce the environmental impacts associated with the bottling stage, thanks to the lower consumption of virgin raw materials and the reduced energy required for glass production. This translates into a decrease in climate-altering emissions and reduced pressure on natural resources.

- The adoption of electricity from renewable sources in the energy-intensive stages (in particular freeze-drying and SFE extraction) would allow a reduction of up to 80% in the carbon footprint and fossil resource use, as indicated by the modelled scenarios. This option, combined with energy efficiency measures on the equipment, represents a strategic lever to align the process with climate neutrality targets.

Overall, these results highlight that the choice of more sustainable materials and energy sources is not a marginal aspect, but a concrete and highly effective lever for improving the environmental performance of the system under study.

As in any LCA study, several sources of uncertainty are present, mainly related to:

- Estimates of energy consumption and process yields, which may vary depending on real operating conditions (plant scale, maintenance status, load fluctuations, etc.);
- Variability of primary data collected at company level, linked for example to differences between production batches, seasonality of raw materials or operational practices;
- Dependence on secondary databases (e.g. Ecoinvent) for the modelling of some material and energy flows, which implies the use of representative datasets that are not always perfectly tailored to the specific context;
- Simplifying assumptions regarding the end-of-life stage of products, necessary to delimit the system boundaries (for example assumptions on glass recycling rates, organic waste management options, and average disposal mixes).

However, thanks to the transparency in the definition of assumptions, the explicit description of data sources and the methodological consistency adopted throughout all phases of the study (goal and scope, LCI, LCIA, interpretation), these uncertainties do not compromise

the robustness of the overall conclusions. The trends identified – the critical role of packaging, the potential of peel valorisation and the benefits of decarbonisation options – can therefore be considered solid indications for guiding future strategies to improve the process.

CHAPTER 6
CONCLUSIONS

The research activities carried out convincingly demonstrate the use of supercritical CO_2 for the valorization of lemon peels, presenting it as a "green" technology in line with the principles of the circular economy, capable of reducing or eliminating the use of conventional organic solvents and, at the same time, sufficiently selective to concentrate the most functionally relevant fractions. This ability to modulate the composition of the extracts by tuning process parameters (pressure, temperature, possible co-solvent) makes $scCO_2$ particularly suitable for the production of high-purity ingredients with a safety profile compatible with high value-added applications, especially in the cosmetic and nutraceutical fields.

The results also highlight that citrus extracts are already supported in the literature by solid evidence of antioxidant, anti-inflammatory and photoprotective activity, as well as by a potentially "skin-friendly" profile that makes them natural candidates for dermocosmetic applications. In this perspective, the orientation toward dermocosmetic applications does not appear as a theoretical stretch, but rather as the natural evolution of a consolidated scientific background: essential oil and fractions enriched in flavonoids and other secondary metabolites can be reinterpreted as functional ingredients for formulations aimed at protecting the skin barrier, preventing oxidative stress and supporting mild inflammatory conditions.

Another relevant aspect is the positioning of Life Cycle Assessment (LCA) as an almost mandatory step to assess the actual sustainability of the proposed model. The valorization of by-products is not, in itself, a guarantee of environmental sustainability: the introduction of highly energy-intensive stages, such as freeze-drying and supercritical CO_2 extraction, may reduce or cancel out the benefits if not carefully designed and managed. LCA therefore becomes the key tool to verify whether the recovery of lemon peels, in an perspective, leads

to an effective improvement in impacts compared to the reference scenario (e.g. disposal or low value-added uses), or whether it is necessary to intervene on the energy mix, process efficiency and plant integration in order to keep the promise of circular economy.

From a forward-looking standpoint, the chapter implicitly suggests the opportunity to evolve from the simple recovery of essential oil to a fully integrated biorefinery concept, in which the same waste biomass is fractionated into multiple product streams: in addition to the lipophilic extracts obtained by $scCO_2$, parallel valorization chains could be developed for dietary fibers, pectins and more polar fractions, for instance through complementary extraction technologies. This would increase the overall efficiency of resource utilization and allow the distribution of process costs across a broader range of marketable products.

At the same time, the need emerges for closer alignment with the cosmetic and nutraceutical regulatory framework, explicitly addressing use limits, safety requirements, required dossiers and the types of claims that are actually admissible. A model such as , in order to be credible at industrial level, cannot stop at the mere techno-scientific demonstration of extract functionality, but must integrate market-driven considerations from the outset, showing how lemon peel-derived ingredients can be positioned on real markets with products and claims that comply with current regulations. In this direction, the integration of LCA with techno-economic assessment tools, up to and including Life Cycle Costing and, in the future, forms of social LCA, represents a natural evolution: only a joint evaluation of environmental impacts, life-cycle costs and social implications can provide a 360-degree view of the feasibility of the model and of its potential transition from demonstration scale to full industrial implementation.

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