UNIVERSITA DEGLI STUDI DI TORINO

UNITA universitas montium

UNIVERSITÉ SAVOIE MONT BLANC

THESIS

To obtain the degree of

DOCTOR OF UNIVERSITÉ SAVOIE MONT BLANC

Subject area: **Chimie**

Arrêté ministériel : 25 Mai 2016

DOCTOR OF UNIVERSITÀ DI TORINO

Subject area: **Scienze Farmaceutiche e Biomolecolari**

Presented by

Lauriane BRUNA

Supervised by **Grégory CHATEL** and **Giancarlo CRAVOTTO**

Prepared within **Laboratoire Environnement, Dynamique et Territoires de Montagnes (EDYTEM)** and **Dipartimento di Scienza e Technologia del Farmaco (DSTF)**

In **École Doctorale Sciences, Ingénierie et Environnement** and **Scuola di Dottorato in Scienze della Natura e Technologie Innovative**

Development of Enabling Technologies for Residual Biomass Valorization in a Context of Circular Economy

Thesis publicly defended on **November 15th, 2024**, before the jury composed of:

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saved me from bothering Alexandre yet again. To Imed and Fabrice, I wish you all the best in your entrepreneurial adventure with KitoSphère! Thank you to Lise and Julia G. for welcoming me into your office this year, it was such a pleasant place to work! I thank Mathieu for helping me with the analytical equipment and the analyses! I'm thankful to Audrey for her help with the bacterial inhibition calculations. She was always patient and willing to explain things multiple times. How can I not thank Camille and Charlotte? There was a time when I spent a lot (maybe too much?) time in your office chatting. Thank you for welcoming me for many hours. You brought me a lot during some difficult periods.

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List of Abbreviations

2-MeOx 2-methyloxolane;

5-HMF 5-(hydromethyl)furfural;

AAE ascorbic acid equivalent;

ABTS antioxidant test using $ABTS^{+\bullet}$;

AE acetone extraction;

ANOVA analysis of variance;

AP apple pomace;

B. cereus *Bacillus cereus*;

BHI brain-heart infusion;

CA citric acid;

CC_{50} cytotoxic mean concentration;

CCRD central composite rotatable design;

CE circular economy;

CfAE caffeic acid equivalent;

ChAE chlorogenic acid equivalent;

ChCl choline chloride;

ChCl:CA choline chloride-citric acid;

ChCl:LA choline chloride-lactic acid;

ChCl:MA choline chloride-malic acid;

ChCl:OA choline chloride-oxalic acid;

ChCl:U choline chloride-urea;

ChCl:U:W choline chloride-urea-water;

DCM dichloromethane;

DES deep eutectic solvent;

DIZ diameter inhibition zone;

DoE Design of Experiments;

DRSC DPPH radical scavenging capacity;

EC_{50} effective concentration at 50%;

E. coli *Escherichia coli*;

EtOH ethanol;

EU European Union;

FAO Food and Agriculture Organization of United Nation;

FAOSTAT Food and Agriculture Organization of United Nation Statistic Database;

FDA food and drug administration;

FFR furfural;

FRAP ferric reducing antioxidant power;

FU functional units;

FW fresh weight;

GAE gallic acid equivalent;

GC-MS gas chromatography mass spectroscopy;

GE glucose equivalent;

G:S:W glucose:sucrose:water;

HBA hydrogen bond acceptor;

HBD hydrogen bond donor;

HIV *Human Immunodeficiency Virus*;

HORAC hydroxyl radical adverting capacity;

HSV *Herpes Simplex Virus*;

IC_{50} inhibition effective mean concentration;

IEA Internation Energy Agency;

IL ionic liquid;

IR infrared;

LA lactic acid;

LCA Life Cycle Assessment;

L. innocua *Listeria innocua*;

M3GE malvidin-3-glucoside equivalent;

MA malic acid;

MAE microwave-assisted extraction;

ME methanol extraction;

MeOH methonal;

M:LA menthol:lactic acid;

MIC minimum inhibitory concentration;

NADES natural deep eutectic solvent;

nd not determined;

OA oxalic acid;

OD optical density;

ORAC oxygen radical absorbance capacity;

o/n overnight;

PBS phosphate-buffered saline;

PD Petri dish;

REACH Registration, Evaluation, Authorization, and Restriction of Chemicals;

RSM response surface methodology;

RT room temperature;

SC-CO_2 supercritical CO_2;

SEM scanning electron microscopy;

SFE supercritical and subcritical fluids extraction;

SPE solid-phase extraction;

SWE Subcritical Water Extraction;

TAC total anthocyanins content;

TE Trolox Equivalent;

THF tetrahydrofuran;

TPC total polyphenols content;

TSC total sugars content;

U urea;

UAE ultrasound-assisted extraction;

US ultrasound.

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INTRODUCTION

According to the European Commission, more than 2.609 million tonnes of waste were produced by the European Union (EU) inhabitants in 2020 including around 88 million tonnes of food loss.[1,2] The Council of the European Union aims to reduce by half the food waste by 2030. To achieve this waste reduction, the European Council has launched the *European Green Deal*. This deal promotes a shortened supply chain from the production to the consumption, called *farm to fork*, or the *circular economy* (CE).[1]

The CE concept has gained popularity worldwide over the last decade. It is based on seven pillars in three areas, the main objective of which is to prevent the generation of waste as part of an overall approach: (1) supply by economic actors (extraction, production and sustainable supply chain; eco-design; industrial and territorial ecology; functional economy), (2) consumer demand and behavior (product life extension; responsible consumption) and (3) waste management (recycling and valorization of materials and organic matter) (Figure I-1).[3]

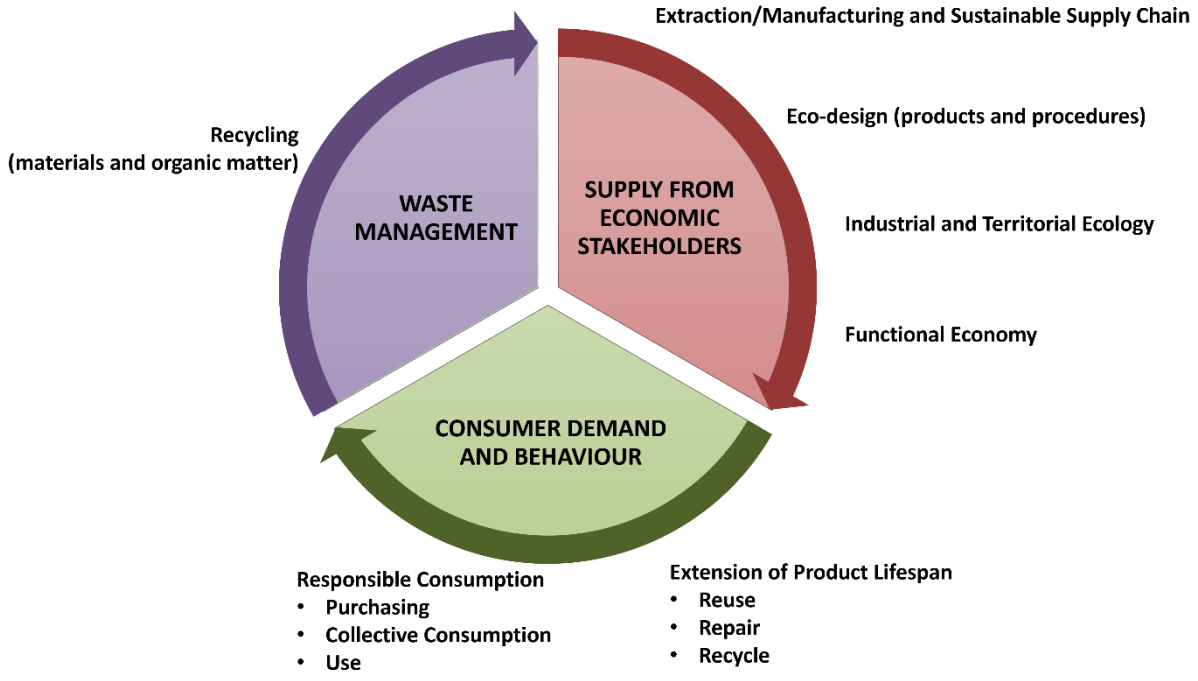


Figure I-1. Circular Economy scheme – ADEME.

In line with sustainable development, the CE has a positive impact on the environment and society, offers new opportunities in terms of consumer behavior and leads to a need for

innovation in every field. CE is highlighted more and more in chemistry, for example for car battery recycling or for cosmetic production. CE was at the heart of the valorization project, choosing the studied waste and extraction methods. The chosen biomass will be part of waste management and the extraction processes tested will have to be eco-designed (supply from economic stakeholders).

At the beginning of this work, several criteria were established to select a biomass to be studied over 3 years, as a waste product to valorize in this context of CE. The first criteria was the availability of selected waste in Savoie and Piedmont territories, as this work is part of a European collaboration, between two Universities of the UNITA *Universitas Montium Alliance*, the Université Savoie Mont-Blanc and the Università di Torino.[4] The second criteria is the large amount in both regions and the final criteria is molecules with high added value in the chosen biomass. For the first criteria, an inventory of production from each region was drawn up. For the Savoie, the products selected were apples, wine, and Crozets (type of pasta), among others, and for the Piedmont, the products were wine, chocolate, rice, pasta, and apple. The production of apples in Piedmont is not very well known internationally, but production is higher than in Savoie. By eliminating the products that did not match and broadening this point, several wastes were selected: waste from the manufacture of pasta, vines, and apples. For the criteria of large amount, pasta waste did not produce enough waste and was not selected for this project. Fortunately, the last two products produce significant amount of non-valorized waste in both regions and contained high added value molecules. Wine waste was not selected because several projects have been carried out and continue to be studied by the two laboratories, in particular the VITIVALO project.[5] With all the information collected, apple waste was chosen, called apple pomace (AP). Apple production in Savoie and Piedmont are 12,000 tonnes (in 2020) and 225,000 tonnes (in 2022), respectively.[6,7] Some of this production is processed into value-added products, such as pies, cider and apple juice. 16.25 to 19.5% of production is processed into apple juice.[8] The estimated production of apple waste is between 585 and 702 tonnes in Savoie and between 10,968 and 13,162 tonnes in Piedmont (Figure 2).

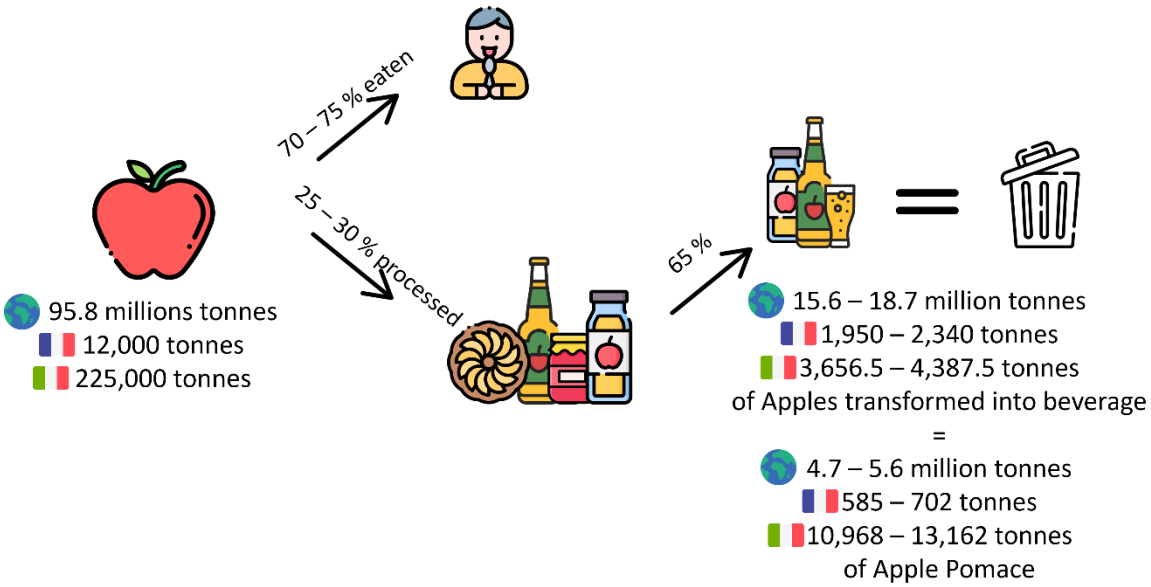


Figure I-2. Recapitulation of production of apple and apple pomace around the world in Savoie and in Piedmont.

The most common way of dealing with this waste worldwide is to bury it in the ground. However, this can cause major problems for human health and the environment.[9] In the European Union, another method is used: methanisation. AP is partially recycled with this solution. It produces electricity and biogas. According to Philippe Bernot, CEO of *Source du Verger*, it is no longer possible in France to feed this waste to livestock. Methanisation is a process that can easily treat several tons of waste (depending on the capacity of the structure) producing biomethane gas. This method valorize C and H but not the value-added molecules. Academia and industry are increasingly looking for ways to recycle and/or valorize AP. For some years now, companies such as *SAMARA*, a North American brand, have been using apple waste to make imitation leather.[10] AP can be sold simply dried and ground. *HUBCYCLE* is a French company that sells various types of waste to compagnies.[11] In this form, AP can be used as a source of sugars or nutrients. Université Aix-Marseille and Symrise have recently been completed a thesis on the recovery of wax for cosmetic purposes to possibly use it as bio-based cosmetic products, avoiding the addition of petrochemical preservatives.[12] These recovery methods are currently pre-industrial or industrial, but do not develop.

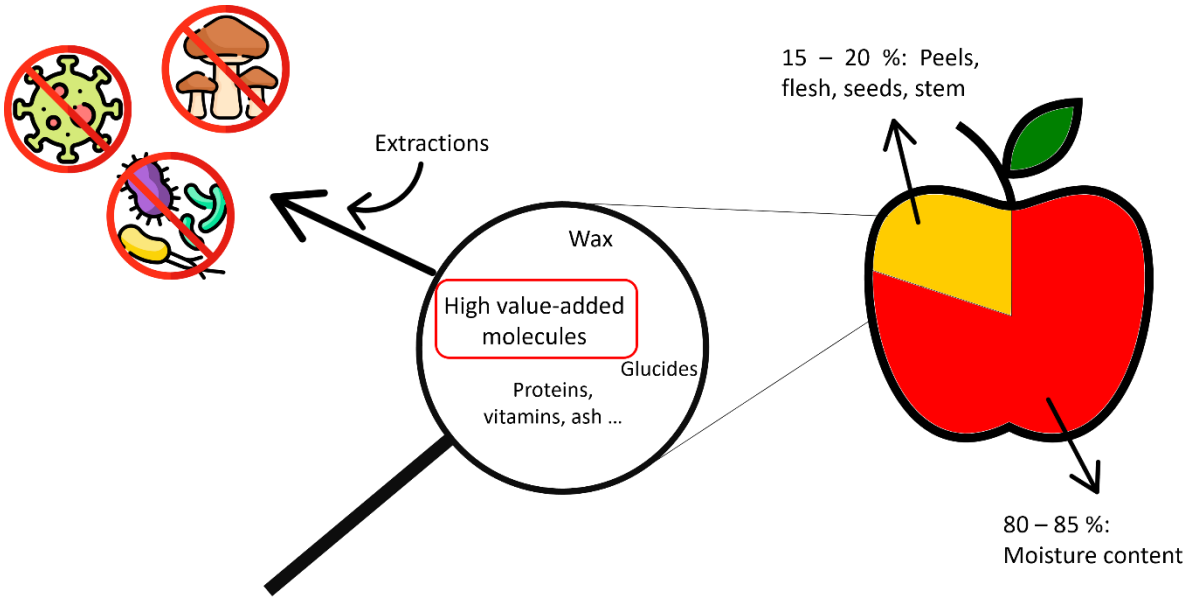
However, the apple polyphenols have very interesting biological properties. These biomolecules have antioxidants, anti-diabetic, anti-cancer, anti-inflammatory and many other activities.[9,13–15] In this project, entitled VAL'Apple, the antibacterial properties of AP

phytomolecules has been using innovative, environmentally friendly techniques. Once the biomass had been selected, the extraction techniques had to be chosen based on innovation and green chemistry (solvent reduction, etc.). A number of extraction processes were identified, including ultrasound, microwaves and supercritical fluids. The least mentioned in the literature for the extraction of polyphenols from AP was chosen. Several extraction methods have been used, including subcritical water and supercritical CO_2 and another eco-solvent, NAtural Deep Eutectic Solvent.

This manuscript is divided into 6 chapters. The first chapter is a review of the state of the art of extractions using the above-mentioned eco-solvents with selected biological, antioxidant and antibacterial activities. The data has been updated and a section on NAtural Deep Eutectic Solvent has been added compared to a review article accepted in 2023. The second chapter is devoted to the materials and methods used during these 3 PhD years. The third and fourth chapters focus on supercritical CO_2 and subcritical water extractions, with optimization of extraction conditions, improvement of mass yields and biological activities. Chapter 5 deals with biological activities of the extracts using NAtural Deep Eutectic Solvent as an eco-solvent. As a final chapter, a life cycle assessment was carried out to determine the feasibility of chemical recovery of these extracts. Finally, a general conclusion is given at the end of the manuscript.

Chapter 1

Waste Management of Apple Pomace: Extraction of Antimicrobial Molecules Using Green Technologies



Chapter 1 is the bibliographic part of the manuscript. This chapter has been published in *Waste and Biomass Valorization* (Springer; 10.1007/s12649-024-02432-4) in 2024. Small modifications were made in this version to be more suitable to the whole manuscript thesis. The last part about eco-solvents was included in the chapter.

This review focuses on the utilization of apple waste for antimicrobial applications, aiming to enhance its value. The use of subcritical and supercritical fluids for extracting biological molecules is emphasized as a promising eco-extraction technology. The study highlights the significant antimicrobial activities observed in the extracts obtained from apple waste. Furthermore, the influence of extraction and storage conditions on the chemical profile and biological activity of these extracts is discussed. Supercritical CO_2 extraction was found to produce higher quality extracts compared to conventional methods, primarily due to the absence of air and light. To maintain the chemical and biological properties of the extracts, it is crucial to carefully control the pretreatments, drying processes, and storage conditions of the apple waste. Lastly, this review explores the potential enhancement of biological activities through physicochemical functionalization methods.

I.Introduction

Over the last decade, the concept of circular economy has gained popularity worldwide. It is based on seven pillars in three areas whose main objective is to prevent the generation of waste as part of an overall approach: (1) supply from economic stakeholders (extraction, production and sustainable supply chain; eco-design; industrial and territorial ecology; functional economy), (2) consumer demand and behavior (product life extension; responsible consumption) and (3) waste management (recycling and valorization of materials and organic matter).[3] In accordance with sustainable development, circular economy has a positive impact on the environment and society and offers new opportunities in terms of consumer behavior and leading to a need for innovation. Depending on the raw material, extractions could meet the expectations of circular economy, especially by taking recycling into account.

Natural products can be extracted in different ways: by conventional extraction (*e.g.*, Soxhlet extraction, maceration, etc.) or by eco-extraction processes (*e.g.*, supercritical CO_2, subcritical water, ultrasound-assisted extraction, etc.) that follow the principle of green chemistry.[16,17] These processes represent a great opportunity to evolve in the face of the environmental context (*e.g.*, global warming), and abandon conventional processes that may be technologically obsolete.

Apples are one of the most widely produced fruits in the world.[18] According to the Food and Agriculture Organization of the United Nations, more than 95.8 million tonnes were produced worldwide in 2022.[19] Nearly 9 kg of apples are consumed per person per year. The largest producer is China, which for many years has accounted for about half of total international production (Figure C1-1). In 2022, Turkey, United States, and Poland are other major producers, accounting for 5.0%, 4.6%, and 4.4% of world production, respectively. According to Kammerer *et al.*, 25-30% of apples production is processed into value-added products, mainly juice (approx. 65%). AP is a left-over obtained after pressing the apples. The waste represents 20-35% of the fresh weight (FW).[20] It consists of 94.5% FW flesh and skin, 4.1% FW seeds and 1.1% FW stem.[21] The moisture content is about 75-80%.[9,21] This non-negligible amount represents a hazardous waste for humans and the environment. According to Bhushan *et al.*, moisture content promotes microbial decomposition, resulting in unpredictable fermentation of AP and the high biodegradable organic load leads to environmental and health problems.[9]

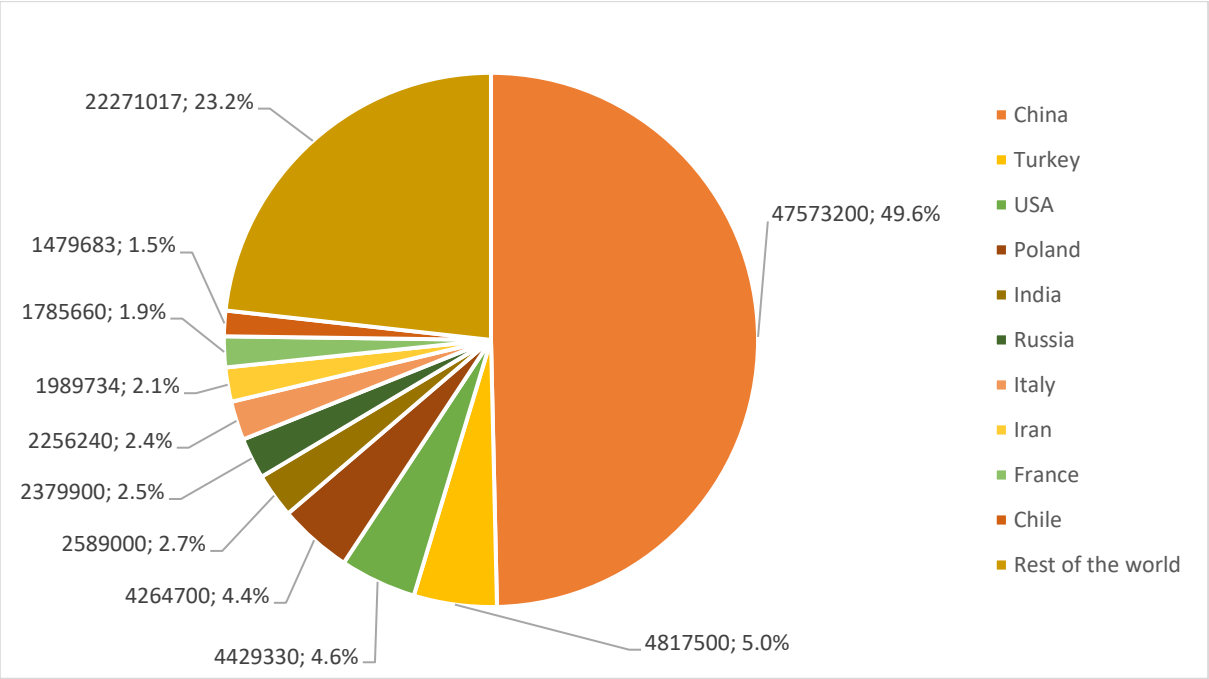


Figure C1-1. Main apple producers in the world in 2022 (amount in tonnes;%).[19]

The literature identifies several potential applications for apple pomace AP. These include animal feed, fuel source, substrate for ethanol production by fermentation, composting material and feedstock for biogas production in waste treatment processes.[21] More recently, AP powder has been utilized to create vegan leather.[22] A prominent method for biogas production is methanation, which is applicable to various types of waste, including crop residues like AP, animal manure, municipal solid waste, and municipal wastewater. The International Energy Agency highlights Europe as the primary region employing methanation. This prevalence is largely due to supportive European policies and financial incentives, making Europe the largest user of this technology.[23]

Apple pomace is no exception in this context, as it is often processed in facilities for gas production. However, it is important to note that apple waste contains high-value molecules, such as polyphenols, which have significant biological activities and positive health effects on humans and animals.[24–26] The primary phenolic compounds in apples include quercetin derivatives, dihydrochalcones, flavanol mono-, di-, and oligomers, as well as esters of caffeic and p-coumaric acids.[27,28] The antioxidant properties of these polyphenols have been extensively researched and documented.[29,30]

This review does not focus on the antioxidant property, but on other biological evidence such as antibacterial, antifungal, and antiviral activities. After a presentation of polyphenols from

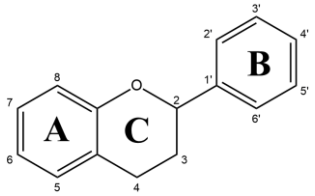
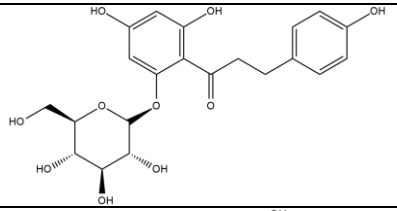
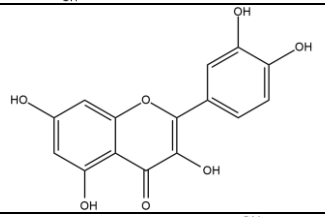
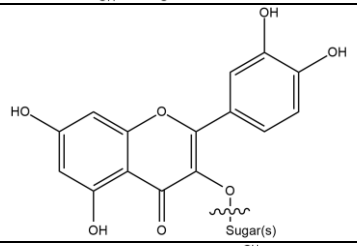
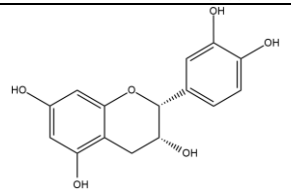
apple pomace and associated biological activities of extracts, the importance of the type of extraction and storage conditions for the preservation of bioactive molecules is explained. Finally, the possibilities of improving biological activities by chemical or physical functionalization are reported.

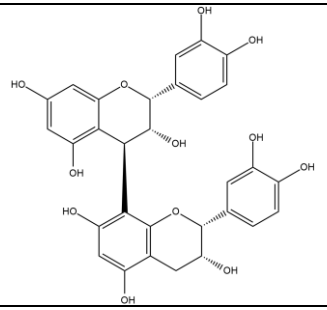
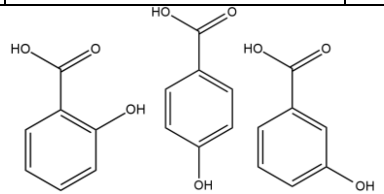
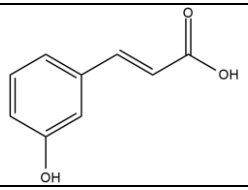
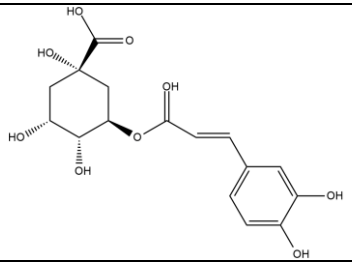
II. Chemical profiles and activities in AP

A. Chemical structures of main molecules of AP

Two main families of molecules are distinguished in AP composition: flavonoids and phenolic acids. Different parameters can modify the composition of apple and/or the apple extract such as weather, cultivar, extractions, pre-treatment *etc.*

Table C1-1. Structures and contents of major phenolic compounds in apples.

Entry	Name	Family	Class	Chemical structure	Contents (mg/kg DM)
1					
2	Phloridzin	Flavonoid	Dihydrochalcone		910[28] 380.05[26] 730[31]
3	Quercetin	Flavonoid	Flavonol		67[28]
4	Quercetin derivatives: Hyperoside, rutin, isoquercetin, quercitrin, avicularin, *etc.*	Flavonoid	Flavonol		880[28] 766.84[26] 5433[31]
5	(-)-epicatechin	Flavonoid	Flavan-3-ol, flavanol		190[28] 88.45[26] 640[31]

6	Procyanidin B2	Flavonoid	Proanthocyanidin		160[28] 74.87[26] 692.4[31]
7					
8					
9	Chlorogenic acid	Phenolic acid	Hydroxycinnamic acid		450[28] 166.12[26] 59.4[31]

1.Flavonoids

The flavonoids family comprises several classes, including anthocyanins, catechins, chalcones, flavones, flavonols, flavanols, flavanones and, flavanonols.[32] The different names are referred to the chemical structure of each subgroup. Flavonoids have a common chemical structure (Table C1-1 entry 1), consisting of two benzene rings (A and B rings) bonded to a pyran (C ring). The heterocyclic pyran-4-one (ketone on C4) and double bond C2-C3 suggest being significant parameters for biological activities. Other modifications further enhanced the bioactivity, such as the number of hydroxyl groups on ring B. This modification is acting as an electron donor and is responsible of the radical scavenging activity.[33] Flavonoids can present glycosidic bonds. In the case of principal flavonoids of apple, the sugar part is linked through the C3 (quercetin derivatives) or the C5 (phloridzin).

The structure of quercetin is a ketone on C4 (C ring), double bond C2-C3 and hydroxyl groups on C3, C5 and, C7 (Table C1-1 entries 1, 3). In food, quercetin is mainly bounded with sugar, phenolic acids, alcohols, *etc.*[34] The C3 hydroxyl group is linked to sugar units in the case of glucosides of quercetin (Table C1-1 entries 1 and 4). Quercetin derivatives are more efficiently absorbed by the organism, unlike rutin which is scarcely absorbed. Glucosides of dietary polyphenols are absorbed faster and more efficiently than the aglycones.[35]

Phloridzin, known as phloretin glucoside, belongs to the dihydrochalcone group. The group does not have a C ring (Table C1-1 entries 1 and 2). Apples are the largest source of dihydrochalcone.[36] The main sources of phloridzin class are tomatoes, peers, strawberries, bearberries and in some products of wheat. It also knows for plenty of biological and health benefits.[32]

Flavan-3-ols family has several names: dihydroflavonols, catechins, flavononols. This group is present in banana, peers, peaches, apples, and blueberries.[32] The chemical structure of catechins is a hydroxyl group on C3, inducing two chiral centers on C2 and C3 (Table C1-1 entries 1 and 5).[37]

Procyanidin B2 belongs to the proanthocyanidin class. This group has the characteristic of being oligomers. This phytochemical is a dimer of (-)-epicatechin. (-)-epicatechin and (+)-catechin are the basic units. The degree of polymerization of proanthocyanidin can be up to 11. Catechin blocks are bounded together by C-C bonds of the C4-C6 and C4-C8 (Table C1-1 entries 1 and 6).[38] The best sources of proanthocyanidin are fruits (plum, apricot) and berries (cranberry, lingonberry).[38]

2.Phenolic acids

Phenolic acids are characterized by a carboxylic acid group and hydroxyl groups attached to a benzene ring (Table C1-1 entry 9).[39] Phenolic acids are separated into two groups: hydroxybenzoic (Table C1-1 entry 7) and hydroxycinnamic acids (Table C1-1 entry 8). Chlorogenic acid belongs to the second group. The chemical structure of phenolic acids, the length of saturated chains, the number of hydroxyl groups on benzene, affect the antimicrobial activity. For instance, longer the saturated chains showed higher antimicrobial activity. They depend on the number of functionalization (hydroxyl and methoxy groups). The increase of double bonds present in chlorogenic acids, enhances the antibacterial property.[14]

B.An overview of biological activities of AP

Apples are composed of many molecules. According to Waldbauer *et al*., eating an apple is beneficial to health while regular consumption of apple juice is controversial about its beneficial value.[40] Dried apples or apple peel have been introduced in teas or decoctions to provide a cough suppressant effect. In the review of Feng *et al*., the location of active biomolecules in apples, mainly in peel and seeds is in agreement with Waldbauer *et al*.[41]

Flavonoids are ubiquitous in vegetables and fruits and of course in apples, and they are the main groups of secondary metabolites. Correct dietary habits (consumption of fruits and vegetables) can prevent cancer deaths.[35] The main advantages of using polyphenols are their low toxicity, high accessibility, the small intake of flavonoids and the diversity of the structures.[34,35] They minimize the food allergies risk.[34] Phytochemicals have very specific functions in the body. They can be pigments, cause an odor or a taste, serve as UV protection *etc.*[42] For example, rutin in sunscreen can increase the Sun Protection Factor effect.[43] The (-)-epicatechin family causes bitterness.[37] The taste of some fruits is due to the high amount of proanthocyanidins. The astringency of the plant is a defense against predators and pathogens.[38] In addition to having properties in the body of the plant, these molecules once extracted keep their properties.

The most known and widespread property is the antioxidant effect. According to Rodríguez-Muela *et al*., adding AP to sheep feed would have health benefits for the animal due to the increase of antioxidant activity in the blood plasma.[25] Some bioactive molecules from AP have higher activity than vitamins C or E.[14,45] AP contains quercetin, one of the best documented

and most potent antioxidant polyphenols.[34] The chemical structure has an impact on the biological activities.[33] Flavonoids without sugar units have a stronger antioxidant capacity than their corresponding glucosides. The degree of polymerization of procyanidins enhances the efficiency of antioxidant activity.[46]

Flavonoids have shown several activities: anticancer, anti-inflammatory, antidiabetic, antiobesity, neuroprotective, *etc*.[46,47] Flavonoids inhibit heat shock proteins (complexes allowing tumors to grow) and thus influence breast cancer, leukemia, and colon cancer.[46] Quercetin combined to medical ultrasound as pre-treatment on cancer cells has been tested on prostate and skin cancer. The results of the *in vitro* study showed 90% mortality on cancer cells within 48 h with no mortality on normal cells at low-frequency (20 kHz, 2 $W.cm^{-2}$, 60 s).[48] Various flavonoids such as catechin, quercetin, rutin have been reported for their hepatoprotective activity.[46] Hypothesis of hepatoprotective capacity of phlorizin is a regulation the metabolism of lipids and oxidative stress and a inhibition of hepatic inflammation and apoptosis.[47] Qin *et al*. reported that, phlorizin and its derivatives from crab-apple leaves showed an antitumor effect on different tumor cell lines (liver, lung, ileocecal, and colon).[49] The results suggest the number of methoxy groups has an influence on the antitumor activity. Several polyphenols from apples are described as agents for the treatment of skin diseases caused by UV radiation. Derivatives of quercetin have been tested in *in vivo* study on UVB irradiated-skin mice model. The molecules have the ability to reduce sunburn symptoms.[24] According to Kano *et al*., the isoflavone (almost same structure of quercetin, except the B ring is on C3) concentration in blood and skin of mice results in photoprotective effect on skin.[50]

Several flavonoids, such as quercetin, have anti-inflammatory effects. Quercetin can affect the functions of enzyme involved in inflammatory system.[46] Phloridzin has the same property by suppressing plasma inflammatory adipokine (adipose protein) level and by reducing inflammation in obese mice.[47]

Table C1-2. Few examples of biological activities.

Entry	Molecules	Biological activity
1	Phloridzin	Antioxidant, antimicrobial[51], antidiabetic[15], hepatoprotective, anti-inflammatory[47], antitumor[49]
2	Quercetin	Antioxidant[34], hepatoprotective, anti-inflammatory[46], anticancer[48]
3	Quercetin derivatives	Antioxidant[33], hepatoprotective[46], UV protector[43]
4	(-)-epicatechin	Antioxidant, antimicrobial, anti-inflammatory, antitumor, cardioprotective, antidiabetic[52]
5	Procyanidin B2	Antioxidant, anticancer[53], antimicrobial[54]
6	Chlorogenic acid	Antioxidant, hepatoprotective, anti-inflammatory, anti-obesity, antidiabetic, antimicrobial[55]

C.Antimicrobial activities of AP

This review focuses on the antimicrobial, antifungal, and antiviral properties of apple pomace (AP) extracts and the phenolic compounds they contain. An antimicrobial agent is chemical compound/extract able to kill microorganisms or to stop their growth. These effects have been increasingly studied in recent years because of their health interest. According to the prediction, microbial pathogens could cause more than 10 million deaths by 2050.[56] In the United States, 70% of people with a bacterial infection have bacteria that are resistant to at least one antibiotic.[57] There are different ways to measure an antibacterial activity: minimum inhibitory concentration (MIC) and diameter inhibition zone (DIZ). The concentration varies depending on bacteria, on compounds and on the infected human. To inhibit *Staphylococcus aureus*, the concentration of phloridzin from AP extract is 0.50 ± 0.05 mg/mL against 0.10 ± 0.02 mg/mL for phloretin.[58] The samples were extracted using ultrasound bath. According to Ganeshpurkar and Saluja, mixture of rutin and other flavonoids enhances the antimicrobial activity synergistically against *Bacillus cereus* and *Salmonella enteritidis.*[59] Flavan-3-ols and flavonols have the highest spectrum of antibacterial activity compared to other polyphenols according to Zardo *et al*.[31] The antimicrobial activity was tested using an AP extract of 1 mg per disk on several bacteria among them *Bacillus subtilis*, *Staphylococcus aureus*, *Methilicillin-Resistant Staphylococcus Aureus*, or even *Escherichia coli* and comparing the pre-treatment of the solid samples (freeze-dried or oven-dried).[60] The extracts have been extracted with maceration. They have a DIZ between 1 - 5 mm on bacteria except for *Pseudomonas putida*

and *Pseudomonas aeruginosa*, no inhibition was observed. The same group enriched their extract with antioxidants *via* combined enzymatic extraction (*Rhizomucor miehei* cellulase and *Aspergillus niger* pectinase). Results showed that the antimicrobial activity remained unchanged or was increased in all antimicrobial tests. The free-enzyme test showed no inhibition but with treatment, inhibition occurred. For example, there was no inhibition on the *Pseudomonas putida* for the oven-dried AP free-enzyme test while in the oven-dried AP cellulase test, the inhibition zone was between 1 - 3 mm. According to Zhang *et al*., gram-positive *Staphylococcus aureus* is more sensitive to dihydrochalcones than gram-negative *Escherichia coli* according to the results (39.17 ± 2.71 mm for *S. aureus* against 28.25 ± 1.67 mm for *E. coli*).[58] The literature suggests that differences in antibacterial efficacy may be attributed to the cellular structure and chemical composition of bacteria. Gram-negative bacteria, characterized by a three-layered structure and an outer membrane composed of lipopolysaccharide, generally exhibit enhanced protection against antibacterial compounds and antibiotics compared to gram-positive bacteria.[61–64] Farooq *et al*. observed the same antibacterial activity in gram-negative and gram-positive bacteria. They extracted polyphenols from different parts of the apple: peels and pomace. They tested extracts on *Escherichia coli* (gram-negative), and on 3 gram-positive bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* and compared to antibiotic (ciprofloxacin). For *Staphylococcus aureus*, the extract from apple peel has antibacterial activity equivalent to the antibiotic (respectively 19.25 ± 0.89 mm and 20.13 ± 0.83 mm). The study by Farooq *et al.* noted similar antibacterial activities in both gram-negative and gram-positive bacteria. In their research, polyphenols extracted from various parts of the apple, including peels and pomace, were tested against *Escherichia coli* (gram-negative), as well as three gram-positive bacteria: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and Enterococcus faecalis, with a comparison to the antibiotic ciprofloxacin. Notably, the apple peel extract demonstrated an antibacterial activity against Staphylococcus aureus comparable to that of ciprofloxacin (19.25 ± 0.89 mm and 20.13 ± 0.83 mm, respectively).[62] The diameter of inhibition zones (DIZ) was found to be greater for phloretin (39.17 ± 2.71 mm) compared to phlorizin (30.15 ± 1.66 mm), a difference attributed to the presence of a glucoside unit. The choice of extraction solvent also appeared to influence the DIZ. The antimicrobial efficacy of (-)-epicatechin, procyanidin B2, and quercetin derivatives was best preserved when extracted in acetone.[31] Giménez-Martínez *et al.* demonstrated that apple pomace (AP) extracts exhibit antibacterial activity

against the gram-positive bacterium *Paenibacillus larvae*, with minimum inhibitory concentrations (MIC) ranging from 20 to 150 µg/mL.[65] Similar findings were reported by Zhang *et al*. for *Staphylococcus aureus*, with MICs between 100 and 500 µg/mL.[58] Additionally, Giménez-Martínez *et al.* found that the antibacterial activity varied among nine tested European apple varieties, each exhibiting different MICs.[65] Švarc-Gajić *et al.* assessed the effectiveness of subcritical water extracts of apple tree bark against several bacteria including *Escherichi coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneunomiae*, *Porteus vulgaris*, and *Bacillus subtilis*, , observing MICs of 19.5 µg/mL for the first two, 80 µg/mL for the third, and 156.2 µg/mL for the remaining bacteria.[66] Fratianni *et al*. reported that apple extracts showed no antibacterial activity against lactic acid bacteria but did exhibit a DIZ against three different strains of Bacillus cereus and two strains of *Escherichia coli.*[67]

Over 1.5 million persons per year perished from fungal illness.[56] In the cases of antifungal agents. As with antibacterial agents, concentrations are an important parameter. Many molecules found in apples have been tested on pathogens. For example, quercetin, a molecule present in apples, is active on different fungi such as *Aspergillus niger* or *Aspergillus fumigatus.*[68] However, there is a lack of studies on molecules derived directly from AP. Oleszek *et al*. tested the antifungal activity of AP extracts on 4 fungi: *Botrytis* sp., *Fusarium oxysporum*, *Petriella setifera* and *Neosartorya fischeri.*[69] The extract with the highest concentration of phlorizin is the strongest antifungal. According to Oleszek *et al*., phloridzin reacts to form phloretin which in turn reacts to form o-quinone, a potent antifungal. The size of the sugars seems to play a role in the antifungal activity, since Oleszek *et al*. obtain a nearly complete inhibition of the growth of *Neosartorya fischeri*, *Botrytis* sp., *Petriella setifera* at a concentration of 100 µg/mL for quercetin hexosides. At the same concentration, quercetin pentosides tend to stimulate the growth of these same fungi. Above this concentration, quercetin pentosides inhibited the pathogens. Švarc-Gajić *et al.* evaluated the efficacy of subcritical water extracts from apple tree bark against two fungi, *Candida albicans* and *Aspergillus niger*, finding their minimum inhibitory concentrations (MIC) to be 156.2 µg/mL and 40 µg/mL, respectively.[66]

Antiviral agents are known for their effects on *Hepatitis virus* (B and C), *Influenza virus* (A and B), *Herpes Simplex Virus* (HSV) and *Human Immunodeficiency Virus* (HIV). Suárez *et al.* tested their AP extracts on HSV-1 and HSV-2, the extracts inhibited each virus.[26] The AP extract allowed a half maximal effective concentration (EC_{50}) of 710.9 ± 1.6 µg/mL for HSV-1 and an

EC_{50} of 629.6 ± 50.7 µg/mL for HSV-2. Suarez's team emphasises that the solvent also plays a role in preserving activity as methanol extraction allows for better preservation and therefore better antiviral activity in maceration extraction. For example, the EC_{50} for HSV-1 is 576.7 ± 17.2 µg/mL for acetone extraction and 710.9 ± 1.6 µg/mL for methanol extraction.

According to the previous examples, antimicrobial activity could be a good solution to valorize AP considering the current difficulties due to the antibiotic resistance. The antimicrobial activities section is summarized in Table C1-3.

Table C1-3. Selected examples of antimicrobial activities.

Entry	Molecules/Extracts	Targeted microorganisms	Quantity used	References
1	Phloridzin	*Staphylococcus aureus*	0.50 ± 0.05 mg/mL (MIC) 30.15 ± 1.66 mm (DIZ)	[58]
2	Phloridzin	*Escherichia coli*	1.50 ± 0.12 mg/mL (MIC) 17.05 ± 1.04 mm (DIZ)	[58]
3	Phloretin	*Staphylococcus aureus*	0.10 ± 0.02 mg/mL (MIC) 39.17 ± 2.71mm (DIZ)	[58]
4	Phloretin	*Escherichia coli*	0.25 ± 0.10 mg/mL (MIC) 28.25 ± 1.67 mm (DIZ)	[58]
5	AP extracts	*Bacillus subtilis*	Oven-dried: 4 – 5 mm (DIZ) 50 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) 100 mg/mL (MIC)	[60]
6	AP extracts	*Bacillus cereus*	Oven-dried: 1 – 3 mm (DIZ) 50 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) 100 mg/mL (MIC)	[60]
7	AP extracts	*Listeria monocytogenes*	Oven-dried: > 100 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) 50 mg/mL (MIC)	[60]
8	AP extracts	*Staphylococcus aureus*	Oven-dried: 1 – 3 mm (DIZ)	[60]

			100 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) > 100 mg/mL (MIC)	
9	AP extracts	*MRSA*	Oven-dried: 1 – 3 mm (DIZ) > 100 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) > 100 mg/mL (MIC)	[60]
10	AP extracts	*Escherichia coli*	Oven-dried: 1 – 3 mm (DIZ) > 100 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) > 100 mg/mL (MIC)	[60]
11	AP extracts	*Staphylococcus enterica*	Oven-dried: 4 – 5 mm (DIZ) > 100 mg/mL (MIC) Lyophilized: 1 – 3 mm (DIZ) > 100 mg/mL (MIC)	[60]
12	AP extracts	*Pseudomonas putida*	Oven-dried: 100 mg/mL (MIC) Lyophilized: 50 mg/mL	[60]
13	AP extracts	*Pseudomonas aeruginosa*	Oven-dried: 100 mg/mL (MIC) Lyophilized: 100 mg/mL	[60]
14	Apple peel extracts	*Escherichia coli*	15.00 ± 1.07 mm (DIZ)	[62]
15	Apple peel extracts	*Pseudomonas aeruginosa*	13.50 ± 1.20 mm (DIZ)	[62]
16	Apple peel extracts	*Staphylococcus aureus*	19.25 ± 0.89 mm (DIZ)	[62]

17	Apple peel extracts	*Enterococcus faecalis*	17.63 ± 1.06 mm (DIZ)	[62]
18	Pomace (seeds and flesh) extracts	*Escherichia coli*	11.50 ± 0.53 mm (DIZ)	[62]
19	Pomace (seeds and flesh) extracts	*Pseudomonas aeruginosa*	9.05 ± 0.71 mm (DIZ)	[62]
20	Pomace (seeds and flesh) extracts	*Staphylococcus aureus*	12.75 ± 0.71 mm (DIZ)	[62]
21	Pomace (seeds and flesh) extracts	*Enterococcus faecalis*	14.00 ± 1.07 mm (DIZ)	[62]
22	Rich-quercetin hexosides franction from AP extract	*Neosartorya fischeri*	100 µg/mL	[69]
23	Rich-quercetin hexosides franction from AP extract	*Botrytis* sp.	100 µg/mL	[69]
24	Rich-quercetin hexosides franction from AP extract	*Petriella setifera*	100 µg/mL	[69]
25	AP extracts	HSV-1	Methanol extraction: 710.9 ± 1.6 µg/mL (EC_{50}) Acetone extraction: 576.7 ± 17.2 µg/mL (EC_{50})	[26]
26	AP extracts	HSV-2	Methanol extraction: 629.6 ± 50.7 µg/mL (EC_{50}) Acetone extraction: 450.7 ± 40.8 µg/mL (EC_{50})	[26]

DIZ: Diameter Inhibition Zone; MIC: Minimum Inhibition Concentration; EC_{50}: Half maximal Effective Concentration; HSV: Herpes Simplex Virus.

III. Pretreatment, extraction methods and storage of AP and extracts

A. Conditions of extraction

Since a few years ago, new techniques have been emerging and improving as alternative techniques to the intensive use of organic solvents, such as supercritical fluids, mechanochemical technology, ultrasound, microwaves, and others. Supercritical CO_2 (SC-CO_2) and subcritical water extractions (SWE) do not use classic solvents. Soxhlet, maceration, and reflux extraction, recognized as conventional methods, typically require substantial amounts of traditional solvents. The Soxhlet method, frequently employed as a reference for plant extractions, often uses ethanol or methanol due to their polar nature.[70] CO_2 and water are available, non-toxic ("Generally Recognized As Safe" substance), non-flammable, and are currently considered as inexpensive solvents.[71] The conditions of SC-CO_2 are above its supercritical point, 73.8 bar and 304 K. For water, supercritical conditions are difficult to achieve. Hence in chemistry field, only subcritical conditions are used. The conditions to obtain subcritical water are: between 373 K and 1 bar and 220 bars and 647 K.[70] The purpose of SC-CO_2 is to extract mainly apolar molecules because the CO_2 molecule is apolar. For example, several authors used SC-CO_2 extraction to obtain oil from apple seeds, wax from apple peel, triterpenic acids, *etc.*[12,72–74] According to the literature, a higher pressure leads to a higher yield in polyphenol extraction, or co-solvents (ethanol, water) can be added to achieve equivalent results.[70,75,76] Kryževičiūtė *et al.* tested different pressures (100, 275 and 450 bar) and obtains an optimum yield at the higher pressure 450 bar with 49.22 °C and 110.27 min for raspberry pomace extraction.[77] On the other hand, SWE is an environmentally friendly industrial process for the extraction of agro-food by-products leading to high-quality extracts with shorter extraction times.[78–80] Several researchers used SWE to extract pectin, polyphenols, *etc.* from apple and apple waste.[80–85] By increasing the temperature, water has a lower dielectric constant at constant pressure. This allows to extract molecules with lower polarity.[79,86,87] Dielectric constant at 200 °C, 250 °C and 300 °C at 100 bar are 35.11, 27.43 and 20.39 F.sr.m^{-1} respectively.[88] They are equivalent to classic organic solvents, acetonitrile (35.11 F.sr.m^{-1}) and acetone (20.39 F.sr.m^{-1}).[87,89] These two methods, while complementary in maximizing the extraction of phytochemicals, are costlier to implement compared to traditional organic solvent techniques. The SC-CO_2 extraction process often necessitates the use of co-solvents or modifiers, depending on the biomass. Additionally, biomass preparation

for SC-CO_2 extraction may require preliminary steps like drying. SWE typically operates at high temperatures, ranging from 100 to 374 °C, which could lead to the degradation of heat-sensitive bioactive compounds.[87] Oliveira *et al.* worked on SC-CO_2 extraction and antibacterial activity on grape pomace.[90] The results showed an antibacterial activity wider for tested bacteria with SC-CO_2 extracts compared to Soxhlet (hexane) extraction. Pedras *et al.* determined MIC from SWE grape pomace extracts on *Escherichia coli* (10.0 mg/mL) and *Staphylococcus aureus* (2.5 mg/mL).[91] Furthermore Oliveira *et al.* added scientific articles with SFE extracts on bacteria are quite uncommon.[90]

Some compounds can be degraded or modified in the presence of air (O_2), or/and light. Ferrentino *et al.* compared Soxhlet (ethanol) and SC-CO_2 extractions from AP (Table C1-4 entries 1 and 11).[92] The Total Content Phenolic (TPC) of the associated extracts is higher for the SC-CO_2 extraction than for the Soxhlet (ethanol) extraction, respectively 8.87 ± 0.17 mg Gallic Acid Equivalent/g of extract and 4.13 ± 0.90 mg GAE/g of extract. This difference can be explained by various factors, in particular the temperature of extraction. Soxhlet (ethanol) extraction is performed at about 78 °C compared to 55 °C for SC-CO_2 extraction. That can degrade phenolic compounds such as catechin.[69] As reported by Suárez *et al*., the solvent can also be an important parameter. Suárez *et al*. compared the antiviral activity of 70% acetone (AE) and 80% methanol extractions (ME) from AP (Table C1-4 entries 5 and 6).[26] The EC_{50} of the acetone/water extraction is lower than the EC_{50} of the methanol/water extraction, respectively 576.7 ± 17.2 µg/mL, and 710.9 ± 1.6 µg/mL. As a result, the AE extract is more potent than the ME extract. On contrary, the acetone extraction presents the highest percentage of inhibition against the strain studied compared to methanol and ethanol extractions.[31] These studies are difficult to compare because of the strains studied, the extraction mode and the cultivar are different. The extractions performed by Ferrentino *et al*. may suggest that the SC-CO_2 solvent is milder than ethanol (Table C1-4 entries 1 and 11).[92] Other factors influencing the recovery of polyphenols are the presence of light and air, leading to oxidation reactions that can degrade the molecules of interest such as with the molecule catechin.[69] The TPC was in favor of SC-CO_2 extractions (Table C1-4 entries 1 and 11). The TPC of supercritical fluid-based extract is 8.87 ± 0.17 mg GAE/g whereas the TPC with Soxhlet (ethanol) is 4.13 ± 0.90 mg GAE/g of extract, explained by the absence of air and light using the SC-CO_2 extraction. Therefore, the bioactive molecules are protected from air and light exposures during supercritical fluid based extraction. Perussello *et al*. demonstrated an

avoided degradation and therefore a better-quality yield with SC-CO_2 extraction because the extract avoids contact with air and light.[93] Raventós *et al*. observed such as Ferrentino *et al*. a better quality of extracts using supercritical fluid extraction.[92,94] Zambrano *et al*. concluded to higher TPC in AP extracts, higher will be antibacterial activity in their study.[60] Regarding to this data, the antibacterial activity is supposed to be higher with SC-CO_2 compared to classic extractions for Ferrentino *et al*. case.[60,92] In their study on extracting bioactive molecules from apple tree bark using subcritical water extraction (SWE), Švarc-Gajić *et al.* observed higher activity compared to Zambrano *et al.*'s apple pomace (AP) extracts.[66] Their minimum inhibitory concentrations (MIC) against Bacillus subtilis were 0.156 µg/mL and 50 µg/mL, respectively. This significant disparity can likely be attributed to the extraction method, as SWE is known to better preserve molecules.[78]

Table C1-4. Examples of extractions: main biological results according to the type of extraction of polyphenols from apple waste.

Entry	Extraction	Parameters	Antioxidant activity	Antimicrobial results	Ref
1	Soxhlet extraction	1:30 (AP:ethanol; w/v) 6 h 78 °C	TPC: 4.13 ± 0.90 mg GAE/g of extract	nd	[92]
2	Soxhlet extraction	1:15 (AP:hexane; w/v) 8 h 69 °C	$IC_{50\%}$: 0.015	nd	[12]
3	Soxhlet extraction	1:15 (apple seeds:hexane; w/v) 6 h 69 °C (boiling point)	DPPH: 22.5 ± 2.5%	nd	[95]
4	Reflux extraction	1:100 (AP:Solvent; w/v) 37 min 100 °C	TPC: 2.37 ± 0.01 mg GAE/g of extract	nd	[92]
5	Maceration	1:10 (AP:70% acetone; w/v) RT in darkness	TPC: 6.48± 0.29 g GAE/kg DM AP	CC_{50}: 5497.9 ± 292.4 µg/mL EC_{50} (HSV-1): 576.7 ± 17.2 µg/mL EC_{50} (HSV-2): 450.7 ± 40.8µg/mL	[26]
6	Maceration	1:10 (AP:80% methanol, w/v)	TPC: 3.63 ± 0.02 g GAE/kg DM AP	CC_{50}: 7281.8 µg/mL	[26]

		RT in darkness		EC_{50} (HSV-1): 710.9 µg/mL EC_{50} (HSV-2): 629.6 µg/mL	
7	Maceration	1:20 (AP:ethanol; w/v) 24 h in darkness RT	TPC: 1.71 mg GAE/g DM AP	nd	[96]
8	Maceration	1:80 (AP:methanol; w/v) 15 min RT 50% solvent concentration	TPC: 5.97 g ChAE/kg DM AP	*S. aureus* (ATCC 25923) inhibition: 9.21% *S. aureus* (ATCC 6538) inhibition: 9.26% *E. coli* (ATCC 25922) inhibition: 12.87% *S. enterica* (ATCC 13076) inhibition: 18.33%	[31]
9	Maceration	1:80 (AP:ethanol; w/v) 20 min 60 °C 50% solvent concentration	TPC: 9.54 g ChAE/kg AP	*S. aureus* (ATCC 25923) inhibition: 9.80% *S. aureus* (ATCC 6538) inhibition: 31.29% *E. coli* (ATCC 25922) inhibition: 19.73% *S. enterica* (ATCC 13076) inhibition: 18.46%	[31]
10	Maceration	1:80 (AP:acetone) 20 min 10 °C 53.50% solvent concentration	TPC: 9.86 g ChAE/kg DM AP	*S. aureus* (ATCC 25923) inhibition: 16.50% *S. aureus* (ATCC 6538) inhibition: 52.98% *E. coli* (ATCC 25922) inhibition: 33.96% *S. enterica* (ATCC 13076) inhibition: 17.96%	[31]
11	Supercritical CO_2	40 g of freeze-dried AP	TPC: 8.87 ± 0.17 mg GAE/g of extract	nd	[92]

		120 min (60 min in static extraction and 60 min in dynamic extraction) 55 °C 30 MPa Co-solvent ethanol 5% (w/w)			
12	Supercritical CO_2	4 – 6 g of freeze-dried AP 95 min 0.14 kg/h_{CO2} 55 °C 30 MPa	$IC_{50\%}$: 0.056 ± 0.15	nd	[12]
13	Supercritical CO_2	80 g of apple seeds (crushed) 140 min 1 L/h_{CO2} 40 °C 24 MPa	DPPH: 20.5 ± 1.5%	nd	[95]
14	Supercritical CO_2	15 g of AP 100 min 10 g/min_{CO2} 30 MPa	ORAC: 525.42 ± 40.41 µmol TE/g extract HORAC: 83.69 ± 5.89 µmol CfAE/g extract	nd	[97]

		46 °C			
15	Subcritical CO_2	1 g of dried AP 40 min 55.7-58.4 °C 54.6-57 MPa 2g/min (CO_2 + ethanol) 20% ethanol	TPC: 0.47 mg GAE/g DM AP	nd	[96]
16	Subcritical water	30 minutes (residence time) 200 °C 5 MPa 1% AP/Solvent (w/v)	TPC: 49.86 mg GAE/g DM AP	nd	[98]
17	Subcritical water	75 min 203.71 °C 1 g of AP/100 mL 500 µm of sample particle size	TPC: 39.08 ± 1.10 mg GAE/g DM AP	nd	[81]
18	Subcritical water	10 min 200 °C 5 g FW of AP	TPC: 13.66 mg GAE/g TEAC: 0.47 mmol/g	nd	[80]

		Water used: 5-10 mL (not detailed)			
19	Subcritical water	1:40 (apple bark:water; w/v) 40 min 150 °C 4 MPa	TPC: 31.47 ± 1.86 GAE/g DPPH-RSA: 22.57 ± 2.24 mg TE/g DW	*E. coli* (ATCC 25,922): 19.5 µg/mL *P. mirabilis* (ATCC 14,153): 19.5 µg/mL *S. aureus* (ATCC 25,923): 80 µg/mL *K. pneumoniae* (ATCC 14,153): 156.2 µg/mL *P. vulgaris* (ATCC 13,315): 156.2 µg/mL *B. subtilis* (ATCC 6633): 156.2 µg/mL *C. albicans* (ATCC 10,231): 156.2 µg/mL *A. niger* (ATCC 16,404): 40 µg/mL	[66]

ChAE: Chlorogenic Acid Equivalent; CfAE: Caffeic Acid Equivalent; CC_{50}: Cytotoxic mean concentration; EC_{50}: Antiviral effective mean concentration; GAE: Gallic Acid Equivalent; TPC: total polyphenol content; nd: not determined; IC50%: inhibition effective mean concentration in percentage; TE: Trolox Equivalent; DPPH-RSA: DPPH radical scavenging activity; ORAC: Oxygen Radical Absorbance Capacity; HORAC: Hydroxyl Radical Adverting Capacity

B. Conditions of pretreatment and storage

In addition to the extraction time, the initial drying of the raw material also affects the yield, oxidizing ability and antibacterial, antifungal and/or antiviral activities. According to Gonelimali *et al.*, oven-dried AP at 60 °C resulted in higher antioxidant activity than at 80 °C with or without vacuum and at 60 °C with vacuum.[99] Zambrano *et al.* tested the antibacterial activity on freeze-dried and oven-dried AP extracts.[60] For example, they noted that oven-dried AP extracts had a lower Minimum Inhibition Concentration (MIC) than freeze-dried AP extracts, respectively 50 mg/mL and 100 mg/mL. Numa *et al.* demonstrated that freeze-drying samples led to enhanced yield and preservation in the extraction process.[12] The drying process after pressing must be immediate and effective to keep the quality of polyphenols and the storage stability and achieve 10% of water content in the total weight of the raw material.[40] Lavelli and Corti studied the storage of polyphenols from AP extracts for 9 months at 30 °C and at different humidity content (2.4 – 4.2%).[100] The antioxidant contents decreased during months. At the beginning of the study, the total phenolics was 5,176 mg/kg DM of AP and at the end it was at between 1,704 and 4,429 mg/kg DM of AP (about 14.4 – 67.1% decrease) depending on the moisture content. The use of extracts from AP for antimicrobial purposes has to be used as soon as possible to avoid the degradation of bioactive molecules and by extension the decrease of antimicrobial potent.

IV. **Polyphenols functionalization**

The biological activity depends on the conservation of the molecules, but they may be improved by functionalization. There are several possibilities for the functionalization of the polyphenols of AP. Phytomolecules can be functionalized by classical organic synthesis or functionalized on specific surfaces to improve biocidal activity.[13,101]

Several researchers have highlighted effects due to the structure of the molecule. They give leads to improve antimicrobial activities. For example, in a recent review of Kumar and Goel several parameters have been highlighted: chain length, chain saturation, pH, position and number of substitution on a benzene ring, etc. The increasing or decreasing of biological activities can be adjusted by these elements.[14] Another example, Cueva *et al*. tested different molecules against different strain of *Escherichia coli.*[102] They found out that the saturated side-

chain length and the substitution on benzene ring affect the antimicrobial effect on *Escherichia coli*.

Baldisserotto *et al.* investigated the enhancement of antifungal activity by functionalization of phloridzin for dermo-cosmetic application.[13] The alcohol functions and the acid function were esterified and, some alcohol functions of the phenolic hydroxyl groups were selectively deprotonated. The stability of this final molecule was measured, and it was stable at 40 °C for 120 days in methanol, ethanol, or in ethanol/water solution (80:20, *v/v*). The phloridzin derivative seems to have a better antifungal effect than phloridzin on most of the fungi tested. For example, the growth inhibition of *Trichophyton tonsurans* is 20.1 ± 1.8% for the derivative and 0.7 ± 0.1% for phloridzin.

Natural compounds present in the AP were functionalized *via grafting onto* chitosan. Before grafting, molecules were oxidized with an enzyme and then grafted.[101] Chitosan was chosen as a support because it has an antibacterial effect. Authors observed an increase of antioxidant activity between 6.55 and 88.46% for all tested molecules that can be found in AP. Quercetin, (-)-epicatechin and, rutin were grafted on chitosan and tested on different gram-negative and gram-positive bacteria. The grafted flavonoids showed antibacterial activity against all tested bacteria, except on *Escherichia* coli. The aforementioned phytocompounds exhibited a higher Diameter Inhibition Zone (DIZ) than the control for Gram-positive bacteria (6.8 ± 0.4 mm for (-)-epicatechin; 5.3 ± 0.2 mm for quercetin and 4.2 ± 0.2 mm for the control for *Staphylococcus aureus*).

Nanoparticles (NPs) are increasingly used in the health field. Some biocompatible metal NPs are known to be used against cancer or to have antibacterial activities like silver.[103,104] Han *et al*. tested the functionalization of quercetin on silver NPs to increase the activity of the NPs.[104] The silver NPs were tested on 4 different bacteria and the results show antibacterial efficacy. The NPs only have a DIZ of 5 mm at 625 µg/mL against a DIZ greater than 10 mm at 62.5 µg/mL on all bacteria tested. In addition, there is a better activity at lower concentration. Functionalization of biomolecules on materials to enhance a biological effect has been much more developed than the functionalization by modifying the functional groups of bioactive molecules. Zambrano *et al*. performed AP extractions with enzyme and free enzyme.[60] For some bacteria, better results were obtained with the use of enzymes. For example, for *Methicillin-Resistant Staphylococcus Aureus* bacteria the DIZ obtained without enzyme is 1-3

mm compared to 6-8 mm in diameter with the use of pectinase and cellulase. This can be explained by the obtention of protoplast, a plant cell without cell wall.

Another way to improve activities is to is to exploit synergies between molecules in the phytocomplex. Better results are often obtained with a crude extract (consisting of several molecules of one biomass) than with fractionation of the same extract. Rutin is known to produce synergistic effects.[59]

V. **Other eco-solvents**

A. Definition

Green solvents (or eco-solvent) are solvents that reduce the environmental impact by using alternative solvents and usually bio-based. For instance, Zaib *et al.* conducted a Life Cycle Assessment (LCA) comparing synthesis in different solvents.[105] The eco-solvents of the study are Deep Eutectic Solvent (DES), methanol (MeOH) and ethanol (EtOH) and the conventional solvents are dichloromethane (DCM) and ethyl acetate. MeOH and EtOH can be considered as green solvents.[106–108] The lowest carbon footprints of the synthesis of 200 g of acetophenone are attributed to MeOH (0.590 kg_{CO2eq}), EtOH (0.967 kg_{CO2eq}) and DES (1.82 kg_{CO2eq}). For DCM and ethyl acetate, the carbon footprints were respectively 8.23 and 3.60 kg_{CO2eq}. The variation of environmental impact of DES and DCM were attributed to the quantity of solvents used (respectively 1 and 2.4 kg for the production of 200 g of acetophenone) and to the carbon footprint of the DCM or DES production (respectively 3.4 kg_{CO2eq}/kg of DCM and 1.8 kg_{CO2eq}/kg of DES).[105] This study shows that green solvents can be more sustainable than fossil-based solvents. Furthermore, green solvents are promoted by European Union regulations such Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). The purpose of REACH is to prevent chemical pollution, to protect the environment and humans and to develop green technologies. Several organic solvents became unauthorized by REACH, and substitutions were necessary. For instance, Bisphenol A, known as endocrine disruptors, has a restricted use due to REACH regulation.[109,110] The allowable amount in food for this molecule is 0.05 mg/kg. Industrial have found a substitute, bisphenol S, for which the health and environmental impact are suspected.[110]

SC-CO_2 and SWE are considered as green solvents attributed to CO_2 and water safety. Both solvents are non-toxic, non-inflammable, cheap and in large amounts. Furthermore, SC-CO_2

technique can be viewed as a method for CO_2 valorization. However, supercritical and subcritical fluids generate expensive costs such as the purchase of the equipment and its maintenance.[111] Several new solvents have been and are still developed with an easy transfer from fossil-based organic solvent to green solvent, and be less expensive. There is a classification of eco-solvents: ionic liquid, (natural) deep eutectic solvent, bio-based solvents, switchable solvents, water, solvent-free (Figure C1-2).[105,111–115]

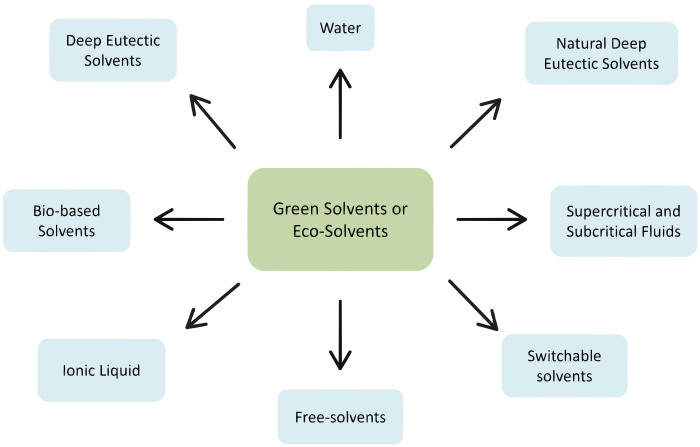


Figure C1-2. Overview of eco-solvents.

1.Bio-based solvents

This class of solvent are produced from feedstock (biomass, biomass waste, etc.). Feedstocks are sent to refinery to be transformed into bio-solvents (EtOH) or in platform molecules (Furfural; FFR).[116,117] As mentioned above, EtOH and MeOH can be seen as green solvents because they can be produced from carbon feedstock such as corn waste. Natural Deep Eutectic Solvents (NADES), 2-methyloxolane (2-MeOx), 1,2-pentanediol, FFR are bio-based solvents for instance.[112,116–118] The case of NADES will be discussed in part *f. Deep Eutectic Solvents*.

Tetrahydrofuran (THF) is a concern for environmental and humans protection because it is carcinogenic, mutagenic or toxic to reproduction (CMR), inflammable and harmful. THF need to be substituted by another solvent. In organometallic-chemistry, THF is usually used as solvent for preparation of catalyst or for reaction.[117,119] Aycock *et al.* compared THF and 2-MeOX behavior in different tests for organometallic-chemistry reaction.[119] 2-MeOX can replace easily THF (Figure C1-3). Hexane (CMR, inflammable, harmful to human and environment) can be as well replace by 2-MeOX (Figure C1-3).[112,115,120,121] Hexane still be used

as extraction solvent for vegetal oil.[112] In order to remove hexane from extraction vegetal oil, researchers are developed and tests different green solvents. 2-MeOX has a small impact on human health compared to hexane.[112] According to Cravotto *et al*., the mass yield of extracted oil from olive pomace, the TPC yield and the antioxidant activity are higher with 2-MeOX (15.68 ± 1.69 g/100 g DM; 21.99 mg GAE/g extract; 26.31 mg Trolox Equivalent/g extract) than hexane (13.87 ± 0.5 g/100 g DM; 0.86 GAE mg/g extract; 0.37 TE mg/g extract).[112] 2-MeOX is a promising green solvent to bioactive molecules from biomass to replace conventional organic solvent.

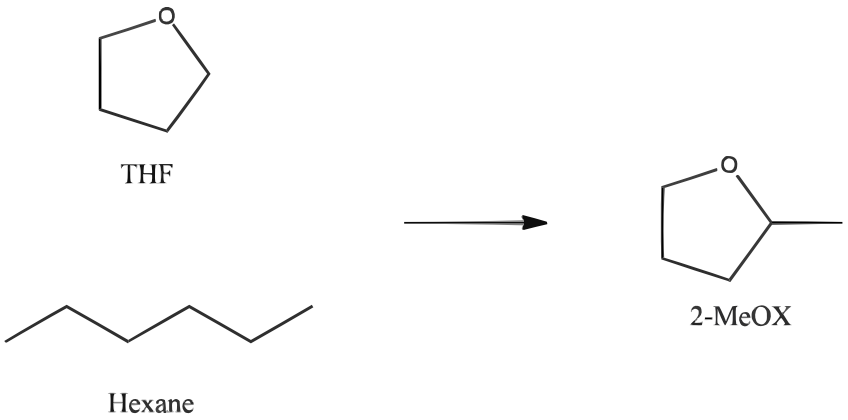


Figure C1-3. THF and Hexane and their substitute, 2-MeOX adapted from Aycock *et al*. and Cravotto *et al*.[112,119]

According to literature, LCAs for bio-based solvents are mostly in their favor (small environmental impact) compared to fossil-based solvents. However, if the bio-based solvents is from carbon feedstock crop/edible food the LCA can be not in their favor especially for the land footprint.[116]

2.Switchable solvents

A switchable solvent, or tunable solvent, is a solvent that can reversibly modify its properties on demand by the addition of chemical agent, or by changing the pressure and temperature. By removing the external trigger, the solvent goes back to its initial properties.[114,122,123] For instance, Jessop *et al*. successfully change the polarity of a solvent, switching from non-polar (an alcohol and an amine salt) to polar solvent (a salt in liquid form) by the addition of CO_2 gas. To return to a non-polar solvent, the researchers replace CO_2 gas to N_2 or Ar gas.[122] Basically

switchable solvents, especially switchable water, are employed for decades. Supercritical and subcritical fluids (increase of pressure and temperature), and reflux extraction (increase of temperature) are examples of tunable solvents commonly used for plant extraction modifying polarity of solvent.

3.Water

In chemistry, water is considered as unlimited raw material and cheap. Water is the greenest solvent due to its safety. It is non-inflammable and non-toxic. Humankind is using water as solvent for centuries.[124,125] However, depending on the method used, water as extraction solvent can be viewed as conventional extraction, and not ecological at all. For example, maceration is usually used as reference for natural products extraction from biomass. Maceration involves soaking biomass in water at room temperature for a certain amount of time (*e.g.* 24 h).[96,125,126] The mass yield and antioxidant activities are not high. It can be attributed to the poor solubility of biomolecules in water in room temperature and pressure.[114,115] SWE technology is modifying the dielectric constant of water by increasing temperature at constant pressure resulting to extract molecules with lower polarity.[79,86,87] For instance, the dielectric constant of subcritical water at 200 °C, 250 °C and 300 °C at 100 bar are 35.11, 27.43 and 20.39 $F.sr.m^{-1}$ respectively.[88] The dielectric constant of acetonitrile and acetone are respectively 35.11 and 20.39 $F.sr.m^{-1}$ corresponding to subcritical water at 200 °C and 100 bar and 300 °C and 100 bar.[87,89] Another method to modify extraction properties of water, ultrasound or microwave techniques can be used.

Applying pressure to water can be expensive and challenging due to the need of specific device. To modify water properties, chemical agent such as pH modifiers, surfactant and enzymes can be used.[115] Adding external triggers transform solvents into switchable or tunable solvents (see *V. b. Switchable Solvents*).

4.Free-solvent

The greenest solvent is no solvent. Free-solvent reaction or extraction respects several principles of Green Chemistry (principles of pollution prevention, atom economy, less hazardous chemical syntheses, reduce derivatives, *etc.*).[17] One of the oldest free-solvent extraction is olive oil extraction.[115] Oil extraction from olive consists to crush and to separate

the oil and olive pomace, purely physical mechanism. In more recent years, reactions without solvent were highlighted, called mechanochemistry.

5.Ionic liquid

Ionic liquids (IL) are a mixture of solid salts, an organic cation and a coordinating anion.[113,127] They are usually liquid at room temperature. The melting point of IL is lower than the two compounds. IL present several advantages as solvent, such as infinite variety of solvents (more than 10^{18} possibilities), tunability, negligible vapor pressure, non-inflammable, stable, detachability.[113,127,128] However, IL are less and less studied over the years for several reasons (according to *SciFinder*). First, they are toxic for humans and the environment, expensive, high viscosity, non-biodegradable, and corrosive. The negligible vapor pressure is an advantage and a drawback at the same time. This property makes evaporation difficult and thus the extraction of molecules challenging with multi-steps.[113,127,128]

6.Deep Eutectic solvent

Researchers found that IL were promising solvents due to their unusual properties. For this reason, they are working on Deep Eutectic Solvents (DES) instead. Il and DES share several properties, such as negligible vapor pressure, tunability, non-inflammable, infinite variety of solvents, high viscosity, *etc*. Nevertheless, DESs are composed of cheaper compounds, are biodegradable, and less toxic. DES is composed of Hydrogen Bond Donor (HBD) and Hydrogen Bond Acceptor (HBA). As IL, the mixture has a lower melting point than HBA and HBA melting point compounds.[129] A special class of DESs is supposed to be even greener than DES, is called Natural Deep Eutectic Solvents (NADES). The difference between DES and NADES are the origin of HBD and HBA compounds, they are bio-based and so renewable. NADES are promising solvents for bioactive molecules from AP with antibacterial application. They are classified in different types of eco-solvents according to Figure C1-2: bio-based solvent, DES, ionic liquid, switchable solvents.

B.Antimicrobial activities from Deep Eutectic Solvent

As previously mentioned, this chapter focuses on the antibacterial, antifungal, and antiviral properties of apple pomace (AP) extracts and the phenolic compounds they contain (Tables C1-2 – C1-4). An antimicrobial agent is chemical compound/extract able to kill microorganisms or to stop their growth.

These effects have been increasingly studied in recent years because of their health interest due to high potential resistance from drugs against pathogens over the years and for the future.[56,57]

NADES are promising solvents for bioactive molecules from AP with antibacterial application. According to our knowledge, any scientific article discusses antimicrobial properties of bioactive molecules extracted from AP (Table C1-5).

Several authors consider DES and NADES as non-toxic solvents and do not attribute their antioxidant or antimicrobial properties as to their results.[126,130–132] However, these solvents exhibit antioxidant property. For instance, Deniz *et al.* determined the antioxidant activity of solvents and extracts from AP (Table C1-5 entries 1 and 2).[133] They observed that the Choline Chloride:Lactic Acid (ChCl:LA) solvent exhibit high antioxidant activity compared to Glucose:Sucrose:Water (G:S:W) solvent, respectively 66.15 ± 5.95 and 15.35 ± 1.21%$_{inhibition}$. ChCl:LA extract is slightly higher than ChCl:LA (respectively 74 and 66.15%$_{inhibition}$). The DRSC of extract is mainly attributed to the solvent. Conversely, the antioxidant activity from G:S:W extract is due to the bioactive molecules extracted, 71%$_{inhibition}$ compared to solvent is 15%$_{inhibition}$. According to these data, we can suppose that antioxidant activity from bioactive molecules from AP are hidden by the solvents for Table C1-5 entries 4 – 7, especially for acid-based DES. Organic acids exhibit strong antioxidant activity.[133–135] Some of them are used as food additives.[134]

However, many authors highlighted that these solvents can be toxic against microorganisms.[134–137] For instance, Alsaud *et al.* extracted phytomolecules from Manuka leaves using menthol:lactic acid (M:LA) (1:2). According to their results, the antioxidant activity (DRSC) provided by the solvent is negligible compared to the extract, respectively under 10 and above 80%$_{inhibition}$ at 20 mg$_{Sample}$/mL. Another antioxidant assay was tested, FRAP. No antioxidant activity from M:LA was observed. Alsaud *et al.* tested extracts and solvents against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermis*. Against *Escherichia coli*, extract and DES have same Diameter Inhibition Zone (DIZ), 25.5 mm. Against

Pseudomonas aeruginosa and *Staphylococcus epidermis*, solvent exhibit higher DIZ than extracts. NADES DIZ were 28.50 ± 0.5 and 33.00 ± 2 mm and extracts DIZ were 27.00 ± 1 and 31.50 ± 1 mm against *Pseudomonas aeruginosa* and *Staphylococcus epidermis* respectively. The antibacterial activity from extracts can be attributed to the solvent and in particular to lactic acid, one of its constituents. Its antibacterial property is due to the pH from the solvent. According to existing literature, acid-based NADES can exhibit toxicity to organisms due to their pH characteristics leading the denaturation of bacterial membranes.[134] The optimal bacterial growth pH is between 6.5 – 7.5.[137] Radošević *et al.* tested 10 NADES against 5 bacterial strains and 1 fungi.[134] All NADES containing acids at 10% of water exhibited antibacterial activity against all the bacteria. As Alsaud *et al.*, the antibacterial property of solvents may be attributed to the pH. Only ChCl:Oxalic acid (ChCl:OA) displays antifungal activity against *Candida albican*. DES and NADES constituted with acids exhibit strong and effective antimicrobial properties.[133,134,137–139]

Table C1-5. Examples of extractions: main biological results according to the type of DES of polyphenols from apple waste.

Entry	DES (molar ratio)	Parameters	Antioxidant activity		Antimicrobial activity		Ref
			Extracts	Solvents	Extracts	Solvents	
1	Glucose:Sucrose:Water (1:1:11)	10 mL (1:1; DES:Water; *v/v*) 1:20 (AP:DES, *w/v*) 60 °C 2 h US bath (140 W, 37 kHz)	ABTS: 730 µM AAE DRSC: 71%$_{inhibition}$ TPC: 9.47 ± 0.10 mg GAE/g AP	DRSC: 15.35 ± 1.21%$_{inhibition}$	nd	nd	[133]
2	ChCh:Lactic Acid (1:1)	10 mL (1:1; DES:Water; *v/v*) 1:20 (AP:DES, *w/v*) 60 °C 2 h US bath (140 W, 37 kHz)	ABTS: 1347.42 ± 15.23 µM AAE DRSC: 74%$_{inhibition}$ TPC: < 1 mg GAE/g AP	DRSC: 66.15 ± 5.95%$_{inhibition}$	nd	nd	[133]
3	ChCl:Ethylene Glycol (1:4)	1:10 (AP:DES; *w/w*) 60 °C 30 min	DRSC: 83.8 – 84.7% $_{inhibition}$ FRAP: 1.10 – 1.12 µM TE/mg dry AP	Consider removed	nd	nd	[140]
4	ChCl:Maleic Acid (1:1 + 20% water)	1:2 (AP:DES, *w/v*) 40 °C 1 h	DRSC: 50 ppm VcE	nd	nd	nd	[141]
5	ChCl:Glycerol (1:2 + 30% water)	1:30 (AP:DES) 40 °C 40 min US probe (400 W, 20 kHz, 70%)	DRSC: 76%$_{inhibition}$ FRAP: 10.7 mM AAE/mg sample TPC: 5.1 mg GAE/g dry AP	nd	nd	nd	[142]
6	ChCl:Lactic Acid (1:3 + 30% water)	1:30 (AP:DES) 40 °C 40 min US probe (400 W, 20 kHz, 70%)	DRSC: 82%$_{inhibition}$ FRAP: 9.1 mM AAE/mg sample TPC: 5.1 mg GAE/g dry AP	nd	nd	nd	[142]

7	ChCl:Citric Acid (1:1 + 30% water)	1:30 (AP:DES) 40 °C 40 min US probe (400 W, 20 kHz, 70%)	DRSC: 72%$_{inhibition}$ FRAP: 8.1 mM AAE/mg sample TPC: 4.6 mg GAE/g dry AP	nd	nd	nd	[142]

AAE: Ascorbic acid equivalent; ABTS: Antioxidant test using $ABTS^{+\bullet}$; ChCl: Choline chloride; DRSC: DPPH Radical Scavenging Capacity; FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalent; nd: not determined; TE: Trolox equivalent; TPC: Total polyphenols content; VcE: Vitamin C equivalent;

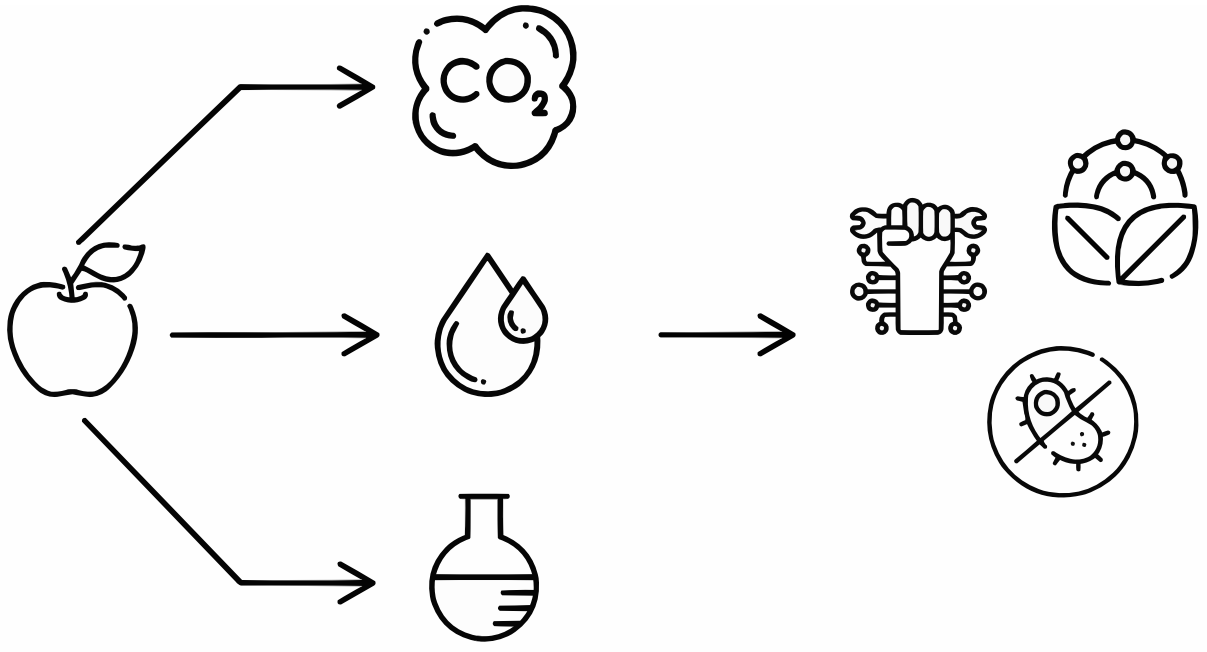
To observe biological activities from bioactive molecules extracted, it is necessary to remove the DES or NADES. Many authors noticed that the MIC values from extracts and solvents are usually the same, masking biological activities from bioactive molecules. As discussed above, the low vapor pressure of these solvents is both an advantage and a drawback. This property makes extraction safer (no risk of solvent evaporation) but it also makes a evaporation ineffective. A few authors removed DES such as Moni Bottu *et al.* using resin (Amberlite XAD-7) (Table C1-5 entry 3).[140] They were able to measure the antioxidant activity from polyphenols from AP. This method involves adsorbing polyphenols onto resin and then desorbing them by using organic solvents such as ethanol or acetone. Several other techniques exist to recover phytomolecules from DES: antisolvent, solid-phase extraction (SPE), back-extraction, *etc*.[143] Every technique presents advantages and drawbacks. The second most used method is antisolvent. This technique requires a large amount of water or organic solvents (ethanol, acetone). The polyphenols are recovered by precipitation. SPE is similar to resin extraction, the first one is usually more adequate for a small amount and resin method can be used in industrial level. Back-extraction technique is equivalent liquid-liquid extraction. For instance, non-miscible organic solvent (ethanol, hexane *etc*.) is added to the extracts, containing polyphenols and DES, to collect phytomolecules.

VI. **Conclusion**

In summary, few papers have been published on the antifungal and antiviral activity of AP, especially for extracts obtained under subcritical and supercritical fluids and Deep Eutectic Solvents. This may be due to the lack of specific equipment for Subcritical and Supercritical Fluids extractions and for microbiological tests. However, the recovery of apple pomace, a significant waste in quantity and available each year (4.7 – 5.6 million tonnes of apple pomace in 2022), remains a promising possibility in a context of circular economy and recovery of bio-waste. As previously reported, even if the antioxidant activity of AP extracts with green solvents was widely studied, the applications involving antimicrobial activities from apple pomace extraction should be carefully explored. Aiming to improve biological activity, polyphenols have been oxidized by enzymes and grafted onto chitosan or synthesized with NPs. A small number of scientific papers use the functionalization of the targeted molecules, but this strategy opens the doors to new and more efficient biological activities.

Furthermore, many Deep Eutectic Solvents exhibit biological activities hiding sometimes the activities from extracts (bioactive molecules), in particular organic acids-based solvents. It can be attributed to the pH, causing the death of microorganisms. Few authors did post-extraction steps, using resin, antisolvents *etc*. But these solutions can be not every time efficient to separate extracts from Deep Eutectic Solvent. To avoid post-extraction steps, researchers are using ready-to-use deep eutectic solvents.

Chapter 2
Materials and Methods



This chapter describes the various methods employed in the preparation of apple pomace, subcritical or supercritical fluids and bio-based solvents extraction techniques, biological tests (antioxidant and antibacterial properties) and the identification of compounds from apple pomace used in the PhD thesis.

I.Materials

A.Apple pomace

Organic apple pomace (*sp. Story*) was obtained from *La Source du Verger* (Tournon, France). The apple pomace (AP) was freeze-dried using a Buchi Lyovapor L-200 until a stable weight was achieved. Subsequently, the dried AP was ground into a powder with a diameter of 500 nm using a Pulverisette 19 from Fritsch. The powdered dried AP was then stored at 4 °C, protected from light.

B.Solvents and reagents

1.Chemistry solvents and reagents in Supercritical CO_2 Extraction

Phloridzin, isoquercetin, quercetin, procyanidin B2, avicularin, hyperoside, (-)-epicatechin were purchased from Extrasynthèse (Genay, France). Rutin dihydrate, gallic acid and quercetin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thermo Scientific Chemicals provided chlorogenic acid, (+)-catechin, and Folin-Ciocalteu reagent. Cellulase, pectinase and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were bought from TCI. Acetonitrile (UHPLC-MS grade), ethanol (≥ 96%), methanol (UHPLC-MS grade) were purchased from Carlo Erba. Liquid CO_2 (50 L) was provided by AirLiquide.

2.Chemistry solvents and reagents in Subcritical Water Extraction

Amberlite XAD-16 resin used for subcrititial water extracts preparations and separation, rutin dihydrate, gallic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phloridzin, isoquercetin, quercetin, procyanidin B2, avicularin, hyperoside, (-)-epicatechin were purchased from Extrasynthèse (Genay, France). Thermo Scientific Chemicals provided chlorogenic acid, (+)-catechin and Folin-Ciocalteu reagent. Cellulase, pectinase and DPPH were bought from TCI. Acetonitrile (UHPLC-MS grade), ethanol (≥ 96%), methanol (UHPLC-MS grade) were purchased from Carlo Erba.

3.Chemistry solvents and reagents in NAtural Deep Eutectic Solvents Extraction

Choline Chloride (≥ 99%) was purchased from Thermo Fisher Scientific. *DL*-malic acid (≥ 99%), citric acid monohydrate, *DL*-lactic acid (~ 90%) and Amberlite XAD-16 resin used for NADES extract preparation and separation were procured from Sigma-Aldrich (St. Louis, MO, USA).

Oxalic acid dihydrate was obtained from Merck. Phlorizin, isoquercetin, quercetin, procyanidin B2, avicularin, hyperoside and (-)-epicatechin were acquired from Extrasynthese (Genay, France). Chlorogenic acid and (+)-catechin were provided by Thermo Scientific Chemicals. DPPH was purchased from TCI. Acetonitrile (UHPLC-MS grade), ethanol (≥ 96%) and methanol (UHPLC-MS grade) were from Carlo Erba.

4.Microbiology part

Bacterial strains (*Bacillus cereus* (CIP 78.3), *Listeria innocua* (CIP 80.11T), *Escherichia coli* (CIP 104049)) were obtained from the Institut Pasteur (Paris, France). Dehydrated brain-heart infusion broth, dehydrated Columbia broth, bacteriological agar, 96-well microplates, and Petri dishes were purchased from Dutscher.

II.Supercritical CO_2 extraction

A.Design of Experiments

Design of Experiments (DoE) was made with Minitab V.17 software (Centre County, Pennsylvania). A central composite rotatable design (CCRD) was chosen as the effective factorial design, as it is consistent with the response surface described in the literature.[76,144,145] Response surface methodology (RSM) is a analytical technique using mathematical model to explore the relationship between variables (dependent and independent). RSM was used to optimize the parameters of bioactive molecules extraction from apple pomace of this study. A screening of three parameters selected, pressure (120 – 250 bar), temperature (45 – 65 °C) and solvent flow rate (10 – 20 g/min), has been performed to determine optimal extraction conditions where the extraction mass yield was investigated (Table C2-1). The DoE consisted of 60 randomized experiments in order to avoid any artefacts. Some experiments were conducted a second time due to absurd results according to the software. The equation of response, R (%), with three variables (x_1, x_2, x_3) was determined using Equation C2-1.

Equation C2-1. Second-order polynomial model equation for AP bioactive molecules with supercritical CO_2 extraction.

$$Y\ (\%) = a + \sum_{i=1}^{3} b_i\, x_i + \sum_{i=1}^{3} b_{ii}\, x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij}\, x_i x_j$$

Where Y (%) is the response variable, here the mass yield; a is the constant coefficient; b_i is the linear coefficient; b_{ii} is the quadratic coefficient; b_{ij} is the two factors interaction coefficient.

In order to determine the optimal conditions for SC-CO_2 extraction, the effects of parameters and interactions between variables on extraction mass yield were statistically studied via analysis of variance (ANOVA). The mathematical model evaluated the accuracy of responses thanks to the coefficients of determination (R^2, adjusted R^2, predicted R^2), and the Lack-of-Fit. Statistical calculations were conducted at a confidence level exceeding 95% ($p < 0.05$).

Table C2-1. Variables and their level (α: 1.68) in CCRD for the optimization of bioactive molecules extraction with supercritical CO_2.

Independent variables	Unit	Level of the factors				
		- α	-1	0	+ 1	+ α
Pressure	Bars	76	120	185	250	294
Temperature	°C	38.2	45	55	65	71.8
Flow	g/min_{CO2}	6.6	10	15	20	23.4

B.Extractions

1.Extraction Without Pretreatment

The extractions were performed using a supercritical CO_2 apparatus from Top Industries (France), a dosing pump with Coriolis debit meter (0-150 $g_{CO2}.min^{-1}$, HP Flow 50 – 1000, serial number 2776 5000), a cooling system set between 0 – 3 °C (Proficool Genius, Germany), a pre-heater with electric heating resistors, an autoclave extractor (500 mL, 600 bars, 150 °C), homemade cellule of extraction (Aluminium, sintered metal disk, Teflon seal), an Automatic Back Pressure Regulator ABPR (689.48 bars, Premier 3000AL, Premier Industries, USA) set with compressed air at 100 psi or 6.89 bars, an autoclave separator (250 mL, 200 bars, 150 °C), bursting disks (650 bars, Sitec, Switzerland) and a touchpad to control the supercritical

apparatus Monitouch TS1070Si. The supercritical CO_2 apparatus was coupled with a co-solvent pump (Series 200 pump, Perkin Elmer).

Lyophilized and crushed AP (20 g) was added to the cellule of extraction and then added into the autoclave reactor. The extraction conditions are 287.7 bar, 12.7 $g_{CO2}.min^{-1}$, 50 °C, 5%$_{EtOH}$, and 1 h. At the end of each experiment, the apparatus was washed with EtOH at 110 bar, 30 $g_{CO2}.min^{-1}$, 2 $mL_{EtOH}.min^{-1}$ until the EtOH solution is clear (around 20 min). The EtOH wash solution was added to the extract. The extractions were performed in triplicate.

2.Extraction With Ultrasound Pretreatment

AP (30 g) were mixed with 420 mL of deionized water. The ratio 1:14 (AP:Water, *w/v*) was chosen because under this ratio, AP was solidified and the ultrasound (US) was inefficient. The US pretreatments were performed using a home-made prototype. The device is a cup-horn reactor of 1000 mL capacity with a NexTgen Ultrasonic Platform (SinapTec) device. The conditions were 10 min, 20 kHz, 50 W. The aqueous solutions containing AP were freeze-dried (Buchi Lyovapor L-200) and stored at 4 °C until supercritical CO_2 extraction.

After the ultrasound pretreatment, the extraction followed the *II.B.1 Extraction without the pre-treatment* section.

C.Sample Clean-Up (Remove of Wax)

The solution containing wax and polyphenols was centrifuged in the co-solvent (ethanol) from SC-CO_2 extraction (4,000 rpm; - 5 °C; 10 min). The procedure was repeated 2 more times. 15 mL of water was added to the solution and ethanol contained was removed by rotary evaporator. The aqueous solutions containing bioactive molecules were freeze-dried (Buchi Lyovapor L-200) until the samples were dried. The dried samples were stored at – 20 °C until analytical analysis and biological tests.

D.FT-IR analysis

The FT-IR spectra of white powder was recorded at room temperature. Before the FT-IR analysis, the ethanol was removed, and the white powder aqueous solution was dried. The powder was conserved at – 20 °C until analysis. The FT-IR instrument was equipped with a detector scanning over the frequency range of 4,000 – 400 cm^{-1}.

E.GC-MS analysis

Qualitative white powder analysis was carried out on a GC-MS (Perkin Elmer) following the method of Dong *et al*.[146] 0.5 mg of the nonpolar fraction was added into 1 mL of hexane and injected (1 µL) into column (30 m x 25 mm x 0.25 µm). Initial oven temperature was 80 °C, raised by 4 °C per min to 290 °C and then held at 290 °C for 30 min. Carrier gas was high purity helium (99.99%) at a flow rate of 1 $mL.min^{-1}$. For MS, EI was used as ion source, transfer line 250 °C, quadrupole temperature 300 °C, ionization energy 70 eV.

F.HPLC-UV analysis

The identification of polyphenols from AP was performed using a Hypersil Gold (100 5 4.6 mm) column from Thermo adapted from Suarez *et al*.'s method.[26] The mobile phases were water (eluent A) and acetonitrile (eluant B). The flow rate was 0.8 mL/min, the injection volume was 10 µL and the column temperature was set to 30 °C. Gradient elution was performed as follows: 95 – 95% A (0 – 2 min); 95 – 65% A (2 – 31 min); 65 – 5% A (31 – 36 min); 5 – 5% A (36 – 38 min); 5 – 95% A (38 – 43 min).

G.Total Polyphenols Content (TPC) – Folin-Ciocalteu Assay

Gallic acid was used as reference and a calibration curve was made from it. The dilutions were between 5 and 250 mg/mL in aqueous solution. Dried extracts were diluted in water at 4 – 6 mg/mL. 250 µL of sample and gallic acid solutions were added into 250 µL of diluted Folin-Ciocalteu reageant (1:1; *v/v*). After 5 minutes, 500 µL of Na_2CO_3 solution (10%, *w/v*) and 4 mL of distilled water were introduced into and vigorously shaken. Gallic acid and sample solutions were analyzed at 740 nm in a quartz cuvette (1 cm) by using a Cary 50 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) after 20 minutes. The TPC was expressed as mg/g of gallic acid equivalents (GAE) over the extract. The measurements were performed in triplicate.[78]

H.DPPH Radical Scavenging Capacity (DRSC)

The antioxidant activity was assessed using a DPPH stock solution prepared by dissolving 4.5 mg of DPPH in 100 mL of methanol, following the procedure outlined by Alsaud *et al.* with slight modifications.[61] Trolox was used as the reference compound and a calibration curve was

generated using Trolox solutions ranging from 0 to 0.150 mg/mL in methanolic solution. The extracts were tested at 50 mg_{Sample}/mL_{DMSO}. In each test tube, 200 µL of each solution and 2 mL of DPPH solution were combined. The mixture was vigorously shaken, and the absorbance was measured after 30 min at 515 nm. The antioxidant activity, expressed as the inhibition percentage of the sample, was calculated using the following equation:

Equation C2-2. Calculation of antioxidant capacity of sample/standard.

$$DPPH\ inhibition\ (\%) = \left(\frac{Ab - As}{Ab}\right) \times 100$$

Where *Ab* is the absorbance of blank solution and *As* the absorbance of sample/standard after 30 min at 515 nm. Collected data were processed by Excel to establish the EC_{50} of each extract.

I.Antibacterial activity

The antibacterial activity of extracts has been tested on 2 Gram-positive *Bacillus cereus* (CIP 78.3), *Listeria innocua* (CIP 80.11T) and on 1 Gram-negative *Escherichia coli* (CIP 104049). All the strains were obtained from Institut Pasteur (Paris, France). *Escherichia coli* and *Bacillus cereus* were grown on Columbia broth at 30 °C; *Listeria innocua* on Brain-Heart Infusion (BHI) broth at 30 °C. Precultures of strains were done. It consists of collecting a bacterial colony of a strain, transferring into 0.5 mL of the corresponding broth and incubating at 30 °C overnight. After overnight incubation, a cascade dilution was performed until the wanted concentration (CFU/mL) was achieved for antibacterial testing.

1.Growth conditions

Growth conditions were determined according to Institut Pasteur and Zambrano *et al*.[60] *E. coli* and *B. cereus* were grown on Columbia broth; *L. innocua* on Brain-Heart Infusion (BHI) broth. *E. coli*, *B. cereus*, *L. innocua* were cultivated at 30 °C. Bacteria were subcultured weekly on the appropriate solid medium. The cultures were incubated overnight and stored at 4 °C. One colony was picked from the solid medium and suspended in 500 µL of the appropriate broth medium overnight before each experiment. The preculture was added to 45 mL of the same broth. The bacteria solution was placed in a microbiology oven at 30 °C. The bacteria were in their growth phase at the end of the incubation period. The number of cells was counted using

a UV-visible spectrometer at 600 nm, and the value given in the optical density (OD) was then converted to CFU/mL (Equation C2-3). A series of cascade dilutions was then made until a concentration of 10^5 CFU/ml was reached for testing on a microplate.

Equation C2-3. Determination of bacteria suspension concentration

$$Concentration_{cells} = 8 \times 10^8 \times OD_{measured}$$

1 of OD corresponding to 8 x 10^8 CFU/mL.

2.Determination of Diameter Zone Inhibition (DIZ)

The antibacterial tests using Petri dishes (PD) were conducted following the method outlined by Alsaud *et al*.[61] with slight modifications. Briefly, cascade dilutions were performed until a concentration of 10^5 CFU/mL was achieved for PD testing. For each PD, 1 mL of bacterial suspension was added onto the appropriate medium PD, and excess liquid was removed. After a brief incubation period, 20 µL of samples (at a concentration of 300 mg/mL, except for oxalic acid at 150 mg/mL) dissolved in Phosphate-Buffered Saline (PBS) and 5 µL of ampicillin (at a concentration of 25 mg/mL) were added. PBS was used as a buffer to maintain a pH of 7.4 and avoid the potential pH effects of NADES on the bacterial strains. In each PD, positive and negative controls (ampicillin and PBS-water, respectively) were included, in addition to the samples. After overnight incubation at 30 °C, the Diameter Inhibition Zone (DIZ) were measured.

3.Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial tests with microplate were performed following the method of Zambrano *et al.* with little modification.[60] MICs were determined using a 96-well microplate. The extracts were diluted in DMSO solution at 50 mg/mL in DMSO. A cascade dilution was performed until a concentration of 10^5 CFU/mL was achieved for microplate testing. For the extract test, 25 µL of extract solution was added to 125 µL of appropriate medium and 25 µL of bacterial suspension, and an extract control was performed, 175 µL of appropriate medium and 25 µL of extract solution. A 25 µL bacterial suspension added to 175 µL of the appropriate medium was considered to be the positive control and the negative control consisted of 200 µL of the

appropriate medium. All experiments were carried out in triplicate. Before incubation, the Optical Density (OD) was measured, and the microplate was sealed and incubated overnight under appropriate conditions. OD was measured after the incubation period. If the percentage of inhibition was greater than 90%, antibacterial activity was considered. The percentage of inhibition was calculated as follows in Equation C2-4.

Equation C2-4. Determination of extract inhibitions on bacteria.

$$1.\ Calculation\ of\ average\ of\ OD\ at\ t_0\ and\ t_{o/n}$$

$$2.\ \Delta OD\ =\ \Delta OD_{t_0} - \Delta OD_{t_{o/n}}$$

$$3.\ Growth\ positive\ contr. =\ \Delta OD_{positive\ contr.} - \Delta OD_{negative\ contr.}$$

$$4.\ Growth\ solvent\ test =\ \Delta OD_{solvent\ test} - \Delta OD_{solvent\ contr.}$$

$$5.\ Growth\ NADES\ test =\ \Delta OD_{NADES\ test} - \Delta OD_{NADES\ contr.}$$

$$6.\ Growth\ ampicill\ test =\ \Delta OD_{ampicill\ test} - \Delta OD_{ampicill\ contr.}$$

$$7.\ \%Inhibition = \frac{Growth\ \text{extracts}\ test}{Growth\ positive\ contr.} \times 100$$

Where ΔOD_{t0} is the average OD at t_0; $\Delta OD_{to/n}$ is the average OD at $t_{o/n}$ (overnight). Collected data were processed by Excel to establish the EC_{50} of each extract.

Table C2-2. Example of microplate experiment (antibacterial test) for extracts obtained with SC-CO_2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	negative contr. (200 µL corresp. medium)			positive contr. (175 µL corresp. medium + 25 µL bact.)			solvent contr. (175 µL corresp. medium + 25 µL DMSO)			solvent test (25 µL DMSO + 25 µL bact. + 150 µL corresp. medium)		
B	contr. [WPSCCO2.1]1 (175 µL corresp. medium + 25 µL WPSCCO2.1 into DMSO)			Test [WPSCCO2.1]1 (150 µL corresp. medium + 25 µL bact. + 25 µL WPSCCO2.1 into DMSO)								
C	contr. [USSCCO2.1]1 (175 µL corresp. medium + 25 µL USSCCO2.1 into DMSO)			Test [USSCCO2.1]1 (150 µL corresp. medium + 25 µL bact. + 25 µL USSCCO2.1 into DMSO)								
D												
E												
F												
G												
H	ampicillin contr. (175 µL corresp. medium + 25 µL ampicillin)			Test ampicillin (150 µL corresp. medium + 25 µL bact. + 25 µL ampicillin)								

Bact.: bacteria; contr.: control; corresp.: correspondoing; DMSO: dimethyl sulfoxide; WPSCCO2: Whitout pretreatment SC-CO_2; USSCCO2: US pretreatment SC-CO_2; [XXSCCO2.X]1: 50 mg/mL

III.Subcritical Water extraction

A.Design of Experiments

A CCRD was chosen as the effective factorial design, as SC-CO_2 extraction for the same reason. The three parameters selected were time (10 – 20 min), temperature (90 – 140 °C) and ratio AP:water (1:10 – 1:15) (Table C2-3). See *II. A. Design of Experiments* for more information.

Table C2-3. Variables and their level (α: 1.68) in CCRD for the optimization of bioactive molecules extraction with subcritical water.

Independent variables	Unit	Level of the factors				
		- α	-1	0	+ 1	+ α
Time	min	6.7	10	15	20	23.4
Temperature	°C	73	90	115	140	157
Ratio	solid:liquid	1:8	1:10	1:12	1:15	1:17

B.Extractions

1.Extraction Without Pretreatment

AP (25 g) were mixed with 200 mL of deionized water. The ratio AP:water (w/v) is determined by the optimal conditions (chapter 3). The mixture was left to impregnate for 5 min in a 1 L Teflon vessel and then was introduced into a Microwave multimodal reactor (SynthWAVE, Milestone, Bergamo, Italy). Ambient air was substituted with N_2 by flushing 3 times with this inert gas and the reactor chamber was pressurized with 5 bar to avoid water ebullition.

2.Extraction With Ultrasound Pretreatment

AP (25 g) were mixed with 200 mL of deionized water. The mixture was left to impregnate for 5 min in a 1 L glass vessel and then, the ultrasonic probe was immersed into the solution of AP and water for 10 min, 20 kHz, 500 W (Hainertec (Suzhou) Co., Ltd). The vessel was put into an iced bath to control the temperature.

After the ultrasound pretreatment, the extraction followed *III.B.1 Extraction without the pre-treatment* section.

C.Sample Clean-Up (Remove of Sugars)

The separation of bioactive molecules and sugars was performed using Amberlite XAD-16 resin. Before use, resin (20 g) was washed with absolute ethanol (200 mL) overnight on an agitate plate (Synthesis 1, Heidolph). The polyphenol compounds were adsorbed onto the Amberlite XAD-16 resin for 3 h on an agitate plate. 20 g of resin are used with 400 mL of extract. Then, the bioactive molecules were desorbed with 80:20 (ethanol:water, *v/v*) for 3 h on an agitate plate. Ethanol was removed by rotary evaporator and the aqueous solution containing bioactive molecules was freeze-dried (LyoQuest, Telstar, Madrid, Spain). The dried samples were stored at -20 °C until analytical analysis and biological tests.

D.HPLC-UV analysis

The protocol of identification of polyphenols from AP using UHPLC technique is described before (*II. D HPLC-UV analysis*).

E.Total Polyphenols Content (TPC) – Folin-Ciocalteu Assay

The protocol to determine TPC is described before (*II. E. Total Polyphenols Content – Folin-Ciocalteau Assay*).

F.DPPH Radical Scavenging Capacity (DRSC)

DPPH assay following the method described by Brand Williams *et al*.[147] The protocol has undergone some changes, according to the procedure reported by Boffa *et al*.[148] The sample solution was between 5 – 10 mg/mL of methanol. DPPH was diluted in methanol (approx. 2%, *w/v*) and the absorbance range is between 0.45 – 0.55 at 515 nm. 700 µL of sample solution in the first cuvette. Starting from the first cuvette, progressive dilutions were prepared by taking 700 µL of solution from the previous cuvette and diluting with 700 µL of methanol. The operations were repeated until the desired number of dilutions. Thus, the sample prepared were then added with 700 µL of DPPH solution. The samples were shaken and covered. DPPH solution addition was made every 30 s between each cuvette. The cuvettes remained dark for 20 min. The samples were analyzed at 515 nm (Cary 50 UV-VIS spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). The wavelength was measured to calculate the EC_{50} (half maximal effective concentration or amount of compound/extract necessary to decrease the

initial concentration of DPPH to 50% at equilibrium). Collected data were processed by Excel to establish the EC_{50} of each extract.

G.Total Sugars Content (TSC)

Glucose was used as reference and a calibration curve was made from it. The dilutions were between 0.01 and 0.15 mg/mL in aqueous solution. The sample solution was between 0.1 – 0.3 mg/mL of water. Anthrone was diluted in concentrated H_2SO_4 (2 g/L). 5 mL of anthrone solution were added into 1 mL of sample solution. The mixture was vigorously shaken and heated at 100 °C for 20 min. The solutions were cooled down and then the samples were analyzed at 620 nm (Cary 50 UV-VIS spectrophotometer, Agilent Technologies, Santa Clara, CA, USA).

H.Antibacterial activity

1.Growth conditions

The protocol described before (*II. G. 1. Growth conditions*).

2.Determination of Minimum Inhibitory Concentration (MIC)

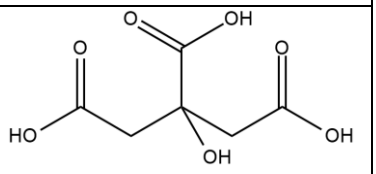
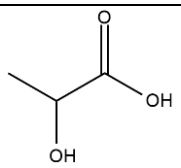
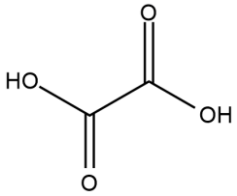
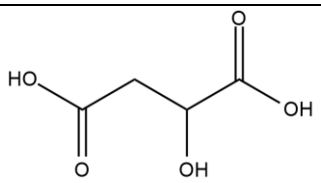
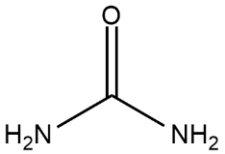
MICs were determined using a 96-well microplate. The extracts were diluted in water solution at 7 different concentrations: 5, 15, 25, 50, 75, 100 and 150 mg/mL. The protocol is described *II. G. 2 Determination of Minimum Inhibitory Concentration (MIC)*.

IV.NAtural Deep Eutectic Solvents extraction

A.Preparation of NADES

NADES were prepared following a molar ratio and 20% (*w/w*) of added water where water content (20%) was reduced to decrease viscosity, except for ChCl:U. The synthesis of NADES was conducted using a heating and stirring method. The compounds were heated to 50 °C and stirred continuously until the solution became homogeneous and transparent (approximately 2 h).

Table C2-4. Natural Deep Eutectic Solvents (NADESs) were used in this study.

HBA	HBD	Acronym	Molar ratio	pKa of HBD[b]	HBD structure
Choline chloride	Citric acid	ChCl:CA	1:1[a]	3.09 4.75 5.41	
	Lactic acid	ChCl:LA	1:1[a]	3.86	
	Oxalic acid	ChCl:OA	1:1[a]	1.23 4.19	
	Malic acid	ChCl:MA	1:1[a]	3.40 5.20	
	Urea	ChCl:U	1:2	0.18	
		ChCl:U:W	1:2[a]		

[a]Addition of 20% (*w/w*) of water. [b]Data from literature (pKa Values in Water Compilation by R. Williams).[149]

B.Characterization of NADES

The viscosity of NADES was measured using a Lovis 2000 M/ME viscometer by Anton Paar. The density of NADES was determined using a DM45 Delta Range densitometer by Mettler Toledo. The pH of NADES and solutions of diluted NADES (1 g_{NADES}/mL_{Water}) was measured at RT using a pH 211 microprocessor pH meter by Hanna Instruments.

C.Extraction of biomolecules

All extractions were conducted in round-bottom flasks at 50 °C using a ratio of 1:10 (*w/w*), with 5 g of AP and 50 g of NADES. The AP was separated from the liquid extracts by centrifugation

(4,000 rpm) and the samples were stored at -20 °C until further separation. Each extraction was performed in triplicate.

D.Total Polyphenol Content (TPC) – Folin-Ciocalteu Assay

The protocol to determine TPC is described before (*II. E. Total Polyphenol Content – Folin-Ciocalteu Assay*). Dried extracts were diluted in water to a concentration of 8 – 20 mg/mL.

E.DPPH Radical Scavenging Capacity (DRSC) – Before Sample Clean-Up

The protocol to determine TPC is described before (*II. F. DPPH Radical Scavenging Capacity Assay (DRSC)*). The extracts were diluted in methanol to yield 13 different concentrations ranging from 0 to 1,000 mg/mL to determine the EC_{50} (half maximal effective concentration or amount of compound/extract necessary to decrease the initial concentration of DPPH to 50% at equilibrium).

F.Sample Clean-Up (Remove of NADES)

The separation of bioactive molecules and NADES was performed using Amberlite XAD-16 resin. The protocol is described in *III. C. Sample clean-up (remove of sugars)*.

G.DPPH Radical Scavenging Capacity (DRSC) – After Sample Clean-Up

The protocol is described above *III. F. DPPH Radical Scavenging Capacity (DRSC)*.

H.Total Anthocyanin Content (TAC)

The TAC was determined following the methods described by Ribéreau-Gayon *et al*.[150] Briefly, for each sample, 100 µL of extract, 100 µL of a 60% (*v/v*) ethanol solution containing 0.1% (v/v) hydrochloric acid and 2 mL of a 2% (*v/v*) hydrochloric acid solution were added to two separate test tubes. In the first test tube, 400 µL of distilled water was added to the mixture, while in the second test tube, 400 µL of a 15% (*w/v*) sodium bisulfite solution was added. Both test tubes were vigorously shaken, and after 15 min, the samples were analyzed at 520 nm using a UV-Vis spectrophotometer with a quartz cuvette (1 cm). The TAC was calculated using the following equation:

Equation C2-5. Equation from calibration curve obtained by Ribéreau-Gayon *et al.* from malvidin-3-glucoside as reference.[150]

$$TAC = 875 \times (D_1 - D_2)$$

Where *D1* is the absorbance of the diluted sample with distilled water, *D2* is the absorbance of the diluted sample with bisulfite sodium after 15 min at 520 nm. The TAC was expressed as µg/mL of malvidin-3-glucoside equivalent (M3GE) per volume of water. All analyses were performed in triplicate.[151]

I.Antibacterial activity

1.Growth conditions

Growth conditions were determined according to Institut Pasteur and Zambrano et al.[60] Escherichia coli and Bacillus cereus were grown on Columbia broth; Listeria innocua on brain heart infusion broth (BHI). *Escherichia coli*, *Bacillus cereus*, and *Listeria innocua* were cultivated at 30 °C. The protocol from growth conditions is described above *II. G. 1. Growth conditions*.

2.Determination of Diameter Inhibition Zone (DIZ)

The antibacterial tests with PD were performed following the method of Alsaud *et al*. with little modification.[61] The extracts were diluted in PBS solution at 300 mg/mL. The protocol is described above *II. G. 2. Determination of Diameter Inhibition Zone (DIZ)*.

3.Determination of Minimum Inhibitory Concentration (MIC)

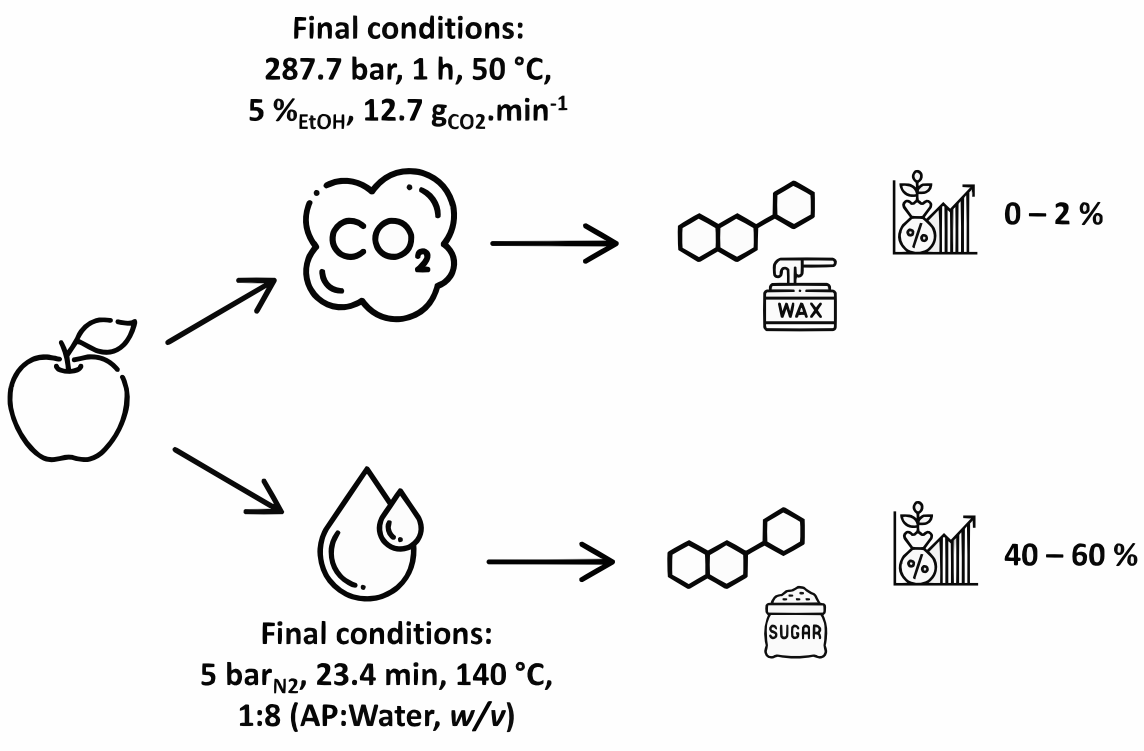
The antibacterial tests with microplate were performed following the method of Zambrano *et al.* with little modification.[60] MIC were determined using a 96-well microplate. The extracts were diluted in PBS solution at 8 different concentrations: 300, 150, 100, 75, 50, 25, 15 and 5 mg/mL in PBS. The protocol is described above *II. G. 3. Determination of Minimum Inhibitory Concentration (MIC)*.

V.Life cycle assessment

The Life Cycle Assessment (LCA) was conducted in accordance with ISO 14040-44 standards to identify and quantify the life cycle phases with the greatest environmental impact, focusing on areas for potential improvement. LCA is recognized as a systematic approach to assess various environmental impacts of a product throughout its life cycle, encompassing aspects such as climate change, resource depletion, and other environmental indicators.[152] The process involves four stages: (i) the determination of the objective and scope to define the goals and boundaries of the assessment; (ii) the conducting of an inventory analysis to compile inputs and outputs (with measurement when it was possible) within the system boundaries; (iii) the performance of an impact assessment to evaluate the potential environmental impacts associated with these inputs and outputs; and (iv) the interpretation of the results to provide insights and recommendations. The LCAs were carried out at the EDYTEM lab, adhering strictly to ISO 14040-44 standards. SimaPro v 9.4 software, along with the ecoinvent v3.8 database, was utilized, ensuring a comprehensive and reliable assessment of environmental impacts.

Chapter 3

Optimal conditions of subcritical and supercritical fluids extraction for bioactive molecules from Apple Pomace



Chapter 3 focused on the determination of the optimal conditions for Supercritical CO_2 (SC-CO_2) and Subcritical Water Extraction (SWE) of bioactive molecules from apple pomace (AP). Supercritical and subcritical fluids extraction (SFE) techniques allow extraction and preservation of phytochemicals more efficiently compared to conventional methods. Design of experiments for SFE were conducted using Central Composite Rotatable Design (CCRD) involving more than 60 experiments determined by Minitab. Several parameters were studied: (1) temperature, pressure, and CO_2 rate flow for SC-CO_2 extraction; (2) temperature, time, and ratio AP:Water (*w/v*) for SWE. The optimal extraction were determined based on the mass yield (%). For SC-CO_2 extraction the optimal conditions are 71.8 °C, 287.7 bar and 12.7 $g_{CO2}.min^{-1}$. However, the final temperature for the evaluation of biological activities (Chapter 4) was adjusted to 50 °C. For SWE, the optimal conditions were 157 °C, 23.4 min and 1:8 (AP:Water, *w/v*). As SC-CO_2 extraction, the determined temperature was modified to 140 °C for extractions from Chapter 4.

I.Introduction

In 2022, an estimation from 4.7 to 5.6 million tonnes of apple pomace (AP) were generated from apple juice and cider production.[8,19,153] As highlighted in Chapter 1 (*Waste Management of Apple Pomace: Extraction of Antimicrobial Molecules Using Green technologies*), biomass waste is typically sent for methanization, and it is converted into biogas in European Union. Around the world, AP is mostly disposed in soil leading to health and environmental concerns.[9] Nevertheless, AP contains bioactive molecules such as phenolic compounds which exhibit biological activities such as antioxidant, antidiabetic, antitumor, antimicrobial properties.[15,48,51,55] Extractions can lead to chemical transformations among others, the transformation of carbohydrates into platform molecules.[98,154–156] Platform molecules are used as starting materials for chemical transformation in different applications for instance pharmaceutical. As an example of transformation of platform molecules, furfural (FFR) can be converted into 1,2-pentanediol, used as monomer, potential fuel, or solvent.[117] The objectives of the thesis are to develop eco-extraction methods for phytochemicals and to explore potential applications for their valorization. This case study focuses on antibacterial application.

Supercritical (and subcritical) Fluids are based on physical properties, on pressure and temperature. In 1822, Charles Cagniard de Latour discovered supercritical fluids with his well-known gun barrel experiment.[157] He filled the cannon with a ball and a liquid (*e.g.* water), sealed and heated it. His experiment consists of hearing the ball falling in a liquid when he is rotated the device. Above a certain temperature, the sound of the ball falling has disappeared. Liquid and gas phases become a homogeneous phase. The solvent achieved its supercritical point (Figure C3-1). Since few years, Supercritical (and subcritical) Fluids Extraction (SFE) is a promising technique to extract molecules. Few industrial processes are using SFE. The main advantage of SFE is the penetration capacity of these kinds of fluids into the matrix. The first example is the DIAMANT® process. For this process, trichloroanisole is removed from cork to avoid cork taste in wine with Supercritical CO_2.[158] One of the most known is the decaffeination of coffee beans.[159–161] SC-CO_2 allows to extract caffeine molecules without the need to crush coffee beans or to use organic solvents due to penetration property of CO_2. Conventional solvents used for caffeine extraction are mostly chloro-based solvents such as dichloromethane (Carcinogenic, mutagenic, or toxic to reproduction) that is removed by distillation from coffee beans. Using classic solvents require additional steps post-extractions,

including solvent evaporation, and quality-control of matrix to verify solvents quantity especially when the solvent is not safe. SFE is considered as eco-extraction because this technique avoids the use of organic solvents. These solvents are regulated by the FDA (*Food and Drug Administration* of USA), or by a European directive for example. Furthermore, SFE allows the extract phenolic compounds with mild conditions compared to conventional extractions. Phytochemicals are extracted at low temperatures (45 – 60 °C), or at high temperatures (> 100 °C) but for a short extraction time (10 – 30 min).[86,153] Green techniques can protect polyphenols from air (O_2) and/or light.[92,153,162] Classic methods use high temperature (*e.g.* reflux, Soxhlet extractions) and/or long extraction time (*e.g.* maceration) leading to degradation of polyphenols.[92,153]

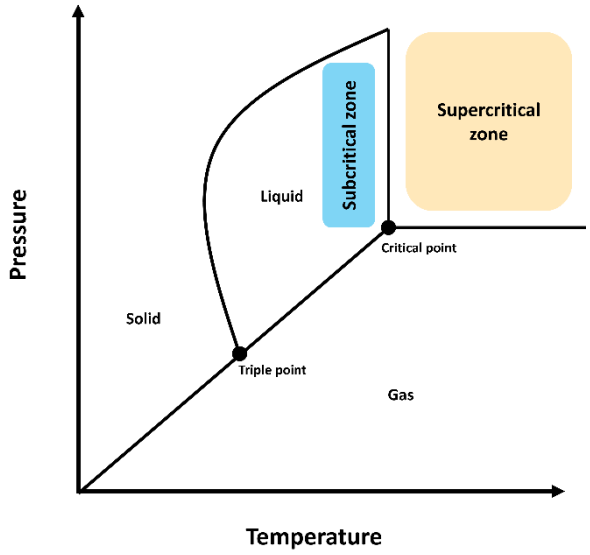


Figure C3-1. Scheme of phase diagram; blue zone: subcritical zone such as used for subcritical water extraction; yellow zone: supercritical zone such as used for supercritical CO_2 extraction.

SC-CO_2 is represented in yellow zone (Figure C3-1). The state of matter of SC-CO_2 is when the critical temperature and critical pressure are achieved which is above 31.1 °C and 73 bar. Above critical point, CO_2 has special properties: viscosity reacts like a gas (0.02 – 0.12 mPa.s at 40 °C) and density like liquids (700 – 1,100 kg.m^{-3} depending on pressure and temperature).[70,163] CO_2 is a great solvent because of its properties and its safety. The solvent is non-toxic, non-inflammable, respect GRAS ("*Generally Recognized As Safe*" by the FDA), cheap and available. CO_2 is an apolar molecule, and by affinity this solvent extracts non-polar such as wax, lipids, oil, *etc.* (Table C3-1).[12,70,72–74] As mentioned in Chapter 1, to improve polar molecules

extractions like polyphenols, it is necessary to use co-solvent such as water or ethanol (EtOH).[153] Addition of large amount of EtOH (or co-solvent) change drastically the critical point (Table C3-2).[96,164] When the concentration of co-solvent exceeds 30%, CO_2 becomes saturated with EtOH. Therefore, it is advisable not to use more than this amount.[96]

Table C3-1. Few examples of target molecules extracted using supercritical CO_2.

Entry	Extraction conditions	Matrix	Target molecules	Ref
1	200 bar, 40 °C, 1 h, 50 $g_{CO2}.min^{-1}$	Spent coffee ground	Lipids (triglycerides)	[161]
2	100 bar, 40 °C, 1 h, 50 $g_{CO2}.min^{-1}$ 300 bar, 40 °C, 50 $g_{CO2}.min^{-1}$	Spent coffee ground	Oil (sterols, tocopherols)	[161]
3	300 bar, 40 °C, 1 h, 50 $g_{CO2}.min^{-1}$	Spent coffee ground	Caffeine	[161]
4	350 bar, 50 °C, 90 min	Apple peel pomace	Wax	[73]
5	240 bar, 40 °C, 0.036 – 0.284 $g_{CO2}.min^{-1}$ (1 – 8 L_{CO2}/h), 140 min 1300 bar, 62.75 °C, 0.013 – 0.021 $g_{CO2}.min^{-1}$ (6 – 10 mL_{CO2}/min), 300 min	Apple seeds	Oil	[72,95]
6	DIAMANT® process	Cork	Trichloroanisole	[158]
7	300 bar, 55 °C, 2.33 $g_{CO2}.min^{-1}$ (0.14 kg_{CO2}/h), 95 min	Apple pomace	Wax	[12]
8	300 bar, 80 °C, 1.08 $g_{CO2}.min^{-1}$ (8.69×10^{-3} L/s), 150 min	Apple pomace	Triterpenic acids, phytosterols	[74]
9[a]	546 – 570 bar, 55.7 – 58.4 °C, 2 $g_{CO2+EtOH}.min^{-1}$, 20%$_{co\text{-}solvent}$ (EtOH, *w/w*), 40 min	Apple pomace	Polyphenols	[96]

10[a]	506 – 510 bar, 50.9 – 52.3 °C, 2 $g_{CO2+EtOH}.min^{-1}$, 20%$_{co-solvent}$ (EtOH, *w/w*), 40 min	Peach pomace	Polyphenols	[96]
11[a]	80 bar, 40 °C, 100 $g_{CO2}.min^{-1}$ (6 $kg_{CO2}.h^{-1}$), 20%$_{co-solvent}$ (*w/w*), EtOH-Water (57%, *v/v*), 30 min	White grape seeds	Polyphenols	[165]

[a]Subcritical CO_2 conditions (According to Table C3-2).

Table C3-2. Critical temperature and pressure of CO_2-EtOH mixtures (calculated by using SF-Solver Software, ISCO Inc. Lincoln, NE, USA)[96]

EtOH concentration (wt.%)	T_c (°C)	P_c (bar)
0	31.1	73.8
5	42.5	73.2
10	53.7	72.7
14	62.8	72.2
17	69.5	71.9
20	76.1	71.5
100	243.3	61.3

Subcritical water is a complementary technique to the SC-CO_2.[70] In order to extract different molecules family, subcritical water extraction (SWE) has been used to compare SC-CO_2 eco-extraction method, and to observe differences. Subcritical water is liquid water above its boiling point and pressurized (Figure C3-1, blue zone).[166] The critical temperature and pressure are respectively 374.15 °C and 221 bar.[70] Under SWE, the decrease in dielectric constant enables the extraction of more molecules compared to water under room pressure.[79,86–88,166] Water is a great solvent because of its properties and its safety as CO_2. The solvent is non-toxic, non-inflammable, respect GRAS, cheap and available. In addition, water is a polar molecule. The purpose of SWE is to extract polar molecules such as polyphenols, carbohydrate, polysaccharide, *etc*.[70,83,86,87,98]

Table C3-3. Few examples of target molecules extracted using subcritical water.

Entry	Extraction conditions	Matrix	Target molecules	Ref
1	50 bar, 200 °C, 30 min, 1:100 (solid/solvent, *w/v*)	Apple pomace	Polyphenols	[98]
2	50 bar, 100 – 150 °C, 20 min, 5:1000 – 95:1000 (solid/solvent, *w/v*)	Apple pomace	Chlorogenic acid and flavonoids	[83]
3	50 bar, 200 °C, 20 min, 5:1000 – 95:1000 (solid/solvent, *w/v*)	Apple pomace	5-HMF, furfural	[83]
4	100 bar, 130 °C, 15 min, 1:22 (solid:water, *w/v*) (lab-scale)	*Orostachys japonicus* A. Berger	Polyphenols, flovanoids, triterpene saponins	[87]
5	Under pressure, 220 °C, 15 min, 3:90 (solid:water, *w/v*) (pilot-scale)	*Orostachys japonicus* A. Berger	Polyphenols, flavonoids, triterpene saponins	[87]

Biomass changes due to different parameters, weather, and soil conditions, plant variety (when the biomass is similar), *etc*.[21,111] Most of them are uncontrollable parameters. In order to perform efficient extractions with AP collected, Design of Experiments (DoE) was used. Several mathematical models are reported in literature, including Broken plus Intact Cell model, Central Composite Rotatable Design, Box-Behnken Experimental Design among others.[12,74,76,81] According to literature, models can be more or less sophisticated. In these case studies, Central Composite Rotatable Design (CCRD) was used. CCRD was chosen as effective mathematical model (20 experiments for 3 parameters in triplicate, in total 60 experiments for 1 DoE) for fitting response surface DoE according to literature.[76,144,145]

II. Supercritical CO_2 Extraction

A. Parameter Choices

As a reminder of Chapter 2 (*Materials and Methods*; II. A. Table C2-1), the studied variables for the SC-CO_2 extraction are **temperature** (T) (45 – 65 °C), **pressure** (P) (120 – 250 bar) and **flow rate CO_2** (F) (10 – 20 $g.min^{-1}_{CO2}$) at constant time (1 h), and at constant amount of co-solvent ($5\%_{EtOH}$). The range of temperature was chosen for multiple reasons. For SC-CO_2 extraction, the temperature needs to be higher than 31.1 °C (critical temperature) and polyphenols are more degraded with high temperature.[70,92,153] In our knowledge, only Adil *et al.* has studied extraction time (10 – 40 min) for AP extraction with SC-CO_2 technique.[96] The conclusion of the study was that the optimal extraction time was 40 min. For other studies with AP, the extraction times were defined and were between 40 min and 150 min (Table C3-1). According to literature, we choose 1 h. One of the purposes of this work is to extract polyphenols. In order to do it with SC-CO_2, polar co-solvent is needed such as water or EtOH. According to Table C3-2, the more EtOH there is, the temperature needs to be higher. Adil *et al.* tested different concentration of EtOH between 14 and 20 wt.% with subcritical CO_2. The optimal concentration is 20 wt.%.[96] At constant pressure, increasing co-solvent results in increasing polyphenols extraction.[164] Addition of large amount of EtOH (or co-solvent) change drastically the critical point (Table C3-2).[96,164] When the concentration of co-solvent exceeds 30%, CO_2 becomes saturated with EtOH. Therefore, it is advisable not to use more than this amount.[96] So, to avoid polyphenols degradation due to temperature, the percentage of co-solvent was fixed at 5%. The critical temperature and pressure of CO_2 at 5% of EtOH are respectively 42.5 °C and 73.2 bar (Table C3-2). According to Table C3-1, the flow rate is between 0.012 and 2.33 $g_{CO2}.min^{-1}$ for AP extraction. The flow rate was chosen between 10 and 20 $g_{CO2}.min^{-1}$ due to the limits of our pump. For the pressure range, the limit of our device was 600 bar. Only Adil *et al.* and Montanes *et al.* tested extraction at more than 500 bar.[96] For other authors, the pressure was between 200 and 300 bar at their optimal conditions (Table C3-1). So, the chosen pressure for our case study is 120 – 250 bar.

B. Results of DoE with SC-CO_2 Extraction

The different results of DoE (modeling, mass yield, impacting parameters) are in Tables C3-4, C3-5 and C3-6. The experimental results about the recovery of mass yield were used to

determine second-order polynomial equations, coded and non-coded (Table C3-4). The equations from Table C3-4 describes the linear effect (T, P, F), the two-ways interactions effects (T*P, T*F, P*F) and the quadratic effects (T^2, P^2, F^2) on the mass yield. A positive coefficient means that the studied parameter has an agonistic effect, and a negative coefficient means that the parameter has an antagonistic effect on the mass yield. The coded equation considers the levels of factors ± α, ± 1 and 0 (Chapter 2; II. A. Table C2-1). The non-coded is limited to the ± 1 and 0 levels (Chapter 2; II. A. Table C2-1).

Table C3-4. Second-order polynomial equations for the response of yield with supercritical CO_2 extraction of AP.

Units	Second-Order Polynomial Equations
Coded[a]	$Y\ (\%) = 1.2499 - 0.0384T + 0.5827P + 0.1919F - 0.0218T^2 - 0.5551P^2 - 0.1034F^2 + 0.3913T*P - 0.0448T*F - 0.2168P*F$
Non-coded[b]	$Y\ (\%) = -0.855 - 0.0284T + 0.01435P + 0.1278F - 0.000077T^2 - 0.000046P^2 - 0.001463F^2 + 0.000213T*P - 0.000317T*F - 0.000236P*F$

Y: mass yield (%); T: temperature (°C); P: pressure (bar); F: flow rate CO_2 ($g_{CO2}.min^{-1}$); [a]Coded levels: - α < T, P, F < + α; [b]Non-coded levels: 45 < T < 65 °C; 120 < P < 250 bar; and 10 < F < 20 $g_{CO2}.min^{-1}$.

The coefficient of determination R^2 is between 0 and 1; 1 corresponds to a perfect correlation.[167] According to the analysis of variance (Table C3-5), the R^2 is 0.9005. It means that the selected theorical model fits with the collected experimental data. Furthermore, R^2 has to be higher than 0.7 to validate DoE.[168] Adjusted and predicted R^2 (respectively 0.8826 and 0.8561) are above 0.7 which means that the mathematical model describes correctly the dependence of the response on process variables and gives good predictions.[81] Nevertheless, the p-value of lack of fit (0.024) is lower than 0.05, which means that the CCRD is not adequate to predict the mass yield from AP with SC-CO_2 extraction.[81] The p-value means that there is strong evidence of lack of fit between independent and dependent variables. Another experimental design model should be tested in order to observe a better fitting between experiments and mathematical model.

Table C3-5. Analysis of Variance (ANOVA) of extraction yield with supercritical CO_2.

Source	DF	Adj Sum of Square	Adj Mean Square	F-value	p-value
Model	9	7.76526	0.86281	50.29	0.000
T	1	0.02136	0.02136	1.24	0.270
P	**1**	**4.91836**	**4.91836**	**286.68**	**0.000**
F	**1**	**0.53344**	**0.53344**	**31.09**	**0.000**
T^2	1	0.00256	0.00256	0.15	0.701
P^2	**1**	**1.66519**	**1.66519**	**97.06**	**0.000**
F^2	1	0.05781	0.05781	3.37	0.072
T*P	**1**	**0.45927**	**0.45927**	**26.77**	**0.000**
T*F	1	0.00602	0.00602	0.35	0.556
P*F	**1**	**0.14107**	**0.14107**	**8.22**	**0.006**
Error	50	0.85781	0.01716		
Lack of fit	5	0.20912	0.04182	2.90	0.024
Pure error	45	0.64869	0.01442		
Total	59	8.62307			

R^2	0.9005
Adj R^2	0.8826
Predicted R^2	0.8561

DF: Total degree of freedom; T: temperature (°C); P: pressure (bar); F: flow rate CO_2 ($g_{CO2}.min^{-1}$); Adj: adjusted; in bold: significative parameters (p-value < 0.05)

According to the results, mass yields are between 0 and 1.45%. In this study, the yields are very low compared to literature.[73,92,97,169] Ferrentino *et al.* obtained 4.41 – 5.85% (mass yield) at 200 – 300 bar, 55 °C, 120 min and 0.071 $g_{CO2}.min^{-1}$.[92] According to literature, authors extracted between 2.08 and 3% at similar conditions.[73,97,169] Mass yield from our case study is still coherent to literature. The difference between literature and our study is may due to different factors. As mentioned above, there are uncontrollable factors from the crop field of studied biomass (weather, soil conditions).[21] The second hypothesis can be due to the apple variety.

Table C3-6. Results (mass yields%) extractions from design of experiments using supercritical CO_2.

Entry	Temperature (°C)	Pressure (Bar)	Flow rate CO_2 ($g_{CO2}.min^{-1}$)	Mass Yield (%)
1[a]	38.2	185	15	1.17 ± 0.13
2	45	120	10	0.68 ± 0.20
3	45	120	20	1.14 ± 0.01
4	45	250	10	1.22 ± 0.20
5	45	250	20	1.26 ± 0.02
6	**55**	**75.7**	**15**	-[c]
7	55	185	6.6	0.93 ± 0.06
8[b]	55	185	15	1.25 ± 0.13
9	55	185	23.4	1.34 ± 0.09
10	55	294.3	15	1.37 ± 0.09
11	65	120	10	0.37 ± 0.03
12	65	120	20	0.66 ± 0.04
13	65	250	10	1.35 ± 0.03
14	**65**	**250**	**20**	**1.45 ± 0.16**
15	71.8	185	15	1.27 ± 0.19

[a]Subcritical conditions (according to Table C3-2); [b]Hold values; [c]no samples were extracted.

The highest yield (Table C3-6 entry 14) is achieved at 65 °C, 250 bar and 20 $g_{CO2}.min^{-1}$ and the lowest (Table C3-6 entry 6) is obtained at 55 °C, 75.7 bar and 15 $g_{CO2}.min^{-1}$. In the case of 0% of yield can be due to the low pressure. At low pressure, the solvation power of solvent (in this case CO_2) is low. The higher is the pressure, the higher will be the solvation power.[12,92] Furthermore, in these conditions (75.7 bar and 55 °C) the solvent is almost in subcritical CO_2 conditions. At 5% of EtOH, the critical conditions of pressure and temperature are respectively 73.2 bar and 42.5 °C (Table C3-2). At low pressure, the CO_2 density is low, and the solvation power is low as well.[12,92] Consequently, the extraction of polyphenols and wax is impossible in these extraction conditions. As previously discussed, the solvation power of the solvent is limited at low pressures. At 75.7 bar, the pressure is insufficient to extract compounds from AP.

When the yield is the highest (Table C3-6 entry 14), it can be explained by different parameters. In this condition, the temperature was 65 °C, which is one of the highest of this study. In

literature, similar results were found. When the temperature is high, the Total Polyphenols Content (TPC) yield and/or the mass yields are higher than at low temperatures.[12,72,96] At constant pressure (200 bar), extraction time (25 min) and co-solvent content (17 wt.% of EtOH), the TPC were 0.168 and 0.143 mg Gallic Acid Equivalent/g of sample at respectively 60 and 40 °C. Another example, Ferrentino *et al.* found 4.05 ± 0.15% at 45 °C and 4.41 ± 0.18% at 55 °C (constant pressure and flow rate CO_2).[92] Furthermore, in this context, the pressure is quite high (250 bar), one of the highest of the study. The solvation power of CO_2 is increasing with the increase of pressure.[12,72] According to the literature, a higher pressure leads to a higher TPC yield and/or mass yield.[72,75,92,96] Kryževičiūtė *et al.* explored the impact of varying pressures on extraction processes. They conducted extractions at three distinct pressures (100, 275, and 450 bar). Their findings indicated that the optimal yield for extracting raspberry pomace was achieved at 450 bar.[77] Adil *et al.* studied pressure between 200 and 600 bar for the polyphenols extraction from AP in subcritical CO_2 conditions. The optimal pressure was found at 546 – 570 bar.[96] The difference between their experiments and ours is that our case study is in supercritical conditions with lower co-solvent (EtOH) content. Furthermore, they tested TPC and antioxidant activity on all their samples. In our case, only the mass yield was considered for DoE. De La Peña Armada *et al.* tested at 300, 425 and 550 bar (and constant temperature) and they found their higher mass yield at 550 bar.[97] Nevertheless, the higher antioxidant (Oxygen Radical Antioxidant Capacity assay) result was found at lower pressure at the same temperature, 425 bar. At 425 bar, the ORAC assay was 609.17 ± 96.11 µmol Trolox Equivalent/g of extract and at 550 bar, it was 506.38 ± 67.74 µmol TE/g of extract.

According to the results from Table C3-5, the most important parameters are pressure, flow rate CO_2, the combination of pressure-temperature, of pressure-flow rate and of pressure-pressure (p-value < 0.05). At 55 °C and 15 $g_{CO2}.min^{-1}$, yield is increasing with the increase of pressure. For example, at 75.7, 185 and 294.3 bar (Table C3-6 entries 6, 8 and 10), yields are respectively 0, 1.25 ± 0.13 and 1.37 ± 0.09%. As explained above, it is due to the solvation power that increases with pressure.[12,92] The second influent parameter is the flow rate CO_2. Extractions were carried out at 3 different flow rates CO_2 (6.6, 15 and 23.4 $g_{CO2}.min^{-1}$) at constant pressure and temperature (Table C3-6 entries 7 – 8). As it shows in Table C3-6, the increase of flow rate CO_2 results in the increase of yield. In several scientific articles, flow rate is not a significant parameter. Our flow rate CO_2 is between 6.6 and 23.4 $g_{CO2}.min^{-1}$. In Ferrentino *et al.*'s study is between 1 and 8 $L.h^{-1}_{CO2}$, or between 0.036 and 0.284 $g_{CO2}.min^{-1}$.[95]

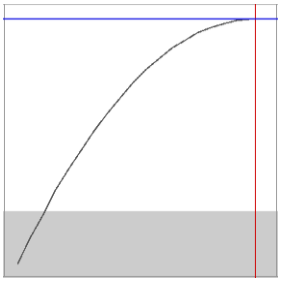
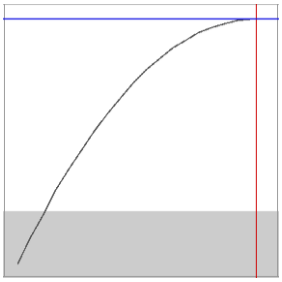
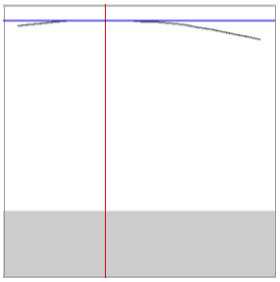
Compared to Ferrentino *et al.*'s greatest flow rate, our smallest is more than 25 times higher than theirs. Furthermore, from 6.6 to 15 $g_{CO2}.min^{-1}$, the increase of yield is 74.4% and from 15 to 23.4 $g_{CO2}.min^{-1}$, the increase yield is 9.32% at constant temperature (55 °C) and pressure (185 bar). So, there is not necessary to use the highest flow rate CO_2.

According to literature, the range of temperature for SC-CO_2 extractions with AP is between 35 and 80 °C.[12,74,97] The temperature is quite low compared to conventional extraction (*e.g.* maceration) but it allows to protect bioactive molecules from degradation.[92] Higher temperature has effect to increase yield significantly in accordance with their DoE.[12,74,92] In our study, it is not the case (p-value > 0.05). The temperature has no effect on the mass yield (Table C3-5). In Table C3-6, extractions of entries 1, 8 and 15 showed similar yield extraction, respectively 1.17 ± 0.13, 1.25 ± 0.13 and 1.27 ± 0.19%. The difference between our study and literature may be due to the device (especially for the flow rate), apple variety, *etc.* Temperature has an antagonistic effect on yield. A negative coefficient is in front of T (temperature) in equations (Table C3-4). At 120 bar and 10 $g_{CO2}.min^{-1}$, yields were 0.68 ± 0.20 and 0.37 ± 0.03% at 45 and 65 °C respectively. There is a decrease of yield with the increase of temperature. However, at high pressure (250 bar) and flow rate CO_2 (20 $g_{CO2}.min^{-1}$), it is not the case anymore. The yields are 1.26 ± 0.02% at 45 °C and 1.45 ± 0.16% at 65 °C.

C.Results of DoE with SC-CO_2 Extraction

To conclude the DoE of SC-CO_2 extraction, the optimum conditions are 71.8 °C, 287.7 bar et 12.7 $g_{CO2}.min^{-1}$ according to the results (Table C3-8). Optimum conditions were achieved for pressure and flow rate (plateau in both cases). Only for temperature, none plateau was reached. According to the curve, the temperature can be much higher than the studied range. Nevertheless, much higher temperatures can lead to the degradation of molecules. For the AP extraction, range temperatures is usually between 40 and 60 °C. In order to avoid bioactive molecules degradation, next SC-CO_2 extraction in Chapter 3 will be fixed at 50 °C. Furthermore, temperature has a small impact and has an antagonistic effect, so it is counterproductive to extract at high temperature such as 71.8 °C (Table C3-4).

Table C3-8. Conditions from optimal mass yield by supercritical CO_2 extraction obtained with Minitab software.

	Temperature (°C)	Pressure (bar)	Flow rate CO_2 ($g_{CO2}.min^{-1}$)
Maximum (+ α)	71.8	294.3	23.4
Optimum	**71.8**	**287.7**	**12.7**
Minimum (- α)	38.2	75.7	6.6
			

Extraction conditions: 15 g of AP (⌀ 500 nm); $5\%_{EtOH}$; 1 h.

III. Subcritical Water Extraction

A. Parameter Choices

As mentioned in Table C3-1, SC-CO_2 extracts apolar molecules such as wax. The polarity of solvent can be modulated by the addition of polar co-solvent (EtOH, water). Subcritical Water Extraction (SWE) is a complementary technique to SC-CO_2 extraction. As a reminder in Chapter 2 (*Materials and Methods*; III. A. Table C2-3), the studied variables for the SWE are **temperature** (T) (90 – 140 °C), **time** (t) (10 – 20 min) and **ratio** (R) (AP:Water; *w/v*) (1:8 – 1:15) at constant pressure (5 bar). The range of temperature was chosen for multiple reasons. For SWE, preliminary tests were conducted at 150, 180 and 197 °C at 1:20 ratio (AP:Water, *w/v*). Above 180 °C AP turned black with a strong burnt smell. It was decided to not exceed 150 °C for the DoE. According to Table C3-3, extraction time is quite short (15 to 30 min). Ibrahim *et al.* tested 4 different extraction times, 0, 20, 30 and 60 min.[98] In this study, the optimal extraction time was found to be 30 min. Nevertheless, the TPC of 20 and 30 min were close, around 46.25 mg GAE/g of dry AP for both residence times. In our case study, the extraction time was chosen between 10 and 20 min. The pressure was not studied in this case. The

purpose of pressure in our case was to keep water in liquid state. By default, extraction was pressurized at 5 bar. The choice for ratio was between 1:8 (AP:Water, *w/v*) and 1:17 (AP:Water, *w/v*) because no authors tested this range.

B.Results of DoE with Subcritical Water Extraction

The different results of DoE (modeling, mass yield, impacting parameters) are in Tables C3-9, C3-10 and C3-11. The experimental results about the recovery of mass yield were used to determine second-order polynomial equations, coded and non-coded (Table C3-9). The equations from Table C3-4 describes the linear effect (T, t, R), the two-ways interactions effects (T*t, T*R, t*R) and the quadratic effects (T^2, t^2, R^2) on the mass yield extraction.

Table C3-9. Second-order polynomial equations for the response of mass yield (%) with subcritical water of AP.

Units	Second-Order Polynomial Equation
Coded[a]	Y (%) = 46.186 + 5.100T + 1.892t + 0.721R + 1.844T² - 0.295t² + 2.763R² + 0.246Txt – 0.896TxR – 0.846txR
Non-coded[b]	Y (%) = 89.0 – 0.325T + 1.35t – 8.10R + 0.0025T² - 0.0118t² + 0.44212R² + 0.00197Txt – 0.0143TxR – 0.0677txR

Y (%): Mass Yield (%); T: temperature (°C); t: time (min); R: ratio (AP:Water, *w/v*); [a]Coded levels: - α < T, t, R < + α; [b]Non-coded levels: 90 < T < 140 °C; 10 < t < 20 min; and 1:10 < R < 1:15 (AP:Water, *w/v*).

According to the analysis of variance (Table C3-10), the R^2 is 0.7222. It is higher than 0.7 and it validates the DoE regression model.[81] It means that the selected theorical model fits with the collected experimental data. Several extractions were reconducted to improve R^2. The best result was 0.7222. Adjusted R^2 and predicted R^2 are lower than 0.7. Predicted R^2 is 0.6154 which means that the predictions will be biased. Adjusted R^2 is 0.6722, the mathematical model is insufficient to describe the dependence of responses on process parameters.[81] Furthermore, the p-value of lack of fit (0.024) is lower than 0.05, which means that the CCRD is not adequate to predict the mass yield.[81] The value means that there is strong evidence of lack of fit between independent and dependent variables. Another experimental design model should be tested in order to observe a better fitting between experiments and mathematical model.

Table C3-10. Analysis of Variance (ANOVA) of extraction mass yield (%) with subcritical water.

Source	DF	Adj Sum of Square	Adj Mean Square	F-value	p-value
Model	9	1731.60	192.40	14.44	0.000
T	**1**	**1065.82**	**1065.82**	**80.01**	**0.000**
t	**1**	**146.67**	**146.67**	**11.01**	**0.002**
R	**1**	21.33	21.33	1.60	0.212
T^2	**1**	**146.98**	**146.98**	**11.03**	**0.002**
t^2	**1**	3.77	3.77	0.28	0.597
R^2	**1**	**330.07**	**330.07**	**24.78**	**0.000**
T*t	**1**	1.45	1.45	0.11	0.743
T*R	1	19.26	19.26	1.45	0.235
t*R	**1**	17.17	17.17	1.29	0.262
Error	50	666.07	13.32		
Lack of fit	5	162.30	32.46	2.90	0.024
Pure error	45	503.77	11.19		
Total	59	2397.67			

R^2	0.7222
Adj R^2	0.6722
Predicted R^2	0.6154

DF: Total degree of freedom; T: temperature (°C); t: time (min); R: ratio (AP:Water, *w/v*); Adj: adjusted

According to the results from Table C3-10, the most important parameters are time, temperature, and the combination of ratio-ratio (p-value < 0.05). According to Table C3-11, mass yields are between 40.90 and 61.03%. The highest yield (Table C3-11 entry 13) is achieved at 140 °C, 20 min and ratio of 1:10 (AP:Water, *w/v*) and the lowest (Table C3-11 entry 6) is obtained at 115 °C, 6.6 min and ratio of 1:12 (AP:Water, *w/v*). For the lowest yield, the temperature and the extraction time were lower than for the highest yield. Ibrahim *et al.* found similar results with longer extraction time and temperature with the TPC. For example, at 100 °C and 10 min, the TPC was 28.2 ± 1.90 mg GAE/g of dry AP and at 150 °C and 20 min, the TPC was higher, 54.3 ± 0.50 mg GAE/g of dry AP. Furthermore, it is consistent with results from our

DoE. Extraction time and temperature are influent parameters due to their low p-values (< 0.05) (Table C3-7).

Table C3-11. Extraction results (mass yields%) from design of experiments using subcritical water.

Entry	Temperature (°C)	Time (min)	Ratio (AP:Water, *w/v*)	Yield (%)
1	73	15	1:12	43.83 ± 0.59
2	90	10	1:10	41.57 ± 2.18
3	90	10	1:15	44.57 ± 1.50
4	90	20	1:10	44.50 ± 3.18
5	90	20	1:15	49.97 ± 2.15
6	**115**	**6.6**	**1:12**	**40.90 ± 1.79**
7	115	15	1:8	53.13 ± 3.44
8[a]	115	15	1:12	44.82 ± 11.34
9	115	15	1:17	50.23 ± 2.03
10	115	23.4	1:12	47.17 ± 1.56
11	140	10	1:10	52.93 ± 3.29
12	140	10	1:15	57.77 ± 2.71
13	**140**	**20**	**1:10**	**61.03 ± 0.59**
14	140	20	1:15	58.30 ± 1.91
15	157	15	1:12	53.30 ± 4.73

[a]hold values

In this case study, the ratio is not influential (p-value 0.212 > 0.05) as independent parameter compared to literature, but it is influential as quadratic parameter (p-value < 0.05) (Table C3-10).[81,83,98] A quadratic parameter indicates a term in the mathematical model (CCRD) that represents the squared effect of independent variables (temperature, extraction time and ratio) on dependent variables (mass yield). Basically, the ratio is not influential in linear relationship but in curve one (upward or downward). Hobbi *et al.* did their experiments in static-mode found that the ratio has a huge impact on TPC (p-value < 0.05).[81] In our study, experiments were made in dynamic-mode, the mixtures were stirred during extraction. According to their results and ours, the influence of linear variable of ratio of Hobbi *et al.* and quadratic variable of ratio of our case study, may be explained by the static and dynamic modes, respectively linear and quadratic (curve) relationships. At 115 °C and 15 min, an

increase of yield is correlated to an increase of ratio. At 1:8 (AP:Water, *w/v*), the yield is 53.13 ± 3.44% and at 1:17 (AP:Water, *w/v*), the mass yield is 50.23 ± 2.03% (Table C3-11 entries 7 and 9). The results are quite similar because the ratio is not important according to Table C3-10.

One of the important parameters is extraction time. The increase of time leads to the increase of yield. At 115 °C and 1:12 (AP:Water, *w/v*), yield is increasing with the increase of time. For example, at 6.6, 15 and 23.4 min (Table C3-11 entries 6, 8 and 10), yields are respectively 40.9 ± 1.79, 44.82 ± 11.34 and 47.17 ± 1.56%. It may be explained by the fact dry AP needs to be rehydrated. There are 3 simultaneous steps: absorption of water by dry AP, increase of AP volume, transfer of molecules into extraction water.[83] Similar results were found in literature. Hobbi *et al.* observed an increase of TPC yield of 11% from 30 min to 75 min, respectively 17.20 and 19.15 mg GAE/g of dry AP.[81] Ibrahim *et al.* found above 30 min, there is no advantage for the recovery of polyphenols. Depending on the temperature, they observed that long extraction time can lead to the degradation of phenolic compounds.[83]

The last important parameter is temperature. The increase of temperature results in the increase the yield. From 73 to 115 °C at constant extraction time and ratio, the increase of yield is negligible, only 2.2% (Table C3-11 entries 1 and 8). At 173 °C, the yield is 53.30 ± 4.73% which means that the yield is increased by 15.9% (Table C3-11 entry 15). Polyphenols from AP were transferred into subcritical water by breaking adhesive and cohesive forces (hydrogen bonding, Van der Waals forces, dipole-dipole interactions).[83] Above 140 °C under SWE, extracts became brown (to dark-brown) and smelling different compared to under 140 °C extracts. It may be due to the degradation of sugars (Figure C3-2). The Story apple variety is sweet.[170] So, the reaction of degradation can may be occurred before compared of Ibrahim *et al.* because it means that our biomass (AP Story) has a lot of sugars. With high temperature a reaction of dehydration of fructose, xylose, glucose, *etc.* has taken place leading to formation of 5-(hydroxymethyl)furfural (5-HMF) and furfural.[70,83,154,171,172]

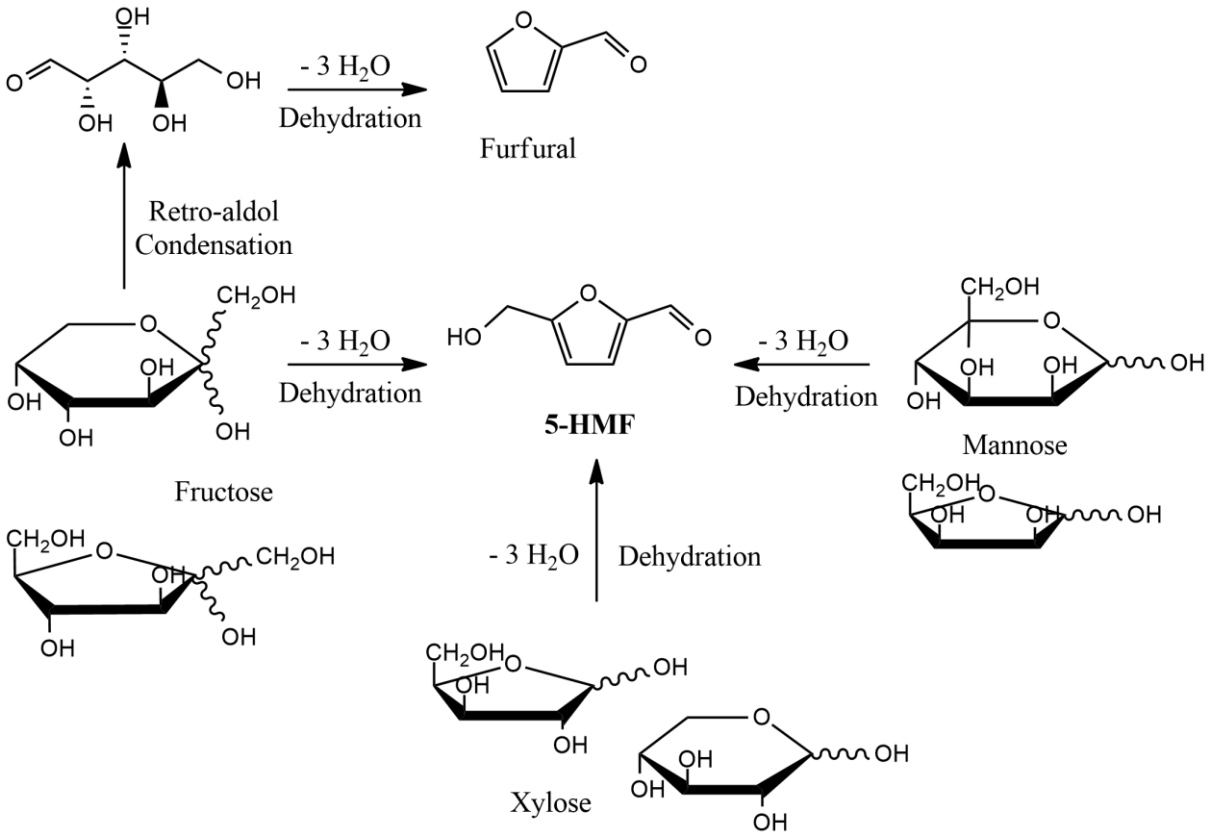


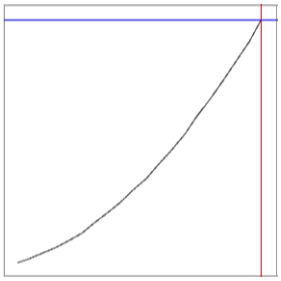
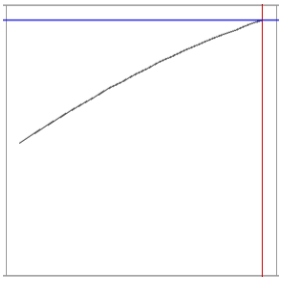
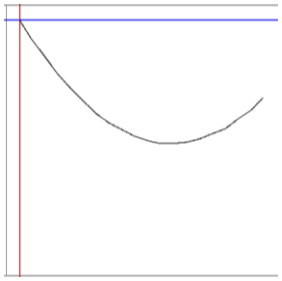
Figure C3-2. Transformation reaction of carbohydrates into 5-HMF and Furfural adapted from Meier *et al.*, Vandeponseele *et al.*, Shapla *et al.*, Roman-Leshkov *et al.*[70,154,171,172]

Ibrahim *et al.* observed similar results above 150 °C by comparing 3 different temperatures, 100, 160 and 200 °C.[83] With the increase of temperature and the smell and the color were stronger. In another study of Ibrahim *et al.*, the formation of these molecules increased with high temperature, long extraction time and low pH.[98]

C.Conclusion of Subcritical Water Extraction

To conclude the DoE of SWE, the optimum conditions are 157 °C, 23.4 min et ratio of 1:8 (AP:Water, *w/v*) according to the results (Table C3-12). Optimum conditions were not achevied for all parameters, because none plateau was reached. According to Table C3-12, the extraction time needs to be longer and at some point, the optimal extraction time should be achieved. Nevertheless, extraction time should not exceed 30 min.[37] Temperature should be as well increase but above 140 °C, the AP is burned. And it could be interesting to test a lower ratio such as 1:30 (AP:Water, *w/v*) to observe what kind of molecules will be extracted.

Table C3-12. Conditions from optimal mass yield by subcritical water extraction obtained with Minitab software.

	Temperature (°C)	Time (min)	Ratio (AP:Water, *w/v*)
Maximum (+ α)	157	23.4	1:17
Optimum	**157**	**23.4**	**1:8**
Minimum (- α)	73	6.6	1:8
			

Extraction conditions: 25 g of AP (⌀ 500 nm); under stirring; 5 bar_{N2}.

IV.Conclusion

As highlighted in the introduction of this chapter, the objectives of the thesis are eco-extraction methods for bioactive molecules and potential applications for their valorization. In this PhD thesis, antioxidant and antibacterial activities are studied. The elements of the studied biomass change due to controllable and uncontrollable parameters, weather, and soil conditions, plant variety (when the biomass is similar), *etc*.[21] DoE was performed in order to extract bioactive molecules efficiently. Several mathematical models are reported in literature, including Broken plus Intact Cell model, Central Composite Rotatable Design (CCRD), Box-Behnken Experimental Design among others. .[12,74,76,81] In these case studies, CCRD was used for both techniques. For each SFE, a total of at least 60 experiments was done and the optimization of conditions was successfully applied for the extraction of biomolecules.

For the SC-CO_2 extraction, the optimal conditions are 287.7 bar, 71.8 °C, and 12.7 $g_{CO2}.min^{-1}$ according to Minitab (DoE software). In these conditions, wax and polyphenols were extracted. In the DoE of SC-CO_2 extraction, the optimal conditions were achieved according to the maximum of mass yield (%) including mass of polyphenols and wax. Authors from literature never distinguished polyphenols, oil, and wax.[12,74,96,97,169,173] The optimal conditions could have been more accurate for bioactive molecules extraction by adding dependent variables such as

TPC, antioxidant activity (*e.g.* ORAC, DPPH assay) or analytical evaluation. In Chapter 4, biological activities (antioxidant and antibacterial) of extracts with optimal conditions will be discussed. For SC-CO_2 extraction, 3 types of extracts will be compared in order to improve the yield and biological activities. The first extract tested will be crude (wax, polyphenols, oil *etc.*), the second one, wax will be removed (post-treatment extraction) and the last one, AP will be pretreated by ultrasound (US) then extracted with SC-CO_2 technique. According to Table C3-8, the temperature can be much higher than the studied range. Bioactive molecules, in particular polyphenols, can be degraded due to temperature. To avoid bioactive molecules degradation, next SC-CO_2 extractions in Chapter 4 will be fixed at **50 °C**, **287.7 bar** and **12.7 $g_{CO2}.min^{-1}$**.

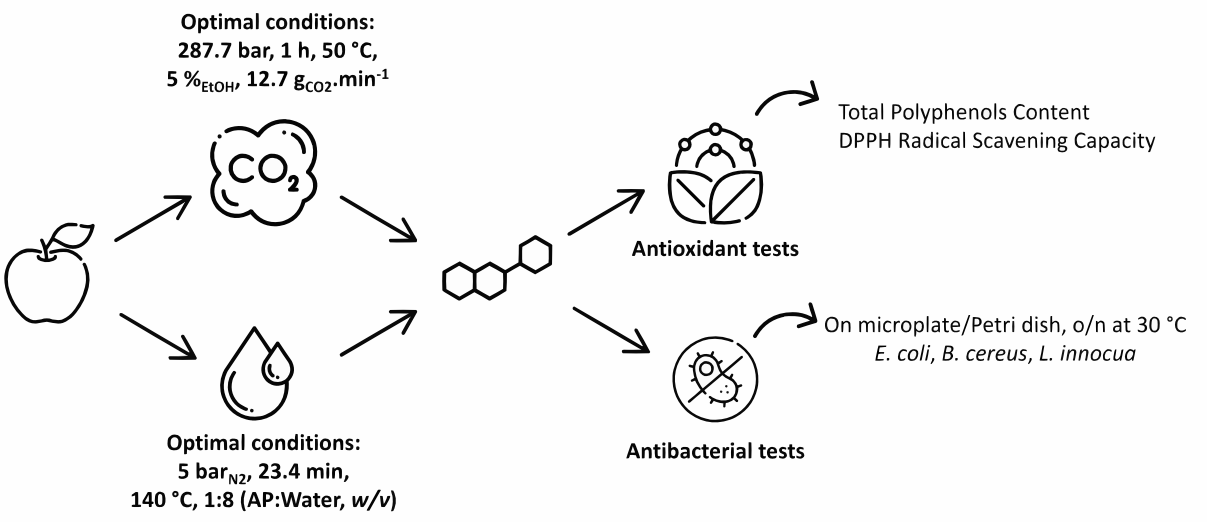
For SWE, the optimal conditions are 157 °C, 23.4 min, and 1:8 (AP:Water; *w/v*). In these conditions, several compounds were extracted, including carbohydrates, undesirable compounds (5-HMF, FFR) and polyphenols. Applications of extracts are antioxidant and antibacterial. For this reason, SWE extracts need to be post-treated with resin to remove sugars to avoid biological activities from sugars, especially for antibacterial tests. Sugars can favorize the growth of bacteria. According to Table C3-12, the extraction time and the temperature need to be longer. Nevertheless, extraction time should not exceed 30 min according to Ibrahim *et al*. and temperature should be as well increase but above 140 °C, the AP is burned.[98] In order to extract more polyphenols, the ratio should be different such as 1:30 to 1:100 should be tested. The optimal conditions were determined thanks to mass yield. As SC-CO_2 extraction, TPC, biological activities or analytical evaluations can be used to establish optimal extraction conditions for bioactive molecules. In Chapter 4, biological activities (antioxidant and antibacterial) of extracts with optimal conditions will be discussed. One of the samples will be extracted with US pretreatment to improve biological activities. For both extracts (with and without US pretreatment), the conditions will be at **140 °C**, **1:8 (AP:Water, *w/v*)** for **23.4 min**.

Different optimal conditions may be determined if TPC, biological and analytical evaluations were considered. As mentioned above, the quantity of extract does not determine the antioxidant capacity of extract. De la Pena *et al.* tested 3 different pressures: at 300, 425 and 550 bar (and constant temperature) using SC-CO_2 technique. They found that higher mass yield at 550 bar than 300 and 425 bar. Nevertheless, Oxygen Radical Antioxidant Capacity assay (antioxidant test) shows that at lower pressure and same temperature, 425 bar was a better choice for antioxidant activity compared to 550 bar. At 425 bar, the ORAC assay was 609.17 ±

96.11 µmol Trolox Equivalent/g of extract and at 550 bar, it was 506.38 ± 67.74 µmol TE/g of extract.[97] For both techniques, kinetic should be done. In the case of SC-CO_2 extraction, it should be interesting to observe the kinetic to optimize extraction time. For the SWE case, the kinetic can provide selective extraction. As an example of selective extraction using SWE, Ibrahim *et al.* found different optimal conditions depending on the molecules.[83] They extracted the maximum amount of phloridzin around 100 – 120 °C and chlorogenic acid at 100 °C but degraded at 112 °C. Between 112 and 120 °C, only phloridzin was extracted compared to chlorogenic acid.

Chapter 4

Antioxidant and Antibacterial Activities of Bioactive Compounds Extracted from Apple Pomace with Subcritical and Supercritical Fluids



Chapter 4 focuses on the biological activities, specifically antioxidant and antibacterial, of Supercritical and Subcritical Fluid Extraction (SFE) of bioactive molecules from apple pomace (AP). SFE techniques allow for more efficient extraction and preservation of phytochemicals compared to conventional methods. Biological tests were conducted in this chapter. For antioxidant activity, Total Polyphenols Content (TPC) and DPPH Radical Scavenging Capacity (DRSC) were measured. Regarding the antibacterial activity of apple pomace extracts, they were tested against three strains: *Listeria innocua*, *Bacillus cereus* and *Escherichia coli*. According to Chapter 3, the mass yield for SC-CO_2 extraction was low (< 2%) and the biological properties were not tested. To improve these results, ultrasound (US) pretreatment was used. The results showed that the mass yield (+ 12.90%), TPC (+ 10.4%), and DRSC (+ 16.6% at 50 $mg_{Extracts}/mL_{MeOH}$) were enhanced for SC-CO_2 extraction. For SWE, TPC and antibacterial activities were improved by 25.5 and 2 – 6% at 50 $mg_{Extracts}/mL_{Water}$ (depending on the strains), respectively. However, DRSC decreased with US pretreatment, attributed to molecule degradation induced by high-intensity cavitation, with a reduction of 13.8% at 15 $\mu g_{Extracts}/mL_{MeOH}$.

I.Introduction

Apple (*Malus domestica* Borkh.) is a common fruit. The fruit originated from Kazakhstan, in Central Asia and for a small part, Europe. The largest city, *Almaty* (known then as *Alma Ata*), means “full of apples” or “father of apples”.[18,174,175] Apples are one of the most produced and eaten fruits in the world, with an average consumption of almost 9 kg of apples per person per year.[18] According to the *Food And Agriculture Organization of the United Nations* (FAO), the production of apples has been increasing every year (Table C4-1).[19] As highlighted in previous chapters, the production of apple juice and cider generated a by-product known as apple pomace (AP). Over the past five years, apple production has grown by 10.4%, consequently leading to an increase in AP. Currently, AP is undervalued and is often disposed of by burial in soil, which poses environmental and health concerns due to its high moisture content (80 – 90%).[9,21] Depending on the country, AP can be used as animal feed or for generating biogas (Chapter 1). Several researchers have sought solutions at laboratory and semi-industrial scales using various green technologies.[12,92,98,140] Moreover, an increasing number of companies are being established with the aim of valorizing AP.[10,11,176,177]

Table C4-1. Worldwide productions (2018 – 2022) of apples and apple pomace according to FAO.[19]

Year of production	Quantity of apples (in million tonnes)	Quantity of apple pomace (in million tonnes)[a]
2018	85.8	4.2 – 5.0
2019	87.5	4.3 – 5.1
2020	90.6	4.4 – 5.3
2021	93.9	4.6 – 5.5
2022	95.8	4.7 – 5.6

[a]Estimation

AP is a great biomass waste to valorize due to its quantity (4.4 – 5.3 million tonnes in 2022) and its composition (Tables C4-1 and C4-2). Polyphenols and more generally phytomolecules are omnipresent in plants, including in apple and AP.[153,178] A well-known saying about the bioactive molecules from apple is “*An apple a day keeps the doctor away*”. Bioactive molecules includepolyphenols, organic acids, triterpenes, sterols, carbohydrates, *etc.*[179] Phytochemical from apple are mainly found in peels and seeds (Table C4-2).[40,41,173,180] Seeds represent a small

part of the total weight of fresh apple and AP (0.7 – 4.1%) but contain large concentration of bioactive compounds.[21,179] Seeds are composed of fatty acids (mainly linoleic acid and oleic acid), tocopherols, phytosterols, polyphenols. Tocopherols and phytosterols have been reported to stabilize oils at high temperatures and prevent polymerization.[179] Seeds also contain polyphenols, with phloridzin being the main polyphenol.. This phytomolecule is well-known for its antidiabetic activity.[15] Its antidiabetic properties on sodium-glucose co-transporter2 (SGLT2) were studied. These proteins transport glucose and sodium, allowing glucose to move from epithelial polarity to peritubular capillaries. contributing to renal glucose reabsorption. Phloridzin shows antidiabetic activity by inhibition of SGLT2 and was the first natural bioactive molecule to demonstrate an inhibition activity against SGLT in 1987.[8,181] Several phenolic compounds have been extracted and analyzed from AP seeds: (-)-epicatechin, phloretin, chlorogenic acid and quercetin derivatives.[72,95] However, the quantity of polyphenols from apple seeds, and more generally from apple and AP, depends on the cultivar, variety on growth conditions.[12,18,21,173] The second part where the molecules are mainly present is the peel.[40,41,173,180] In apple/AP peels, wax is the main component. Terpenoids from apple wax have been reported as antiproliferative component against human cell lines of liver, breast and colon cancers.[73] Additionally, wax (fatty acids, fatty alcohols, alkanes, triterpenoids) finds application in pharmaceutical and cosmetic industries.[73] Its use derived from apple or AP offers a sustainable alternative to fossil-based wax.[12] The moisture content is higher in AP flesh than in other parts of AP, around 90%. The AP flesh does not contain a lot of bioactive molecules such as polyphenols.

AP contains bioactive molecules such as phenolic compounds which exhibit biological activities such as antioxidant, antidiabetic, antitumor and antimicrobial properties (Chapter 1, Table C1-2 and Table C4-2).[15,48,51,55]

Table C4-2. Composition and biological activities depending on different parts of apple adapted from Kennedy *et al.*, Numa *et al.*, Tian *et al.*, Kumar *et al.*, Massias *et al.*, Li *et al.*, Francini et Sebastiani, Woźniak *et al.*, and Perussello *et al*.[12,21,73,74,93,173,178,179,182]

Part of apple pomace	% of wet weight mass of components	% of dry weight mass of components[a]	Main components	Biological activities
Seeds	2 – 4.1	0.2	Oil, triterpenic acids, polyphenols	Antioxidant, antimicrobial, anti-inflammatory, anti-atherosclerotic, anti-cancerous, cholesterol-lowering effect
Peels	94.5 – 95	99.8	Triterpenic acids, sterols, fatty acids, polyphenols	Antioxidant, antiproliferative, anti-inflammatory, cardiovascular protective effect, tyrosinase inhibition
Flesh			Polyphenols, sugars	Antioxidant, antiproliferative, cardiovascular protective effect
Stem	1 – 1.1	0.04	nd	nd

[a]Measured values with commercial apples; nd: not determined.

In the last decades, alternative techniques have been developed in order to reduce the use of organic solvents (one of the 12 principles of Green Chemistry) and to preserve bioactive molecules. New techniques include Subcritical and Supercritical Fluids Extraction (SFE), ultrasound, microwave among others. Supercritical CO_2 (SC-CO_2) and Subcritical Water Extractions (SWE) do not use classic solvents such as hexane or dichloromethane. Nevertheless, *green* solvents such as ethanol can be used as co-solvent.[96,108,165] Soxhlet, maceration and reflux extraction, recognized as conventional methods, typically require substantial amounts of traditional solvents which do not adhere to several principles of Green Chemistry. As mentioned in Chapter 3, subcritical and supercritical fluids were discovered 200

years ago by Charles Cagniard de Latour.[157] Since 1822, scientific articles are published every year about subcritical and supercritical fluids according to the database *SciFinder*. However, it was not until the 1970s and 1980s that subcritical and supercritical fluids gained popularity for their properties with industrial and academic applications.[161] Supercritical fluids, as CO_2, have viscosity like a gas and density like a liquid at the same time. The viscosity allows to enhance the mass transfer and to penetrate porous biomass to easily extract biomolecules such as gas. Furthermore, as discussed in Chapter 3, the increase of pressure (and consequently density) improves the extraction of bioactive molecules due to the solvating power.[12,92]

Extraction and conservation can lead to the modification of biological activities due to the degradation of molecules and environmental issues such as catechin.[16,69,153] Light, air, solvent and high temperature can also damage the quality of extracts. SFE allow to extract and preserve sensitive bioactive molecules, especially from light and air (O_2).[78–80,92,153] Mašković *et al.* extracted polyphenols from *Satureja hortensis* plant using different techniques, such as Soxhlet, SWE, maceration, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE).[183] The most efficient extraction method was SWE, with a Total Polyphenols Content (TPC; indicative of antioxidant activity) of 151.54 ± 0.85 Gallic Acid Equivalent mg/g dry matter. In comparison Soxhlet extraction (used usually as reference) exhibits a TPC at 119.25 ± 0.50 mg GAE/g dry matter. The difference between these two TPC can be explained by several hypotheses: SWE protects the molecules from light, has a shorter extraction time than Soxhlet method (30 min *vs*. 8 h), change the solvating properties of water. According to Zeković *et al.* SWE extracts from *Urtica dioica* leaves exhibit more antimicrobial property than ultrasound-assisted extraction and microwave-assisted extraction according to their Minimum Inhibitory Concentration (MIC) against *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli*. Against *Proteus vulgaris*, SWE extracts exhibit the same MIC as UAE. Furthermore, the TPC of SWE extracts was the highest compared to UAE and MAE. Even if the polyphenol contents are higher with UAE and MAE, polyphenols seem to exhibit more biological activities.

The objective in this chapter was to recover the bioactivity of molecules extracted from AP obtained by SFE. In this study, the characterization of extract has been conducted through analytical studies and antioxidant (DPPH Radical Scavenging Capacity and TPC) and antibacterial (on *E. coli*, *Listeria innocua* and *Bacillus cereus*) assays. To our knowledge, it is the

first time where the antibacterial property of extracts from AP obtained by SFE techniques have been investigated.

II.Supercritical CO_2 Extraction

As a reminder of Chapter 3, the optimal conditions for SC-CO_2 extraction are 71.8 °C, 287.7 bar and 12.7 $g_{CO2}.min^{-1}$ obtained by Minitab using AP particles at 500 nm, $5\%_{EtOH}$ for 1 h of extraction time. These conditions were determined by the mass yield (%). To preserve the biological activities from bioactive molecules, the temperature was reduced to 50 °C. Yields obtained by SC-CO_2 extraction were low, ranging 0 and 1.64% (Chapter 3, Table C3-5).

A.Improvement of Mass Yield with US pretreatment

In order to enhance mass yield, pretreatments were considered. Several pretreatments exist, such as enzymatic, US, microwave, NADES, *etc.*[184–189] Enzymatic pretreatment (with cellulase and pectinase) was initially considered (a few experiments were conducted) but was ruled out due to time constraints. Liu *et al*. chose US pretreatment (probe, 1.25 $W.mL^{-1}$) before SC-CO_2 extraction of *Iberis amara* seeds oil. The extraction oil yield was higher with US pretreatment than untreated SC-CO_2 extraction yield, respectively 25.28 ± 0.39 and 19.73 ± 0.44% (*w/w*, dry basis).[187] According to Oancea *et al.*, phenolic content extraction increases by increasing the extraction time and amplitude of US.[188] *EDYTEM laboratory* is an expert in ultrasound field. Several patents and scientific articles from the laboratory were produced and published.[190–195] Given this expertise, ultrasound pretreatment was chosen to enhance both the mass yield and biological activities in the case of SC-CO_2 extraction. Several US systems exist: cup-horn, probe, bath, whistle reactor, *etc*.[191,196–198] Each system has advantages and drawbacks (Table C4-3).

Table C4-3. Selected examples of characteristics of main US systems adapted from De La Calle *et al*., Chevallier *et al.*, Cheng *et al.*, Behling *et al.*, Golmohamadi *et al*. and ICMS equipment.[191,198–202]

Characteristics	Bath	Probe	Cup-horn
Distributed energy	Irregular	Regular	Regular
Amplitude	Fixed	Adjustable	Adjustable
Type of sonification	Indirect	Direct	Indirect/Direct[a]
Contamination risk	No	Yes	No/Yes[a]
Cost	Low	High	High
Frequency (kHz)	20 – 60	20 – 40	20 – 1000
Acoustic intensity (rough estimation) ($W.cm^{-2}$)	1 – 5	100	50

[a]Depending if the ceramic is in contact with the mixture.

The US pretreatments conditions were 10 min, 20 kHz and 50% amplitude using a cup-horn homemade system. The cup-horn system was chosen for its efficiency and capacity, with our homemade vessel having a volume of 1 L. For the pretreatment, 420 mL of water was used with 30 g of dry AP. The probe system was not efficient enough for this volume. According to *De La Calle* et al., a US probe system can be suitable for a volume ranging from 1 and 50 mL.[199] Furthermore, watered AP was too dense for effective US to penetrate through the biomass. Although adding more water could enable the use of the probe, it would complicate the freeze-drying process necessary for SC-CO_2 extraction, as AP needs to be dry. In the case of homemade cup-horn, the system is schemed in Figure C4-1. The cup-horn has three 20 kHz-emitting surfaces, which homogenize and increase the contact surface between solution of AP and US. US baths were not under consideration due to the way they distribute energy (Table C4-3). The selected frequency was 20 kHz, a common for extraction using US-assisted extraction or pretreament.[151,187,196,200] The last parameter was time. According to Golmohamadi *et al*., the TPC increased by 6% (respectively 1529 ± 28 and 1628 ± 30 mg GAE/L) and the TAC decreased by 22% (respectively 317 ± 8 and 248 ± 8 mg cyanidin-3-glucoside equivalent (C3GE)/L) between 10 and 30 min of UAE.[200]

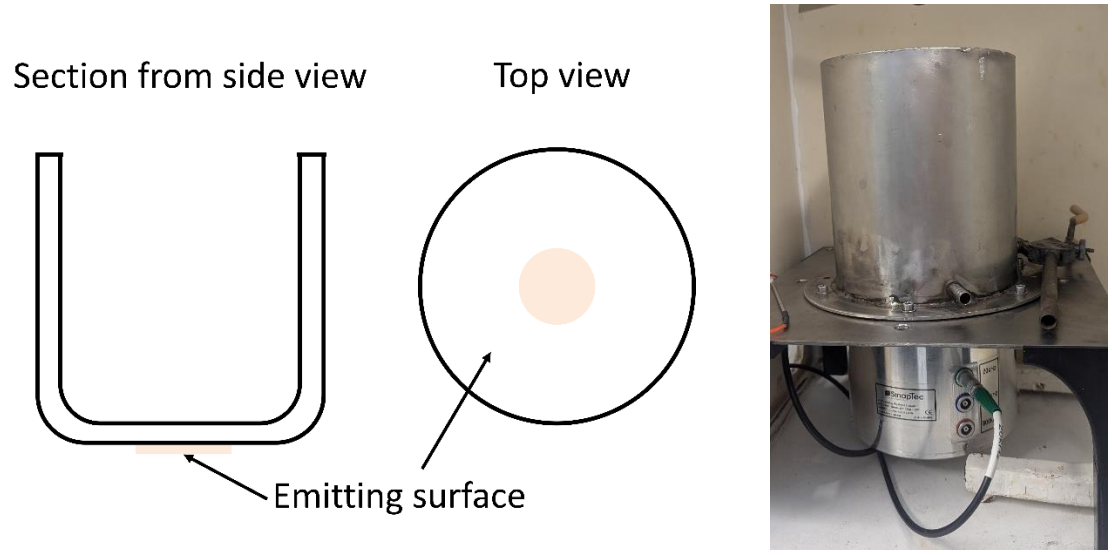


Figure C4-1. Scheme of homemade cup-horn, indirect sonification (1 L).

As discussed above, one of the aims of using US pretreatment was to improve the mass yield of bioactive molecules from AP with SC-CO_2 extraction. The mass yield was 1.12 ± 0.07% without pretreatment. An increase of 12.90% was observed with US pretreatment (10 min, 50 W, 20 kHz) (Figure C4-2). The improvement of mass yield can be caused the several factors induced by US pretreatment. During the pretreatment, the temperature is locally higher attributed to acoustic and cavitation streaming.[203] The increase of temperature appears to be beneficial to the improvement of mass yield. Another effect from US pretreatment is the breaking of cell walls of biomass. Liu *et al*. analyzed *Iberis amara* seeds by Scanning Electron Microscopy (SEM) images after different types of extraction. The treated biomass by US pretreatment was porous compared to untreated seeds.[187] The morphology has changed attributed to the cavitation bubbles induced by US. The new porosity of seeds allows the SC-CO_2 to penetrate easier into the treated biomass, leading to improvement of mass yield.[204] The morphology of treated biomass with US was more irregular and disorganized surfaces with cracks.[187] Furthermore, the US treatment reduce bond energy between molecules (*e.g.* polyphenols) and plant biomass. The US caused the desorption of molecules absorbed on the plant matrix due to the micro-agitation effect. The objective of mass yield improvement was achieved.

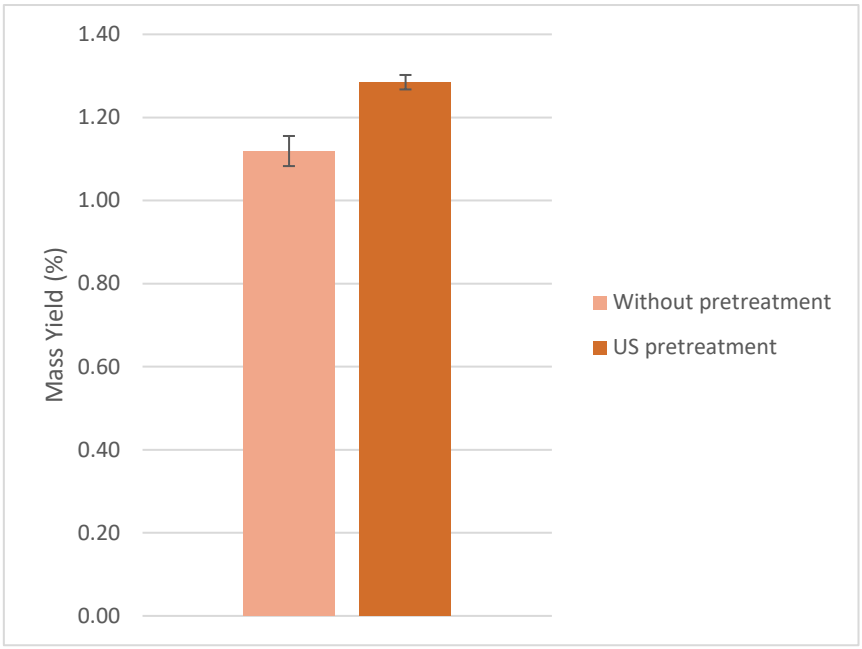


Figure C4-2. Impact of US pretreatment.

To improve the mass yield even further, amplitude, frequency and time can be modified. However, according to Golmohamadi *et al.*, longer time pretreatment can be inefficient for TPC.[200] They conducted a test with several frequencies: 20, 490 and 986 kHz. After 10 min of UAE of bioactive molecules from red raspberry puree, the TPC were respectively 1529 ± 28, 1130 ± 28 and 1103 ± 14 mg GAE/L. Only TAC was higher at 490 kHz than with other frequencies after 10 min (341 ± 4 against 317 ± 8 and 315 ± 2 mg C3GE/L at respectively 20 and 986 kHz). Golmohamadi *et al*. did not consider the improvement as significant (only 9% of increase). However, they suggested the improvement of TPC at 986 kHz after 30 min of UAE attributed to the temperature. If the temperature is not controlled, the vibration from US will increase the temperature of extraction solvent. This phenomena was observed by Barba *et al*. with the extraction of high-values added molecules from blackberries.[196] Da Porto *et al*. extracted molecules (oil) from *Cannabis sativa L.* seeds using SC-CO_2 and US pretreatment.[205] They observed above 10 min of pretreatment, the mass yield decreased. The effect of US pretreatment time can degrade the biomass before extraction due to degradation reaction such as oxidation, and polymerization.[205] In a study of Egüés *et al*., they optimized UAE of bioactive molecules from AP.[206] Among all the parameters, they tested extraction time (5 – 20 min) and amplitude (50 and 70%) regarding TPC and DRSC. At constant time, the maximum TPC was obtained at 50%, except at 15 min, TPC was higher at 70%. However, regarding the DRSC, the antioxidant activity was preserved at 50%. The maximum DRSC observed were 50%

at 10 min, 1.39 ± 0.13 mg Trolox Equivalent (TE)/g dry AP. According to these studies, there is no need to change the parameters of US pretreatment.

B.Characterization of extracts

In Chapter 3, different pressures, temperatures and flow rates of CO_2 were tested to determine the optimal extraction conditions. No characterization of extracts was discussed. Mass yield is considered polyphenols, wax and oil masses. During Design of Experiment (DoE) experiments, several observations were made during extractions and post-extractions steps. Depending on the pressure, several observations on sample were noted. For instance, no bioactive molecules were extracted, resulting in a mass yield of 0% (Table C3-6). At 120 bar, the extract was white and at 185 bar, it was yellow. The variation in color can be explained by the fact that the polyphenols were extracted at and above 185 bar. According to the literature, higher pressure leads to a higher yield in polyphenol extraction.[70,75,76] In our case study, higher pressure allowed for the extraction of polyphenols.

The white powder obtained at 120 bar, 65 °C, $5\%_{EtOH}$, and 20 $g_{CO2}.min^{-1}$ was analyzed by Gas Chromatography – Mass Spectrometry (GC-MS). The major peak corresponded to *hexatriacontane*, an alkane, according to the intern library of software (Figure C4-3). Only the non-polar fraction of wax was analyzed by GC-MS. To determine the polar fraction of wax (fatty acids, fatty alcohols, triterpenoids), the sample should have been derived. Infrared (IR) spectroscopy was used for the identification of the white powder (Figure C4-4). With this analysis, no specific molecule can be identified. However, the families of molecules can be recognized. According to the IR spectrum, the presence of alkanes is confirmed. Nevertheless, the bands at 2915 and 2848 cm^{-1} indicate C-H from alkane which aligns with the GC-MS chromatogram (Table C4-4). Li *et al.* scanned their sample using IR spectroscopy. A similar spectrum was observed.[73] Only a few peaks were too weak to be considered. For instance, at around 3400 cm^{-1}, the peak is too flat. However, it can correspond to O-H stretching vibration. This hydroxyl group can be associated with fatty acids and alcohols. Li *et al*. observed a peak at 1627 cm^{-1} corresponding to C=C bond. In our case study, this band was not observed. The peaks at 1377, 730 and 719 cm^{-1} can be associated respectively with C=O from carboxylic acid and C-H from long straight carbon chain. No further investigations were carried out about the white powder. However, this powder was considered as “wax”. Furthermore, SC-CO_2 is well

known for the extraction of non-polar molecules, such as waxes (Table C3-1).[12,73,153,161] Li *et al*. used SC-CO_2 for the extraction of wax from AP peel without mentioning polyphenols.[73] In AP peel, polyphenols are present (Table C4-2). As a test, AP extraction with SC-CO_2 at 250 bar without co-solvent (EtOH), wax and polyphenols were extracted. As mentioned above, high pressure enables to extracts polar molecules such as polyphenols.[70,75,76] According to their study of wax obtained by SFE, alkane, fatty alcohols and fatty acids are majors which is quite coherent with our results. They are supposed to not only extract wax but also polyphenols according to Table C4-2. Constituents of peels include alkane, fatty acids and alcohols but also polyphenols.

According to the GC-MS chromatogram, there is no molecules with oxygen but according to IR spectrum, there are molecules with that element (Figures C4-3 and C4-4). Additionally, there is a strong possibility that the identified molecule does not correspond to reality. According to the literature, no author who has extracted and identified molecules from apple wax has observed *hexatriacontane*. When comparing the GC-MS chromatogram and the IR spectrum of the same sample, differences are observed. For instance, the IR spectrum shows the presence of molecules containing oxygen. In contrast, only an alkane was observed on the GC-MS chromatogram.

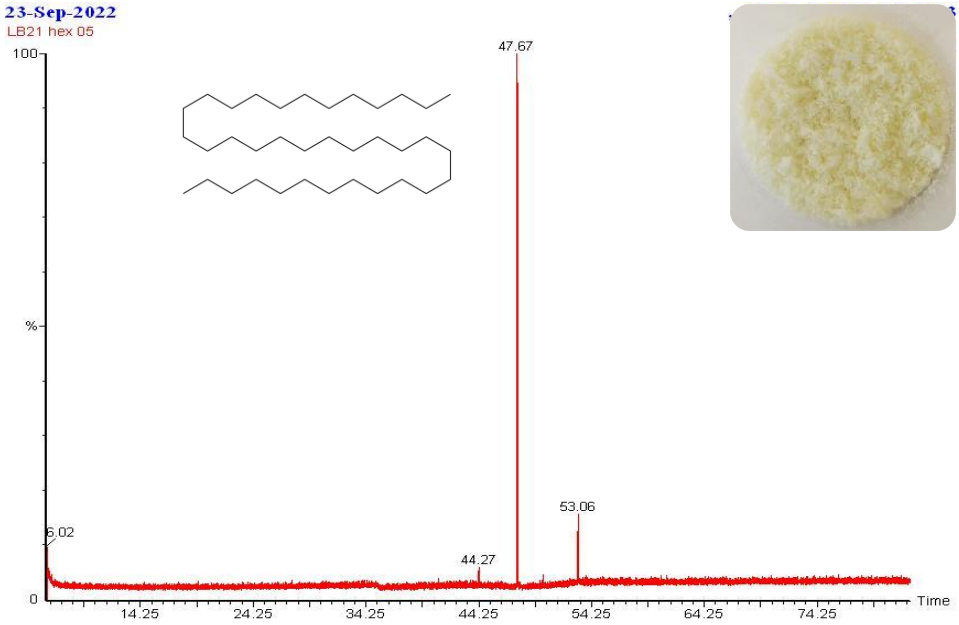


Figure C4-3. GC-MS chromatogram of SC-CO_2 extracts (0.5 mg_{Sample}/mL_{Hexane}) extracted at 120 bar, 65 °C, 5%$_{EtOH}$, 20 $g_{CO2}.min^{-1}$.

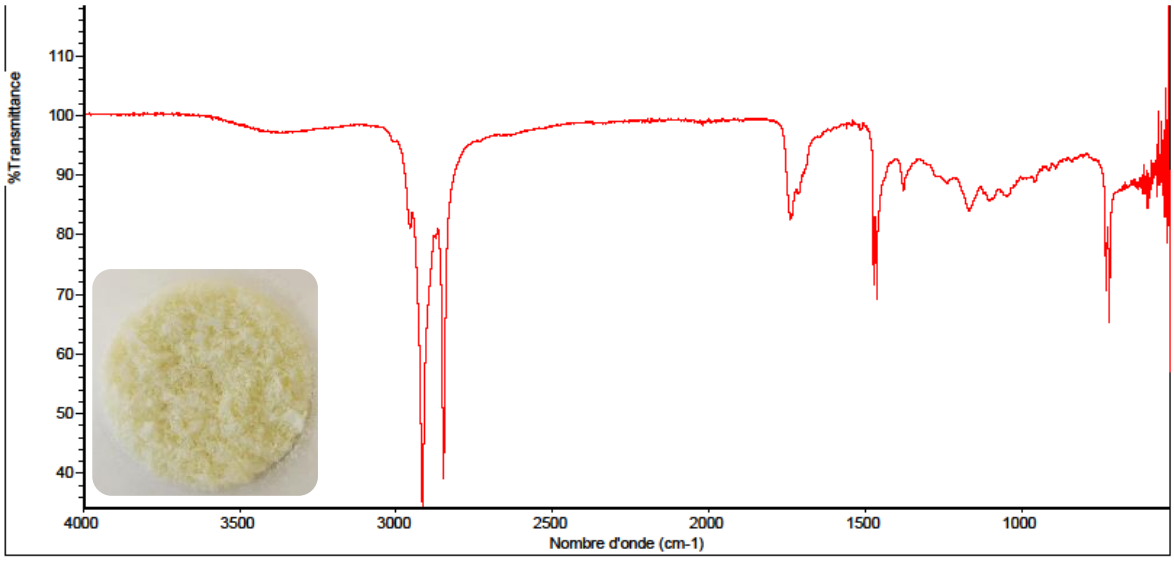


Figure C4-4. Infrared spectrum of white powder obtained at 120 bar, 65 °C, 5%$_{EtOH}$, 20 $g_{CO2}.min^{-1}$ with SC-CO_2 extraction.

Table C4-4. Identification of peaks obtained by infrared spectroscopy of white powder obtained at 120 bar, 65 °C, 5%$_{EtOH}$, 20 $g_{CO2}.min^{-1}$ with SC-CO_2 extraction.

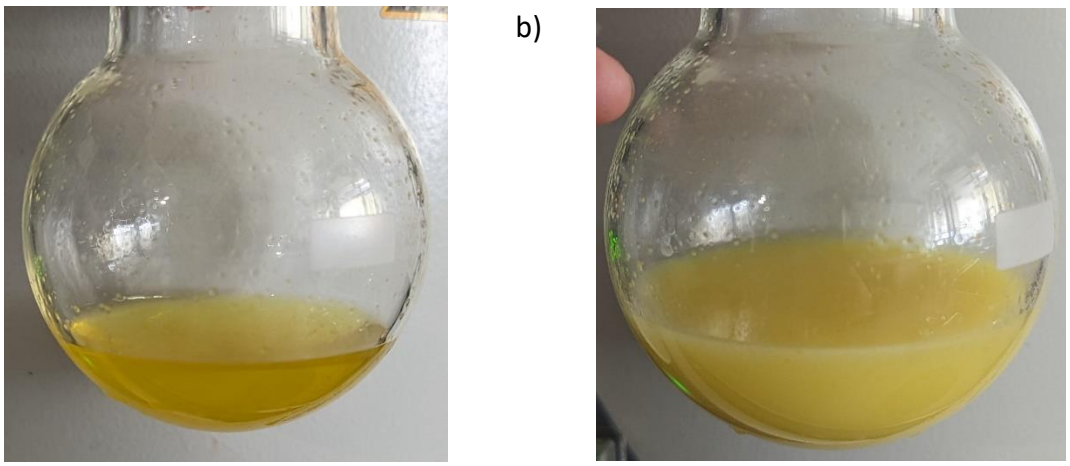
Frequency (cm^{-1})	Identification
2915	C-H; stretch
2848	
1739	C=O; stretch
1473	C-H, alkane; bend, rock
1462	
1377	C=O, carboxylic acid; stretch
1167	C-O-C, ester; stretch
730	$-(CH2)_n-$
719	

After the discovery of selective extraction with AP, several tests were conducted or considered to separate wax from polyphenols: two consecutive extractions, post-extraction steps such as solid – liquid extraction, resin use, or centrifuge. The first option was a first extraction at 120 bar for wax extraction and a second one with the same AP at 185 bar for polyphenols extraction. It was unsuccessful. Although the first extraction at 120 bar effectively extracted the wax, the subsequent extraction at 185 bar extracted both, wax and polyphenols. Solid –

liquid extraction method proved ineffective as well. Several organic solvents (hexane, EtOH) were tested in order to dissolve wax or polyphenols. Resin was considered but dismissed due to the length of protocol with a small quantity of polyphenols. Finally, the last solution was the use of centrifugation. The wax sedimented at the bottom of the Falcon tube. After overnight at – 4 °C, a small amount of white particles was in the solution of ethanolic polyphenols. Nevertheless, the best results were obtained with centrifugation.

In this project, the antibacterial properties of AP phytomolecules were the focus. SC-CO_2 extracts contain EtOH which needed to be removed due to its high antibacterial property. To recover only bioactive molecules from AP (wax, polyphenols, oil), a solvent change was conducted, replacing EtOH with water, which has no effect solvent on bacteria. In order to remove EtOH, a rotary evaporator was used. As the quantity of EtOH decreased, water was added. The ethanolic solution was clear and limpid (Figure C4-5, a), the solution turned opaque after the addition of water (Figure C4-5, b).

a) b)



Before the addition of water | After the addition of 15 mL of water

Figure C4-5. Presence of oils in supercritical CO_2 extract (287 bar, 12.7 $g_{CO2}.min^{-1}$, $5\%_{EtOH}$, 50 °C) from apple pomace; a) extract after removing wax in ethanol; b) extract in mixture ethanol-water.

One of the hypotheses for turbidity is that the solution contains natural surfactants. Natural surfactants include fatty acids or alcohols, and they auto-assemble themselves to form micelle. Micelle formed a colloidal suspension (Figure C4-6, a). Surfactants are composed of hydrophilic head and hydrophobic tail. For instance, a fatty acid has carboxylic acid (hydrophilic part) and an aliphatic chain (hydrophobic part). In our case study, the solvent is a mixture of water and ethanol. Instead of micelle, liposome could have been assembly (Figure C4-6, b).

Our AP is composed of flesh, peels, seeds and stems. The white power is considered as wax. According to results from the study of Li *et al*., around 57% of extracted molecules from AP peels are surfactants.[73] Furthermore several authors extracted fatty acids from seeds.[72,95] In the white powder extracted, it is possible that fatty acid and alcohol have been extracted as oil. However, the amount of seeds in our AP is small, only 0.2% of dry weight mass of components (Table C4-2).

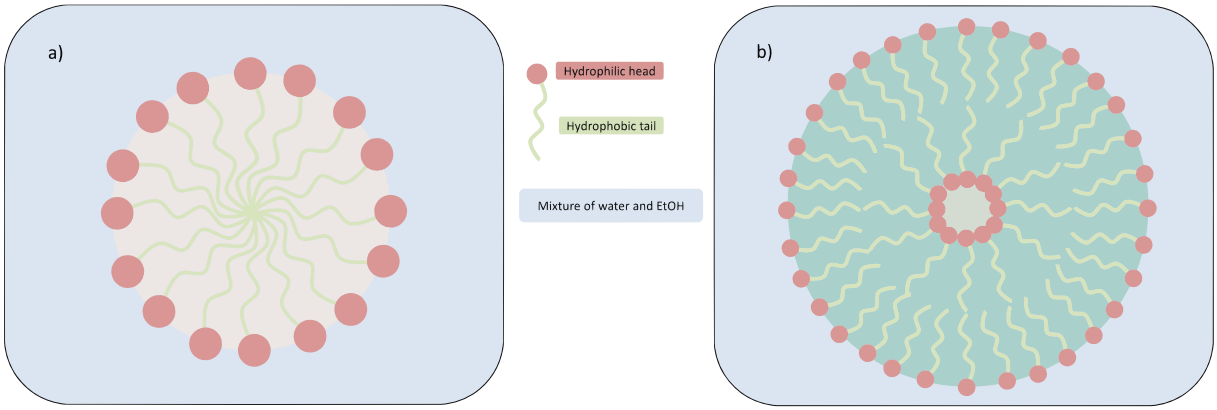


Figure C4-6. Schemes of a) micelle; b) liposome.

C.Antioxidant activity

Antioxidant activity was measured using DPPH Radical Scavenging Capacity (DRSC) and Total Polyphenols Content (TPC). These assays are commonly used to determine antioxidant activity of extracts. SC-CO_2 extracts are dissolved into DMSO (dimethyl sulfoxide). Biomolecules (polyphenols, wax and oil) are soluble into this solvent but have difficulty dissolving in organic solvents (*e.g.* acetonitrile or ethanol) at room temperature and ambient pressure. DMSO do not exhibit antioxidant activity in either assay.

According to Figure C4-7, the extracts *without wax* demonstrated higher antioxidant activity compared to *crude* extracts at 50 $mg_{Extracts}/mL_{DMSO}$. At this concentration, the extracts *without wax* are supposed to contain more polyphenols than *crude* ones. The variation between these two extracts is about $18\%_{inhibition}$. The DRSC results of both extracts can be explained by the contribution of wax. Wax exhibits a small impact on the antioxidant activity (DRSC) and the activity can be attributed mainly to polyphenols. For TPC assay, the difference between *crude* extract and extract *without wax* is low (< 2%) (Figure C4-2). Wax contribution cannot be

considered on the impact of TPC. Its exhibition is too low which is in accordance with the DRSC assay.[207,208] Wax exhibits minimal antioxidant properties according to both antioxidant tests.

As mentioned, several times in Chapter 3 and above, the purpose of US pretreatment was to enhance, both the mass yield and the biological activities of extracts from AP obtained by SC-CO_2 extraction. The DRSC ($\%_{inhibition}$) from *US pretreatment* extracts increased compared to *crude* extracts, respectively 45.78 ± 3.68 and 38.16 ± 3.63$\%_{inhibition}$ (Figure C4-7 and Table C4-5). The increase was about 16.6% between these two extracts. The *US pretreatment* extracts showed 51.68 ± 3.77 mg GAE/$g_{extract}$ for TPC assay. The TPC values of crude extracts was 46.30 ± 2.11 mg GAE/$g_{extract}$. The variation between these two extracts were 5.38 mg GAE/$g_{extract}$, representing an increase of 10.4% (Figure C4-8 and Table C4-5). The improvement of antioxidant activity can be caused the several factors induced by US pretreatment. During the pretreatment, the temperature was locally higher attributed to acoustic and cavitation streaming.[203] The increase of extraction temperature appears to be beneficial to the improvement of antioxidant activity.[196] Barba *et al*. observed that the extraction temperature can have a positive effect on extraction of polyphenols. For instance, at 20 °C with US (probe, 24 kHz into water), TPC from blackberries extracts was lower than at 50 °C. The increase was around 50% after 5 h.[196] On the contrary Egüés *et al*. determined that high temperature has positive and negative effects on antioxidant activity of polyphenols from AP using US (probe, 20 kHz). At high temperature (65 and 90 °C), the TPC was higher, but the DRSC was lower. The antioxidant activity was damaged with higher temperature. According to Egüés *et al*., the TPC could have been influenced by extracted sugars.[206] Folin-Ciocalteu reagent shows low selectivity and reacts with phenolic and non-phenolic compounds (reducing agent). For instance, sugars are non-phenolic reducing agent.[209] Another effect from US pretreatment is the break of wall cell of biomass. In a study of Liu *et al*., SEM images were used to compare treated by US and un-treated biomasses. According to these images, new porosity of treated seeds allows the SC-CO_2 to penetrate more easily, leading to an increase in antioxidant activity.[204] Furthermore, the US treatment reduced bond energy between molecules (*e.g.* polyphenols) and plant biomass. The US caused the desorption of molecules absorbed on the plant matrix attributed to the micro-agitation effect. In this case study, the improvement of antioxidant activity was correlated with the improvement of mass yield.

The increase of antioxidant activity was achieved according to our results. Table C4-5 summarized antioxidant activity.

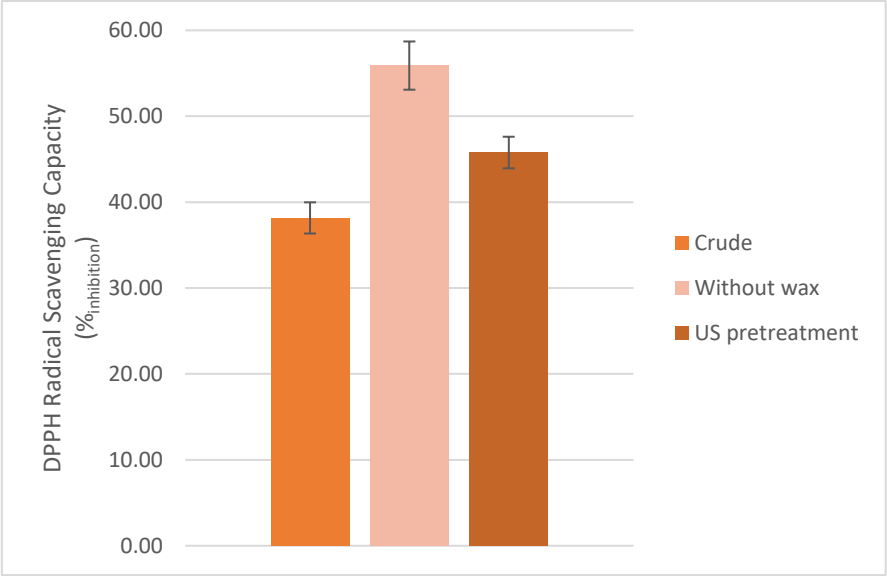


Figure C4-7. DPPH Radical Scavenging Capacity activity from apple pomace extracts from supercritical CO_2 at 50 $mg_{Extracts}/mL_{DMSO}$.

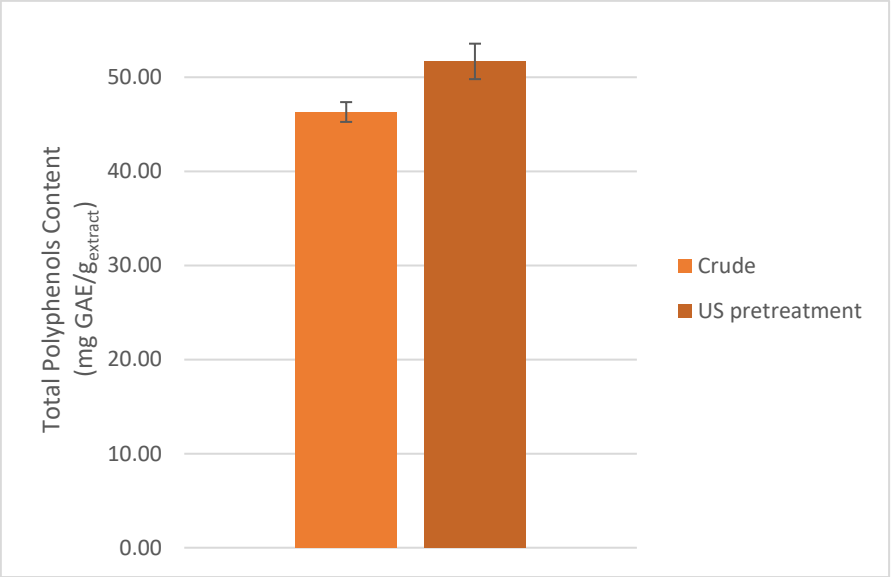


Figure C4-8. Total Polyphenols Content from apple pomace extracts from supercritical CO_2 extraction (mg $GAE/g_{extract}$)

Table C4-5. Summary of antioxidant activity of supercritical CO_2 extracts from apple pomace.

Samples	DRSC		TPC (mg GAE/$g_{extract}$)
	Trolox® eq. (µg TE/$mg_{extract}$)	$\%_{inhibition}$[a]	
Crude	1,036.87 ± 92.71	38.16 ± 3.63	46.30 ± 2.11
Without wax	1,489.72 ± 143.40	55.90 ± 5.62	48.16 ± 3.55
US pretreatment	1,231.29 ± 93.85	45.78 ± 3.68	51.68 ± 3.77

[a]with a concentration of 50 $mg_{Extracts}$/mL of apple pomace extracts

D.Antibacterial activity

Petri dish and microplate assays were used to evaluate the antibacterial activity of the extracts. Both tests indicated no antibacterial activity. As mentioned above, the biomolecules extracted do not dissolve in water, so DMSO was used as solvent. Water is the best solvent for antibacterial tests as it does not exhibit a solvent effect in the antibacterial activity tests. Ethanol can be a solution, but its solvating power is not strong enough for biomolecules from AP and this solvent exhibits a strong antibacterial activity. Several tests were done with diluted acetonitrile against strains on Petri dishes, but they were not successful. On a Petri dish, DMSO has no Diameter Inhibition Zone (DIZ), indicating no biological activity against *L. innocua*, *E. coli* and *B. cereus*. In the case of microplate, the DMSO exhibited an antibacterial activity against all the strains.

According to the previous discussion, the antibacterial assay chosen was the Petri dish method. SC-CO_2 extracts were placed on a Petri dish at a concentration of 100 $mg_{Extracts}/mL_{DMSO}$. For all the different extracts, none exhibited DIZ.

III.Subcritical water extraction

As a reminder of Chapter 3, the optimal conditions for SWE are 157 °C, 23.4 min and 1:8 (AP:Water, *w/v*) obtained by Minitab using AP particles at 500 nm and under pressure at 5 bar_{N2}. These conditions were determined based on the mass yield (%) as mentioned in Chapter 3 (III. C). To preserve the biological activities from bioactive molecules, the temperature was reduced to 140 °C. In Chapter 3, above 150 °C, the biomass turns black and emits a burnt smell. In the case of SWE, only the improvement of biological activities was desired. Furthermore,

the Design of Experiment (DoE) was validated to describe SWE of bioactive molecules from AP using Central Composite Rotatable Design (CCRD) model, achieving an R^2 value greater than 0.7.[81] However, the lack-of-fit (0.024 < 0.05) indicated that the chosen mathematical model is not adequate to predict the mass yield (%).[81] For this reason, the improvement of mass yield with SWE was not studied.

A.US pretreatment

US pretreatment was selected for this study, as it was for SC-CO_2 extraction. According to Grillo *et al*., using high power (500 W) was more efficient for anthocyanidin extraction than 100 W.[151] After 10 min of UAE using NADES as solvent, the Total Anthocyanidins Content (TAC) increased by 13% with a power of 500 W compared to 100 W. Additionally, after 40 min, the TAC increased by 30%. In our study, a US probe was used. Probes deliver US energy directly to the mix of water and biomass enhancing the intensity of cavitation (Table C4-3). This allows for better and faster extraction of biomolecules. The direct type of irradiation facilitates a deeper US penetration into the mixture of AP and water increasing the breaking of cell wall. The combination of direct irradiation and the high power used allow to extract more bioactive compounds. The pretreatment conditions were 10 min, 20 kHz and 500 W with the US pretreatment outlined in Figure C4-9. An ice bath was used to control the temperature, avoiding bioactive molecules degradation.

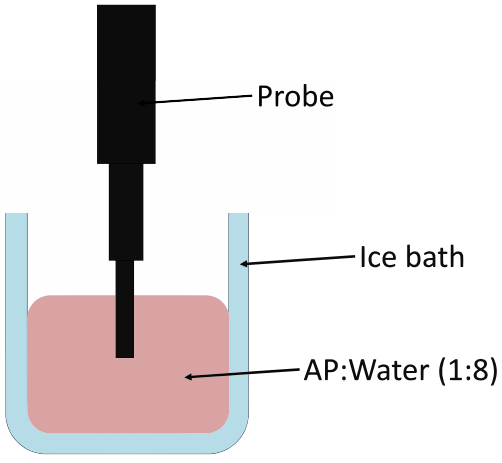


Figure C4-9. Scheme of US pretreatment system (1 L).

B.Characterization of Extracts

Samples were analyzed by HPLC-DAD, and 8 molecules were identified. The *in-situ* production of 5-HMF in the sample without pretreatment was observed. This can be explained by the fact that at high temperatures, the Maillard reaction occurs. This means that sugars (fructose, glucose) undergo a reduction, transforming into 5-HMF (Figure C3-2). In the literature, the thermal transformation of sugars into 5-HMF using SWE technology has been reported.[210] In our case, the temperature used was 140 °C, which explains the formation of 5-HMF in the sample without pretreatment. Moreover, the higher the temperature, the greater the amount of 5-HMF produced.

Between the two samples, the quantities of polyphenols are relatively similar. Regarding the amount of 5-HMF, an increase of about 31% was observed using US pretreatment. Pedreschi *et al*. reported that the use of US treatment increased the amount of 5-HMF produced from potato chips.[211] Sugars are highly soluble in water. This increase in 5HMF is explained by the selectivity of US treatment of certain conformations of glucose due to cavitation, which enhances interactions with water and thus increases their extraction.

Table C4-6. Phenolic composition of apple pomace.

Molecules	Without pretreatment (%)	US pretreatment (%)
5-HMF	1.04 ± 0.28	1.50 ± 0.14
Phloridzin	0.92 ± 0.65	0.85 ± 0.13
Chlorogenic acid	0.21 ± 0.13	0.20 ± 0.01
Catechin	Detected	Not Detected
Hyperoside	0.21 ± 0.16	0.17 ± 0.01
Isoquercetin	Detected	Not Detected
Avicularin	Detected	Not Detected
Quercitrin	0.72 ± 0.50	0.64 ± 0.08
Total Polyphenols (+ 5-HMF)	2.06 (3.1)	1.86 (3.36)

Results in% w/w of dry extract

C.Antioxidant activity

As mentioned several times in Chapter 3 and above, the purpose of US pretreatment is to enhance the biological activities of SWE extracts from AP. The TPC values of with and without US pretreatment were respectively 148.08 ± 7.25 and 110.33 ± 11.55 mg GAE/$g_{extract}$ resulting to an increase of 25.5% in the presence of US irradiation (Figure C4-10). According to the TPC results, there was a higher polyphenols extraction with US pretreatment. The variation

between these two extracts can be explained by the fact that US generate bubble cavitation leading to break cell walls from AP.[187,205,212,213] SEM images from *Siah-Sardasht* grape skin before and after SWE and US pretreatment revealed that the US pretreatment damaged the biomass as excepted but SWE even more.[213] Liu *et al*. observed similar results with US pretreatment.[187] These damages lead to the improvement of biomolecules extraction by increasing the solute/solvent interaction.[212]

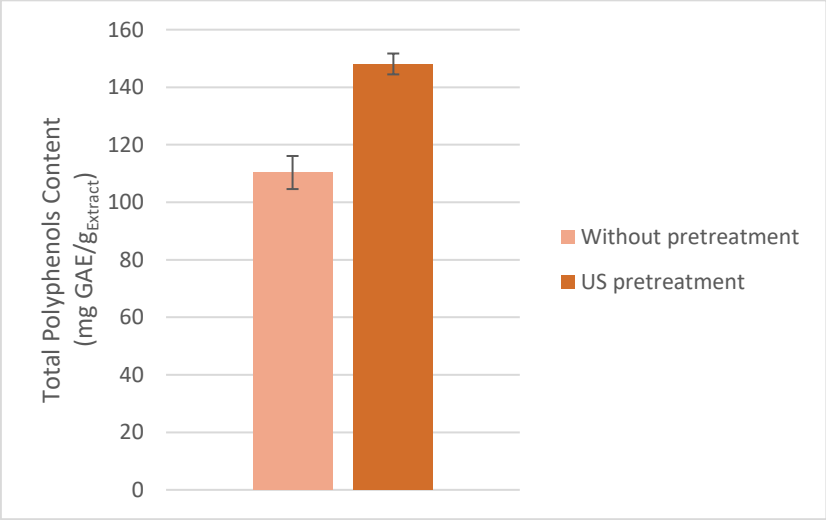


Figure C4-10. Total Polyphenols Content from apple pomace extracts from subcritical water extraction (mg GAE/$g_{extract}$).

The second method to measure antioxidant activity was using DPPH, described by the percentage of DPPH inhibited (%$_{inhibition}$) or by EC_{50} (µ$g_{Extract}$/mL_{MeOH}). As a reminder, the lower EC_{50}, the higher antioxidant activity of extracts. The EC_{50} values of different treatments were 6.6 ± 0.7 and 7.6 ± 1.8 µ$g_{Extracts}$/mL_{MeOH} for *without pretreatment* and *US pretreatment* extracts respectively (Table C4-7). According to Figure C4-11, the values of%$_{inhibition}$ of *without pretreatment* and *US pretreatment* extracts were 70.43 ± 9.69 and 60.68 ± 4.87%$_{inhibition}$ at the same concentration of extracts (15 µ$g_{Extract}$/mL_{MeOH}). The extract with the higher antioxidant activity was the *without pretreatment* extract. The variation between these two extracts decreased by 13.8%.

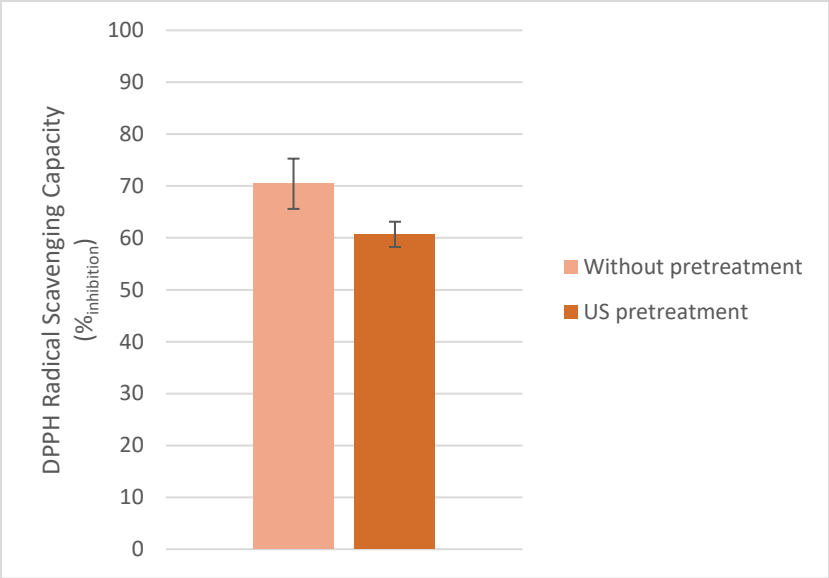


Figure C4-11. DPPH Radical Scavenging Capacity activity from apple pomace extracts from subcritical water at 15 $\mu g_{Extract}/mL_{MeOH}$.

According to Figures C4-10 and C4-11, *US pretreatment* extract contained more polyphenols, but its antioxidant activity was lower than *without pretreatment* extract. Usually, TPC and DRSC are correlated (Table C4-5). When the TPC value is high, the DRSC value is also high. Several factors can lead to this kind of difference. Egüés *et al.* observed similar results, high TPC but low DRSC.[206] For instance, they measured for the TPC and DRSC values respectively 3.54 ± 0.33 $mgGAE/g_{Dry\ Weight}$ and 1.11 ± 0.14 mg Trolox® Equivalent (TE)/$g_{Dry\ Weight}$ at 70%, 15 min and 20 kHz (probe). For the same condition at the exception of 10 min instead of 15 min, the TPC and the DRSC values were respectively 3.00 ± 0.30 mg GAE/$g_{Dry\ Weight}$ and 1.29 mg TE/$g_{Dry\ Weight}$. The explanation for the variation was that the polysaccharides are accounted for the TPC, increasing the TPC values.[206] In our case study, sugars were supposed to be remove by the use of resin. Furthermore, US pretreatment generated locally high pressure and temperature leading to the degradation of bioactive molecules.[187,205] US created degradation reaction such as oxidation, polymerization, non-enzymatic browning.[205] These explanations can fully account the contradiction from the results from our case study. For the without pretreatment extract, the total sugar content was 10.3 ± 2.4 mg Glucose Equivalent (GE)/$mg_{Extract}$.

According to Table C4-6, the total amount of polyphenols and 5-HMF is lower in *without pretreatment* extract than in *US pretreated* extract (respectively 3.1 and 3.6% w/w of dry extract). This is consistent with the TPC values. In the case of the TPC, the *US pretreated* extract contains more compounds compared to the *without pretreatment* extract (respectively 148.08

± 7.25 and 110.33 ± 11.5 mg GAE/$g_{extract}$). According to Chen *et al*., 5-HMF is accounted for in the TPC test starting at a concentration of 2.5 mg/mL, with a value of 3.97 ± 0.08 mg GAE/g.[214] In our samples, the amount of 5-HMF is below the minimum concentration found by Chen *et al*. However, it is possible that monosaccharides remain in our samples despite the post-extraction step (resin), which may influence the TPC values.

Regarding the DRSC test, the *without pretreatment* extract has a higher antioxidant capacity. In literature, several authors reported that 5-HMF possesses antioxidant activity.[214–217] However, the difference between *without* and *US pretreatments* extracts can be explained by the fact that 5-HMF has a lower antioxidant capacity than polyphenols. Indeed, if only the quantity of polyphenols was considered, the *without pretreatment* extract contains more polyphenols (2.06% w/w of dry extract, excluding detected but unquantified polyphenols) compared to the *US pretreatment* extract (1.86% w/w of dry extract). According to Tables C4-6 and C4-7, the values obtained for the DRSC are consistent with the quantification.

The increase in antioxidant activity was not achieved according to our results. Table C4-7 summarized antioxidant activity from AP extracts with SWE.

Table C4-7. Summary of antioxidant activity of subcritical water extracts from apple pomace.

Samples	DRSC		TPC (mg GAE/$g_{extract}$)	TSC (mg GluE/$mg_{extract}$)
	EC_{50} ($\mu g_{Extract}/mL_{MeOH}$)	$\%_{inhibition}$[a]		
Without pretreatment	6.6 ± 0.7	70.43 ± 9.69	110.33 ± 11.5	10.3 ± 2.4
US pretreatment	7.6 ± 1.8	60.68 ± 4.87	148.08 ± 7.25	-

[a]with a concentration of 15 $\mu g_{Extracts}$/mL of apple pomace extracts

D.Antibacterial activity

The antibacterial activity of the extracts is evident from the EC_{50} and Minimum Inhibitory Concentration (MIC) values presented in Table C4-8. All extracts display antibacterial activity against *E. coli*, *L. innocua*, and *B. cereus*. The EC_{50} for antibacterial properties means that to kill or inhibit the growth 50% of bacteria, a smaller quantity is needed (lower concentration is more effective).

According to Table C4-8, the MIC of all the SWE extracts from AP are 50 $mg_{Extracts}/mL_{Water}$. When the EC_{50} is calculated, variations are observed, and extracts are discriminated against each other. Previous studies have indicated that gram-positive bacteria (*e.g. L. innocua* and *B. cereus*) are generally more sensitive compared to gram-negative bacteria (*e.g. E. coli*) due to differences in cell wall structure. Gram-negative bacteria possess an additional lipopolysaccharide membrane, providing an extra layer of protection to the cell wall.[134,137] Our results are consistent with the literature. Extracts exhibit lower EC_{50} against *L. innocua* and *B. cereus*. For instance, the value of EC_{50} from *without pretreatment* extracts against *E. coli* is 22.0 ± 1.2 $mg_{Extracts}/mL_{Water}$ compared to the same extracts with EC_{50} values of 15.9 ± 3.1 and 11.3 ± 4.7 $mg_{Extracts}/mL_{Water}$ against respectively *L. innocua* and *B. cereus*. For the same inhibition (50%), higher concentration of extracts is needed to be effective against *E. coli*.

Table C4-8. EC_{50} and minimum inhibitory concentration of subcritical water extracts from apple pomace against 3 strains.

Samples	*Listeria innocua*		*Escherichia coli*		*Bacillus cereus*	
	EC_{50} (mg_{Sample}/mL)	MIC (mg_{Sample}/mL)	EC_{50} (mg_{Sample}/mL)	MIC (mg_{Sample}/mL)	EC_{50} (mg_{Sample}/mL)	MIC (mg_{Sample}/mL)
Without pretreatment	15.9 ± 3.1	50	22.0 ± 1.2	50	11.3 ± 4.7	50
US pretreatment	11.8 ± 2.3	50	19.3 ± 4.3	50	14.6 ± 2.6	50

EC_{50} in $mg_{Extract}/mL_{Water}$; Ampicillin 25 mg/mL > 90% of inhibition

Another way to present the inhibition from extracts against bacteria is the percentage of inhibition at a fixed concentration. In this case, the chosen concentration is 50 $mg_{Extracts}/mL_{Water}$ corresponding to the MIC. According to Figure C4-11, the values of $\%_{inhibition}$ of *without pretreatment* and *US pretreatment* extracts are respectively 82.15 ± 7.51 and 86.54 ± 2.33$\%_{inhibition}$ against *L. innocua*, 74.69 ± 7.41 and 79.25 ± 3.74$\%_{inhibition}$ against *E. coli*, and 76.63 ± 3.36 and 78.39 ± 6.88$\%_{inhibition}$ against *B. cereus* at 50 $mg_{Extracts}/mL_{Water}$. The extract with the higher antibacterial activity is the *US pretreatment* extract. In Figure C4-12, *US pretreatment*

extract exhibits the highest antibacterial activity against all the studied strains. The variation between these two extracts increase between 2 and 6% depending on the strain. According to Figure C4-10, *US pretreatment* extract contains more polyphenols. Antibacterial activity seems to be correlated with the TPC values. As noted earlier, 5-HMF seems to be influenced by TPC values. The antibacterial study suggests that 5-HMF may possess antibacterial properties. According to Sánchez-Hernández *et al*., they reported antimicrobial activities of 5-HMF against *Erwinia amylovora* and *Erwinia vitivora*.[218]Moreover, a correlation was observed between antibacterial activities, TPC values, and 5-HMF content. 5-HMF may induce stronger antibacterial activity compared to polyphenols against all strains studied.

The increase of antibacterial activity was achieved according to our results. Table C4-8 summarized antibacterial activity from AP extracts with SWE.

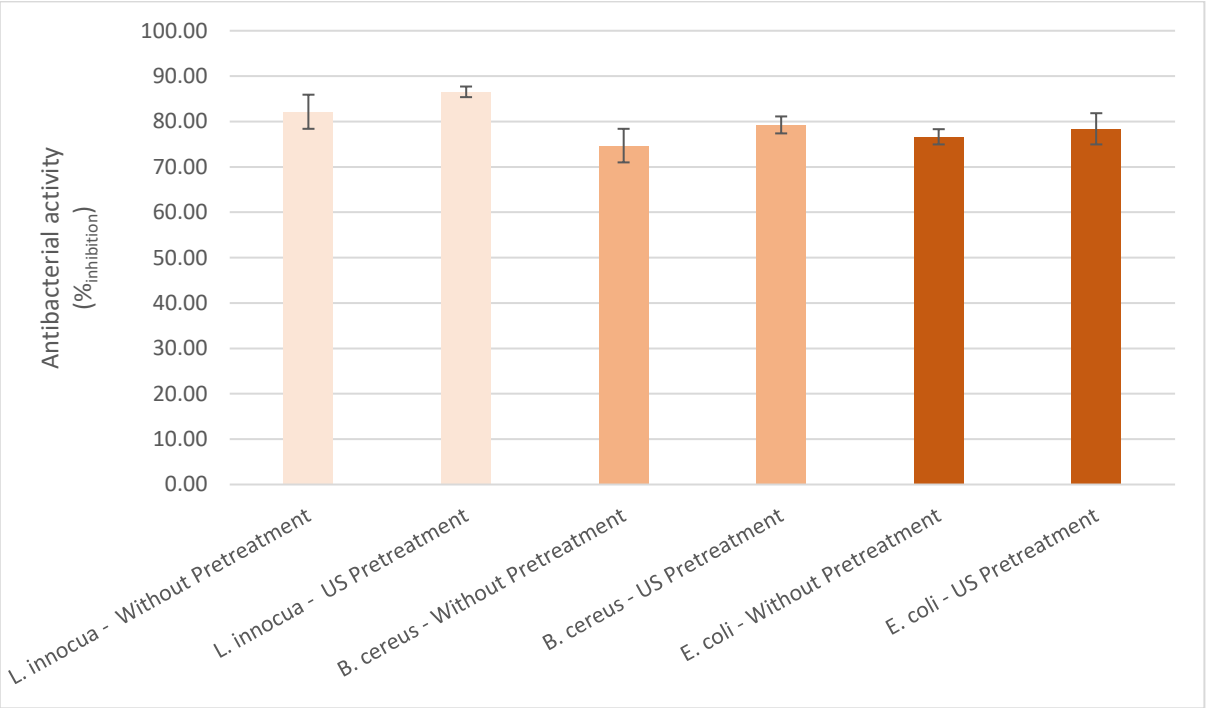


Figure C4-12. Antibacterial activity from apple pomace extracts from subcritical water at 50 $mg_{Extracts}/mL_{Water}$.

IV.Conclusion

As highlighted in the introduction, the objectives of this chapter are the biological activities of bioactive molecules and their improvement. Bioactive molecules from apples or AP are polyphenols, organic acids, triterpenes, sterols, carbohydrates *etc.*[179] Biomolecules from AP

such as phenolic compounds exhibit biological activities including antioxidant, antidiabetic, antitumor and antimicrobial properties (Chapter 1, C1-Table C4-2).[15,48,51,55] For both extraction methods, supercritical CO_2 and subcritical water, US pretreatment has been chosen based on the expertise of the laboratories (*EDYTEM* and *DSTF*) and existing literature.[187,190–196,200,205,206,212,213] According to the literature, there is no article about the US pretreatment for the extraction of AP polyphenols and their increase of biological activities using SFE.

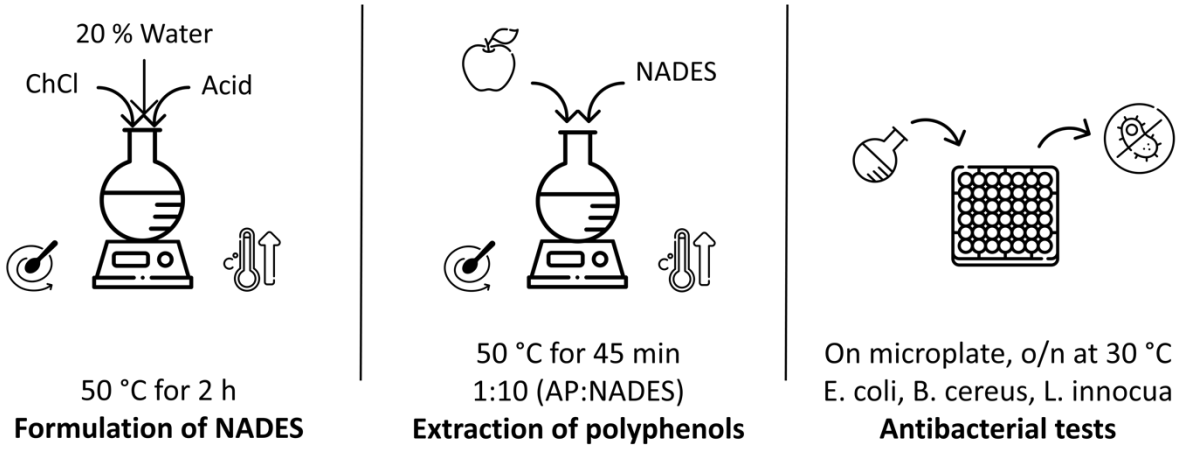
For SC-CO_2 extraction, bioactive molecules containing polyphenols, wax and oil were extracted. According to Figure C4-2, US pretreatment increased the mass yield, which was one of the objectives discussed in this chapter. The morphology of biomass treated by US changed due to the cavitation bubbles, creating new porosity that allowed the SC-CO_2 to penetrate easier into the biomass, improving mass yield. Liu *et al*. observed similar results regarding the improvement of mass yield with US pretreatment.[204] The second improvement was the antioxidant activity increase of extracts. Antioxidant activity was measured using DPPH Radical Scavenging Capacity (DRSC) and Total Polyphenols Content (TPC). The DRSC inhibition of *crude* extract was lower than *US pretreatment* extract with an increase of 16.6%. Similarly, TPC values from *US pretreatment* extract were higher than *crude* extracts, showing a 10.4% increase. The improvement in antioxidant activity of US pretreatment extracts was attributed to the new morphology of AP as mentioned above, which improved solutes/solvent interaction. The morphology of treated biomass with US was more irregular and disorganized surfaces with cracks. Additionally, the US treatment reduce bond energy between molecules (*e.g.* polyphenols) and plant biomass leading to a better biomolecule extraction and thus higher TPC. The purpose of using US pretreatment was effectively achieved for the antioxidant activity (TPC and DRSC) and mass yield.

For SWE, only the improvement of biological activities was assessed. As with SC-CO_2 extract, antioxidant activity was measured by TPC and DRSC. For TPC assay, values for *US pretreatment* extract was 25.5% higher than *without pretreatment* extract (Figure C4-10). At 15 $\mu g_{Extracts}/mL_{MeOH}$, the DRSC inhibition was 13.8% lower for *US pretreatment* extract compared to *without pretreatment* extract (Figure C4-10). Usually, TPC and DRSC are correlated. In this study case, the TPC could be overestimated due to the polysaccharides in the extracts. Nevertheless, sugars should have been removed using resin as post-extraction step. US treatments generate locally high pressure and temperature leading to the degradation of bioactive molecules and thus reducing antioxidant activity. According to Figure C4-10, *US*

pretreatment extract exhibited higher antibacterial activity on all the studied strains compared to *without pretreatment* extract. The purpose of using US pretreatment was effectively achieved for TPC and antibacterial activity. Only DRSC for antioxidant activity was not successful. For prospect experiments, other types of pretreatments or eco-solvents can be tested such as enzymatic pretreatment and Natural Deep Eutectic Solvents. Furthermore, it is necessary to test another pretreatment to compare with US pretreatment especially for SWE process and its antioxidant activity.

Chapter 5

Extraction of Polyphenols and Anthocyanins from Apple Pomace with Natural Deep Eutectic Solvents: Evaluation of their Antioxidant and Antibacterial Activities



Chapter 5 is another use of green solvent, Natural Deep Eutectic Solvents. This chapter has been submitted in *Journal of Food Science and Technology* (Springer) in 2024.

The aim of this study was to evaluate the antioxidant and antibacterial properties of apple pomace (AP) extracts using Natural Deep Eutectic Solvents (NADES). Six NADES, based on choline chloride (ChCl), were used as environmentally friendly solvents. Four of these were combined with organic acids while the other two were combined with urea; they were then used to extract bioactive compounds form AP. ChCl:urea mixture proved to be the solvent with the highest Total Polyphenols Content (TPC), with 13.15 ± 4.70 mg gallic acid equivalent/mL. Antioxidant activity and Total Anthocyanidins Content (TAC) were also assessed. ChCl:oxalic acid recorded the highest values with 35.59 ± 9.53 mg extract/mL and 64.81 ± 4.65 malvidin-3-glucose equivalent µg/mL respectively. Solvent pH plays a crucial role in selective extraction; an acidic pH facilitates selective anthocyanidins extraction, while a basic pH does not. Anthocyanidin extraction correlated with extract antioxidant activity and solvent viscosity. In addition, the antibacterial activity of the extracts against *Bacillus cereus*, *Listeria innocua* and *Escherichia coli* strains was studied. All extracts showed antibacterial properties against the strains tested. The ChCl:oxalic acid extracts showed particularly low Minimum Inhibitory Concentrations (*e.g. 25* $mg_{extract}$/mL for *B. cereus*) and EC_{50} values (*e.g.* 6.0 ± 0.3 $mg_{extract}$/mL for *B. cereus*).

I.Introduction

With a global production of 95.8 million tonnes in 2022, apple is the fourth most produced and one of the most consumed fruits in the world.[18,19] According to Kammerer *et al*., around 25 – 30% of apples are processed into value-added products such as jams, pies and so on. Of these processed products, 65% are juices and cider, corresponding from 16.3 to 19.5% of total apple production processed into apple beverages. This estimate corresponds to a range of 15.6 to 18.7 million tonnes. However, apple juice and cider production generate a significant amount of apple pomace (AP) as by-product. Based on 2023 apple production, the amount of AP has been estimated at between 4.7 and 5.6 million tonnes. At present, AP is still under-exploited. In many countries, AP, like many other plant waste, is disposed of in soil, posing health and environmental problems due to its high moisture content, which encourages bacterial decomposition.[9] In the European Union, biomass waste is mainly converted to methane anaerobic digestion, but this process does not allow phytochemicals to be recovered. These compounds are interesting molecules to extract because of their diverse biological activities, including antioxidant, antimicrobial, anticancer, and antidiabetic properties.[15,48,51,55] AP contains several classes of polyphenols, including dihydrochalcone (Phlorizin), flavonol (quercetin and its derivatives), hydroxycinnamic acid (chlorogenic acid), proanthocyanidin (procyanidin B2), and anthocyanin (ideain chloride).[28,153,219]

Phytochemicals are sensitive compounds, and their extraction and preservation methods can have a significant impact their biological activities, due to degradation of the molecules.[16,153] Exposure to air, light, solvents and temperature variations can adversely affect extract quality. For instance, Ferrentino *et al.* showed that the Total Polyphenol Content (TPC; indicator of antioxidant activity) of Soxhlet extracts was lower than that of Supercritical CO_2 (SC-CO_2) extracts, measuring 4.13 ± 0.90 mg gallic acid equivalent (GAE)/g of extract and 8.87 ± 0.17 mg GAE/g of extract, respectively.[92]

The differences in polyphenol content between the two extraction methods can be attributed to the temperature and exposure to light and air (O_2) during the extraction steps. In the case of SC-CO_2 extraction, the biomass was processed at lower temperatures in the absence of light and air, compared with Soxhlet extraction, where the temperature was high, and compounds exposed to air (O_2). Lavelli and Corti monitored the antioxidant activity of AP extracts over a nine-month period under varying humidity conditions (2.4 – 4.2%). At the start of the study,

total phenolic content was 5,176 mg/kg of dry AP, decreasing to 1,704 to 4,429 mg/mg dry AP at the end of the study, depending on the moisture content.[100]

Researchers continue to explore new solvents for the extraction of phytochemicals in order to eliminate the use of volatile organic solvents and preserve polyphenols during extraction processes. Traditional solvents have a number of drawbacks, including high toxicity, non-biodegradability, high cost and a significant tendency to accumulate in air, water, and soil.[127] In recent decades, subcritical and supercritical fluids have been studied for environmentally-friendly extractions. The influence of physical parameters such as pressure and temperature has been studied. A well-known process, the DIAMANT® process, is used to remove trichloroanisole, the molecule responsible for cork taint in wine.[158] However, the use of subcritical and supercritical fluids requires substantial expenses for acquisition and maintenance of the devices.[111]

Ionic liquids, Deep Eutectic Solvents (DES) and bio-based solvents are being explored as alternatives to conventional solvents such as ethanol and hexane due to their ease of use. Natural Deep Eutectic Solvents (NADES) made from compounds of natural origin are a promising class of solvents. NADES offer advantages such as affordability, renewability, low toxicity, biodegradability, extraction selectivity and low vapor pressure. However, challenges remain, notably with regard to their higher viscosity than the majority of traditional solvents, and the post-extraction separation of phytochemicals.[111] NADES are made up of a Hydrogen Bond Acceptor (HBA) compound and a Hydrogen Bond Donor (HBD) compound, both of biological origin. In particular, the melting point of NADES is lower than that of HBA and HBD compounds of which they are composed.[140] For example, NADES ChCl:urea (1:2) has a melting point of 12 °C, while Choline Chloride (ChCl) melts at 302 °C and urea at around 134 °C.[129] Depending on the specific formulation of NADES, its use can enable selective extraction of family compounds and minimizes the need for additional reaction steps such as purification. In previous research, Yu et Bulone successfully extracted and de-glycosylated quercetin derivatives from AP in a one-pot reaction using organic acid-based DES.[141] In this study, ChCl was chosen as the HBA compound due to its well-documented efficiency for polyphenol extraction.[220,221] Organic acids are among the most polar compounds, followed by amino acids, sugars, and polyalcohols. Existing literature suggests that DES based on organic are generally the most efficient solvents for extracting bioactive compounds.[127,133,142,220,221] Urea-based solvents were employed as reference. These solvents are usually used in scientific research for

plant extraction, gas capture, electrodeposition, battery technology, hydrogen production with enzyme and so on.[162,222] The aim of the work described in this article was preserve the bioactivity of molecules extracted from AP *via* eco-friendly solvents, NADES. Several NADES were tested for the extraction of compounds of interest, in particular for anthocyanins. In this study, the antioxidant (DPPH Radical Scavenging Capacity and TPC) and antibacterial (on *E. coli*, *L. innocua* and *B. cereus*) properties were investigated. To our knowledge, this is the first scientific paper on the antibacterial activity of AP extracts using NADES.

II. Physico-chemical characterizations

A. Viscosity effect

Due to the high viscosity of some NADES at room temperature (RT) even with the addition of water (20%) to reduce viscosity, measurements were carried out at 50 °C. As shown in Table C5-1, ChCl:CA had the highest viscosity (162.6 ± 21.2 mPa.s), while ChCl:U:W and ChCl:LA were the least viscous (9.32 ± 0.13 mPa.s and 13.64 ± 1.05 mPa.s, respectively). The characterization temperature chosen was identical to the extraction temperature, to reduce the NADES viscosity. In agreement with Oomen *et al.*, viscosity was controlled by adding water. However, under these experimental conditions, the addition of water was not sufficient, and an increase in temperature was necessary.[223] The results obtained in this study are in line with literature. Notably, the addition of 20% water to ChCl:U reduced its viscosity by 91.1%, resulting in a final viscosity of 9.32 ± 0.13 mPa.s for ChCl:U:W.

Equation C5-1. Viscosity of liquids.

$$\mu = Ae^{\frac{b}{T}}$$

where μ is the viscosity (mPa.s), *A* and *b* are constants and *T* is the absolute temperature (K).[224]

The viscosity of ChCl:OA is comparable to that of ChCl:MA (33.49 ± 0.18 mPa.s and 49.14 ± 15.14 mPa.s, respectively). However, it should be noted that ChCl:OA solidifies at RT (< 18 °C), whereas ChCl:MA remains liquid. The high viscosity of ChCl:CA is attributed to intermolecular and Van der Waals bonds between NADES molecules and ions.[225,226] According to Oomen *et*

al., water content significantly influences NADES extraction capacity, with optimal extraction occurring at concentrations below 30%, as higher concentrations lead to a decrease in hydrogen bond availability.[142,223] Nevertheless, extraction efficiency remains relatively stable when water content is kept below 30%.

B.Density effect

Density measurements were carried out at RT (25 °C). As with viscosity, the NADES exhibit variable densities, with the highest density being that of ChCl:CA (1.28438 ± 0.00294) and the lowest that of ChCl:LA (1.13269 ± 0.00832). Notably, ChCl:OA and ChCl:MA show comparable densities (1.22439 ± 0.00085 and 1.23429 ± 0.00201, respectively). NADES density can be influenced by a variety of factors, including the nature of the HBD and the molar ratio between HBD and HBA.[227] The addition of water can also significantly alter NADES density. In this study, although ChCl:U and ChCl:U:W have the same molar ratio of HBD and HBA, their densities vary, with ChCl:U showing a density of 1.19219 ± 0.00201 and ChCl:U:W a density of 1.16191 ± 0.00025. This difference underlines the influence of compositional variations on NADES density, and the importance of understanding and controlling these factors in NADES synthesis and utilization.

C.pH effect

The pH measurements were carried out at RT. Attempts to measure the solvent pH directly were unsuccessful, probably due to factors such as high viscosity, low freezing point, or solvent acidity.[225] NADES solutions were then diluted to a concentration of 1 g_{NADES}/mL_{Water} for pH measurement. The pH values obtained ranged from 0.35 to 9.43. Although these values do not accurately reflect the pH at the time of extraction, they do provide valuable information. pH is temperature-dependent, decreasing as temperature increases (Equation 2).[226] For instance, pH values reported by Skulcova *et al.* ranged from 1.2 and 2.74 at 23 °C, but decreased to 0.05 and 2.09 when the temperature was raised to 60 °C.

Equation C5-2. pH of solution as a function of temperature.

$$pH = a + b \times T$$

where *a* and *b* are constants, *T* is temperature (°C).

The pH measurements obtained in this study align with existing literature.[134] However, pH can vary depending on factors such as composition and temperature, highlighting the need to consider and control these parameters when using NADES.[226] Table C5-1 provides values of the viscosity, density, and pH characteristics of the NADES studied. The viscosity was measured under the experimental temperature conditions used due to the high viscosity at 25 °C. However, it is offering a comprehensive overview of their properties.

Table C5-1. Characterization of viscosity, density, and pH of studied NADES.

NADES	Dynamic viscosity (mPa.s)[a]	Density[b]	pH of NADES solution[c]
ChCl:CA	162.6 ± 21.2	1.28438 ± 0.00294	1.10
ChCl:LA	13.64 ± 1.05	1.13269 ± 0.00832	1.70
ChCl:OA	33.46 ± 0.18	1.22439 ± 0.00085	0.35
ChCl:MA	49.14 ± 15.14	1.23429 ± 0.00449	1.26
ChCl:U	104.3 ± 7.8	1.19219 ± 0.00201	8.60
ChCl:U:W	9.32 ± 0.13	1.16191 ± 0.00025	9.43
Water	1.002[d]	1.00000[d]	6.34

[a]Dynamic viscosity was measured at 50 °C. [b]Density was measured at 25 °C. [c]Measurements of solution of diluted NADES (1 g_{NADES}/mL_{Water}) at RT. [d]Data from literature.

III. Biological Characterizations

A. Antioxidant activity

The results showed that the Total Polyphenols Content (TPC) values are consistent with existing literature, confirming the robustness and reliability of our experimental approach (Table C5-2).[133,221] The range of values provided by the literature is between 1 and 9.5 GAE mg/g extract.[221] The observed variation in TPC values between different NADES with extracts, ranging from 2.04 ± 0.19 to 13.15 ± 4.70 GAE mg/g extract, highlights the influence of pure

NADES composition on phenolic compound extraction efficiency. Notably, ChCl:U with extracts showed the highest TPC, while ChCl:MA with extracts showed the lowest. The variation in TPC values between literature and our case study could be attributed to several factors, including variations in biomass composition (due to climatic environment and variety) and differences in the extraction process.[21,111] In addition, the impact of pH on the stability of phenolic compounds cannot be overlooked, except for anthocyanins. The pigments are degraded when the pH is over 7. Under our conditions, the pH is low thus anthocyanins are stable. Previous studies, such as the work by Ruesgas-Ramón *et al*., have highlighted the potential for phenolic compound degradation under low pH conditions.[127] This could potentially explain the higher TPC observed in ChCl:U with extracts, given its relatively higher pH values compared to pure acid-based NADES, which ranged from 0.35 to 1.70. In addition, the polarity index of the solvent, as pointed out by Deniz *et al*., has an influence on the extraction of phenolic compounds. Urea, with its relatively high polarity index of 89.63 kJ/mol compared to oxalic acid (the most efficient HBD compound of one of the NADES studied) of 78.59 kJ/mol, may contribute to the higher TPC observed in ChCl:U extracts.[133] Despite the high viscosity of pure ChCl:U (104.3 ± 7.8 mPa.s), its superior extraction capacity, as reflected in the TPC results, highlights the influence of polarity index on phenolic compound extraction. This suggests that factors other than viscosity, such as solvent polarity, play a crucial role in determining extraction efficiency. Golmohamadi *et al*. extracted high-value added molecules from red raspberries puree using ultrasound-assisted extraction.[200] They observed that anthocyanidins had minimal impact on TPC results. This explains why TPC values for were highest for ChCl:U extracts but antioxidant test values were lowest.

EC_{50} values obtained from the DPPH Radical Scavenging Capacity (DRSC) antioxidant assay range from 35.59 to 425.28 mg extract/mL (Table C5-2). As a reminder, EC_{50} represents the half effective concentration required to decrease the initial concentration of DPPH to 50% at equilibrium. Lower EC_{50} values indicate higher biological activity of the sample tested. According to Table C5-2, the ChCl:OA extract is the most effective extract in terms of antioxidant activity. However, it should be noted that NADES solvents are primarily responsible for antioxidant activity.[228] EC_{50} values (ChCl:OA) for extracts and pure NADES are 35.59 ± 9.53 and 21.55 ± 1.40 $mg_{Samples}$/mL respectively. Notably, all acid-based solvents show antioxidant activity, while urea-based solvents do not, which is consistent with the existing literature.[133–135] Radosevic *et al.* have previously suggested that ChCl and urea lack antioxidant properties,

which was confirmed by the results of the Oxygen Radical Absorbance Capacity test (antioxidant test) and corroborated by results of our DRSC assay.[134]

Interestingly, both urea-based NADES extracts demonstrated notable EC_{50} values, with ChCl:U and ChCl:U:W extracts reaching 176.71 ± 16.82 and 111.91 ± 25.06 $mg_{Extracts}$/mL respectively. The difference between these two samples is the addition of 20% water, which results in a decrease in viscosity (from 104.3 ± 7.80 to 9.32 ± 0.13 mPa.s).[221] In addition, pure NADES do not exhibit antioxidant activity. This reduction in viscosity improves diffusion rates and mass transfer, which may enhance antioxidant activity. Extracted molecules in ChCl:LA and ChCl:MA solvents show similar EC_{50} values, 308.06 ± 56.38 and 268.09 ± 26.35 $mg_{Extracts}$/mL respectively. However, the EC_{50} values of their solvents vary significantly; the ChCl:MA solvent shows a higher antioxidant property (656.92 ± 72.82 $mg_{Solvent}$/mL) than the ChCl:LA solvent (1,930.44 ± 140.29 $mg_{Solvent}$/mL). This variation suggests that the extracts in ChCl:LA may possess higher antioxidant properties than the ChCl:MA with extracts. In contrast, the extracts in ChCl:CA showed lower antioxidant activity (425.28 ± 51.22 $mg_{Extracts}$/mL), mainly due to its solvent (441.34 ± 25.37 $mg_{Solvent}$/mL). As previously mentioned, viscosity plays a crucial role during extraction and, consequently for antioxidant activity. The EC_{50} values appear to correlate with the pH of pure acid-based NADES, except for ChCl:CA solvents, for which lower pH values correspond to lower EC_{50} values, indicating better antioxidant activity. Overall, the order of antioxidant activity of the NADES observed is as follows: ChCl:OA > ChCl:CA > ChCl:MA > ChCl:LA (Table C5-2), with corresponding pH values of the solvent: ChCl:OA < ChCl:MA < ChCl:LA < ChCl:CA (Table C5-1). The exception of ChCl:CA may be attributed to the high viscosity of this solvent.

Using Amberlyte XAD-16 (resin) to remove solvents, we were able to measure the EC_{50} of extracts, even in the presence of residual solvent (Appendix – Table A-2). Remarkably, after partial purification of extracts, the solvent had no noticeable effect on antioxidant activity. These results suggest that the antioxidant activity observed is mainly attributed to the bioactive compounds extracted from the AP rather than the solvent itself.

Table C5-2. Characterizations of antioxidant activities of NADES containing an extract and pure NADES.

Sample	DRSC EC_{50} (mg_{Sample}/mL)		TPC (GAE mg/g of extract)	
	With extract	Pure	With extracts	Pure
ChCl:CA	425.28 ± 51.22	441.34 ± 25.37	4.09 ± 0.87	2.00 ± 0.56
ChCl:OA	35.59 ± 9.53	21.55 ± 1.40	3.05 ± 0.53	2.33 ± 0.16
ChCl:MA	268.09 ± 26.35	656.92 ± 72.85	2.04 ± 0.19	1.20 ± 0.11
ChCl:LA	308.06 ± 56.38	1,930.44 ± 140.29	5.30 ± 1.40	1.67 ± 0.47
ChCl:U	176.71 ± 16.82	-[a]	13.15 ± 4.70	1.36 ± 0.34
ChCl:U:W	111.91 ± 25.06	-[a]	2.98 ± 0.72	1.32 ± 0.13

[a]Antioxidant effects were not observed.

B.Total Anthocyanins Content Determination

The Total Anthocyanins Content (TAC) of the solvents was measured to observe their impact on the TAC (Figure C5-1). However, no significant responses were observed. It should be noted that acid-based NADES are well-known for their ability to extract anthocyanins.[139,140,221] Among them, ChCl:OA extracts exhibited the highest TAC, with a value of 64.81 ± 4.65 malvidin-3-glucoside equivalent (M3GE) µg/mL. Interestingly, ChCl:LA and ChCl:U:W extracts gave similar results, with TAC values of 33.46 ± 3.87 and 31.41 ± 11.13 M3GE µg/mL, respectively. Similarly, ChCl:MA extracts and ChCl:U extracts produced comparable results, with TAC values of 18.89 ± 5.56 and 16.63 ± 2.44 M3GE µg/mL, respectively. Notably, ChCl:CA extracts presented the lowest TAC, with a value of 9.68 ± 2.23 M3GE µg/mL.

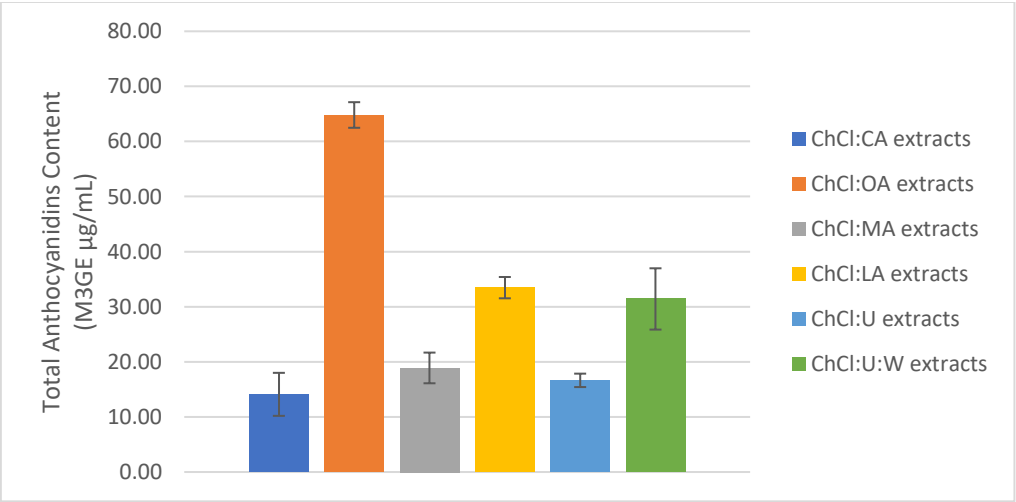


Figure C5-1. Total Anthocyanidins Content (TAC) of extracts in malvidin-3-glucoside equivalent µg/mL.

Pure ChCl:CA appears to be the the NADES with the highest viscosity (162.6 ± 21.2 mPa.s) and the highest EC_{50}, and the lowest TAC value from NADES with extracts. This observation points to the limitation of high-viscosity NADES, which poses a significant problem for anthocyanidins extraction of by reducing mass transfer.[221] Moreover, the high viscosity of pure NADES may dissuade manufacturers from using them.[111,221] High viscosity results to use more power to stir for instance.

The results show a clear correlation between TAC and the EC_{50} of extracts, except for ChCl:U extracts (Table C5-3). The relatively low TAC of ChCl:U extracts could be attributed to the absence of additional water and the high viscosity (104.3 ± 7.8 mPa.s) of the pure NADES. Similarly, ChCl:CA extracts showed poor anthocyanidins extraction due to the highly viscous nature of the solvent, which is in accordance with the literature.[221] Furthermore, pure ChCl:U is not commonly used for anthocyanidin extraction compared to acid-based NADES, as anthocyanidins are more easily extracted with low pH solvents.[139,140,221] The pure ChCl:U seems to be more suited for extracting polyphenol (such as Phlorizin, quercetin, *etc*.), which explains a higher TPC despite its high viscosity and low TAC from its extracts. The variation in TAC between the ChCl:U extract (16.63 ± 2.44 M3GE µg/mL) and the ChCl:U:W extract (31.41 ± 11.13 M3GE µg/mL) is explained by the addition of water, anthocyanins being water-soluble molecules.[151]

The color of the extracts also provides information (Figure C5-2). Extracts in acid-based NADES, particularly the extract in ChCl:OA (with the highest TAC), show a dark red color. In contrast, extracts in ChCl:U:W and ChCl:U appear yellowish-orange. Given that the apple variety used in the study is Story, known for its dark red color, the observed color of the extracts is in agreement with the results obtained for TAC.[170] In addition, the color of anthocyanins is pH-dependent. Under acidic conditions, anthocyanins are red or pink, and in basic solutions, these pigments are blue or purple.[151] Extracts in ChCl:U and ChCl:U:W are under alkaline conditions and both of them are neither blue nor purple, reflecting the low concentration of anthocyanins in the solvents. According to Golmohamadi *et al*., the solvent is darker when there are more anthocyanidins extracted.[200] This observation is in good agreement with our results.

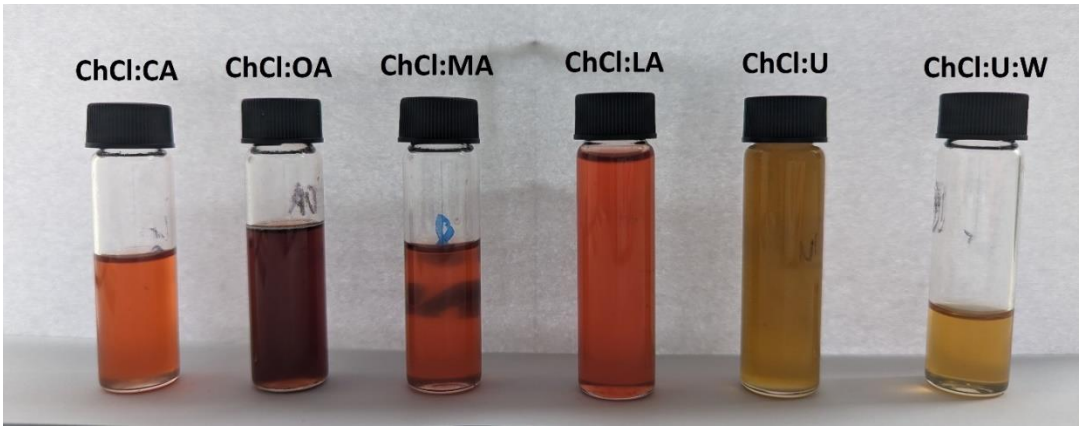


Figure C5-2. Extracts color related to anthocyanidins extraction.

As previously indicated, there appears to be a correlation between TAC and EC_{50}, suggesting that the antioxidant activity of extracts is influenced by both the solvent used and the concentration of anthocyanidins extracted. Similar results were observed by Zannou *et al.*, who also noted a relationship between antioxidant activity and TAC.[221]

Interestingly, TAC also appears to correlate with viscosity (Table C5-3). According to Zannou *et al*., high viscosity tends to limit anthocyanidins extraction capacity.[221] Comparing acid-based NADES with each other's, NADES ChCl:CA appears to be the most viscous (162.6 ± 21.2 mPa.s) with a low TAC (9.38 ± 2.23 M3GE µg/mL), which is consistent with the results of Zannou *et al*. On the other hand, the extract in NADES ChCl:OA, which has the lowest viscosity (13.64 ± 1.05 mPa.s), has the highest TAC (64.81 ± 4.65 M3GE µg/mL). Although NADES ChCl:LA (49.14 ± 15.14 mPa.s) has a NADES higher viscosity than ChCl:MA (33.46 ± 0.18 mPa.s), it extracts anthocyanidins more efficiently (33.46 ± 3.87 and 18.89 ± 5.56 M3GE µg/mL, respectively). This may be attributed to the ability of NADES ChCl:LA to readily form hydrogen bond networks with polyphenols, thus facilitating anthocyanidins extraction despite its higher viscosity than NADES ChCl:MA.[221]

Table C5-3. Comparison of Total Anthocyanidins Content (TAC) and DRSC (EC_{50}) NADES with extracts and dynamic viscosity from pure NADES.

Sample	TAC (M3GE µg/mL)[a]	DRSC EC_{50} ($mg_{Extracts}$/mL)[a]	Dynamic viscosity (mPa.s)[b]
ChCl:CA	9.68 ± 2.23	425.28 ± 51.22	162.6 ± 21.2
ChCl:OA	64.81 ± 4.65	35.59 ± 9.53	13.64 ± 1.05
ChCl:MA	18.89 ± 5.56	268.09 ± 26.35	33.46 ± 0.18
ChCl:LA	33.46 ± 3.87	308.06 ± 56.38	49.14 ± 15.14
ChCl:U	16.63 ± 2.44	176.71 ± 16.82	104.3 ± 7.8
ChCl:U:W	31.41 ± 11.13	111.91 ± 25.06	9.32 ± 0.13

[a]NADES with extracts; [b]Pure NADES.

C.Antibacterial activity

The antibacterial activity of the extracts is evident from the EC_{50} and the Diameter of Inhibition Zone (DIZ) values presented in Tables C5-4 and C5-5. All extracts show antibacterial activity against *Escherichia coli* (*E. coli*), *Listeria innocua* (*L. innocua*), and *Bacillus cereus* (*B. cereus*). Extracts in ChCl:CA and in ChCl:MA exhibited larger DIZ than their respective solvents for all strains studied (Table C5-4). Furthermore, the extract in ChCl:LA leads to larger DIZ the pure than ChCl:LA for *L. innocua* and *B. cereus*, while the extract in ChCl:OA and pure ChCl:OA show this trend only for *E. coli* (Table C5-4). The concentration of 300 mg_{Sample}/mL_{PBS} was chosen according to preliminary tests.

Analysis of the Minimum Inhibition Concentration (MIC) and DIZ values (Tables C5-4 and C5-5) shows that *B. cereus* is the least sensitive of the strains studied. Interestingly, *E. coli* and *L. innocua* show similar MIC values, except for extract in NADES ChCl:LA and pure NADES, where the MIC for *L. innocua* is higher than that of *E. coli*, at 100 and 75 mg_{Sample}/mL, respectively. It should be noted that *E. coli* is gram-negative bacteria, while *L. innocua* and *B. cereus* are gram-positive bacteria. Previous studies have indicated that gram-positive bacteria are generally more sensitive than gram-negative bacteria due to differences in their cell wall structure. Gram-negative bacteria have an additional lipopolysaccharide membrane, which forms a protective layer for the cell.[134,137]

Table C5-4. Diameter inhibition zone (mm) of samples on different bacterial strains at 300 mg_{Sample}/mL.

Sample	Diameter Inhibition Zone (mm)					
	Escherichia coli		*Listeria innocua*		*Bacillus cereus*	
	With extract	Pure	With extract	Pure	With extract	Pure
ChCl:CA	16.2 ± 1.0	14.7 ± 2.1	16.7 ± 2.1	13.3 ± 1.5	14.8 ± 2.0	12.7 ± 0.6
ChCl:OA	18.0 ± 0.5	13.3 ± 0.6	14.2 ± 1.0	20.0 ± 1.0	14.3 ± 1.5	19.7 ± 0.6
ChCl:MA	17.3 ± 1.3	15.3 ± 0.6	16.0 ± 1.0	13.2 ± 0.3	16.7 ± 0.8	16.3 ± 1.5
ChCl:LA	14.8 ± 0.3	15.0 ± 2.0	13.2 ± 0.3	12.3 ± 0.6	14.5 ± 1.5	13.8 ± 0.8
ChCl:U	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]
ChCl:U:W	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]
ChCl	-[b]		-[b]		-[b]	
CA	17.0 ± 1.0		21.3 ± 0.6		23.7 ± 2.1	
OA[a]	20.7 ± 0.6		20.0 ± 1.0		24.3 ± 1.5	
MA	19.7 ± 0.6		15.3 ± 2.1		24.5 ± 0.5	
LA	12.7 ± 0.6		16.0 ± 1.0		22.0 ± 1.0	
U	-[b]		-[b]		-[b]	
Ampicillin	21.0 ± 2.0		28.7 ± 0.6		12.3 ± 1.7	
PBS in water[c]	-[b]		-[b]		-[b]	

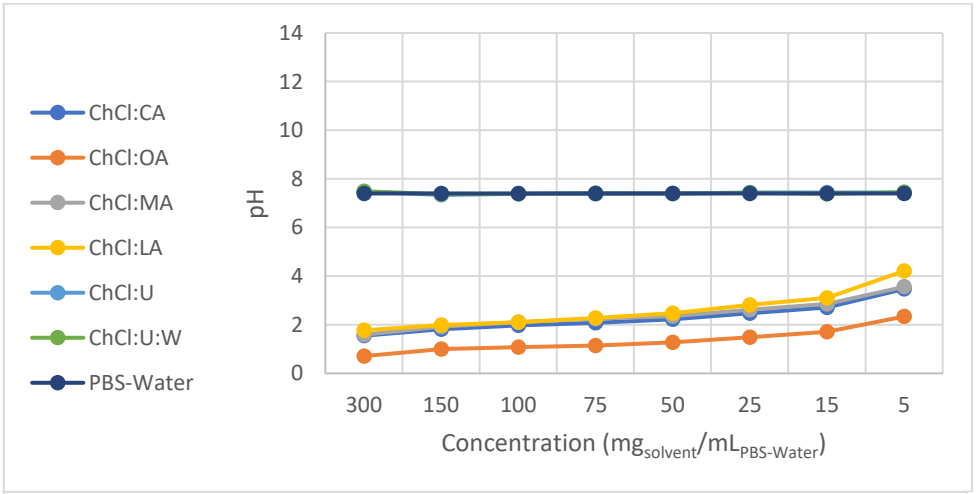
[a]Concentration at 150 mg/mL; [b]Antibacterial effects were not observed; [c]PBS: phosphate-buffered saline, pH 7.4.

The antibacterial properties observed are mainly attributed to the NADES used. According to existing literature, pure acid-based NADES can exhibit a certain toxicity to organisms due to their pH, which denatures bacterial membranes.[134] The optimum pH for bacterial growth lies between 6.5 and 7.5.[137] In particular, oxalic acid shows the highest antibacterial activity, followed by malic acid and citric acid.[134,137] In our study, pure ChCl:CA displays greater antibacterial activity than pure ChCl:MA, with EC_{50} values of 24.1 ± 0.3 and 30.7 ± 1.4 $mg_{Solvent}$/mL for *E. coli*, respectively (Table C5-5). This difference in activity could attributed to variations in the formulation method of the NADES.[137] Indeed, Bedair *et al.* observed that the

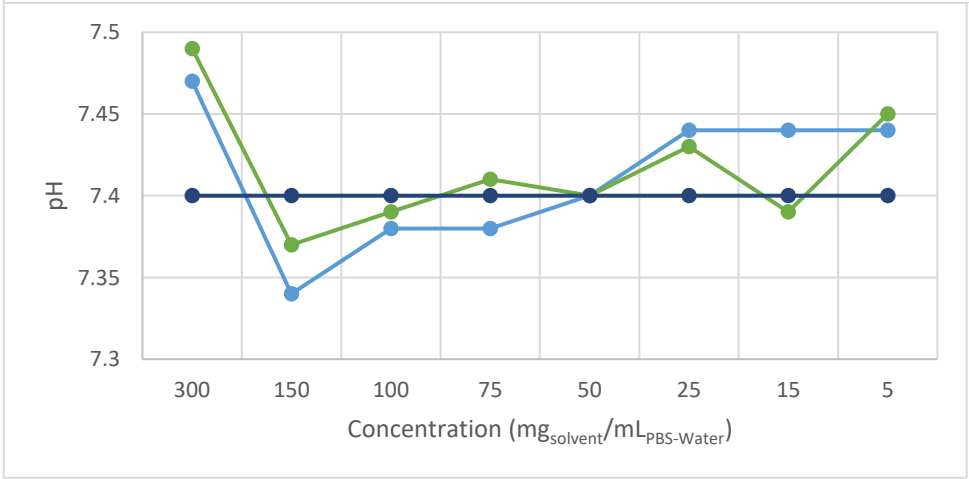
same NADES can exhibit different toxicities depending on its method of preparation. The higher antibacterial activity observed with pure ChCl:CA may be attributed to antibacterial impurities present in the solvent, or conversely, pure ChCl:MA may contain impurities that promote bacterial growth.

Potential causes of bacterial mortality are pH, viscosity of solvents, osmolality, chelation of membrane-bound divalent cations, or interactions of ChCl in aqueous media, such as electrostatic interactions or hydrogen bonding with polysaccharide chains.[134,136,137] In our case study, several different concentrations of NADES with extracts in phosphate-buffered saline (PBS) was tested against bacterial strains. PBS was added to achieve pH of 7.4 to avoid pH effect against bacteria. Unfortunately, the aqueous solution of PBS was not enough to modify the pH of NADES with extracts because it was not the case with pure acidic NADES (Figure C5-3). There appears to be a correlation between the EC_{50} of solvents and pH. Lower pH values for pure NADES correspond to lower EC_{50} values, as shown in Tables C5-1 and C5-5.

a)



b)



c)

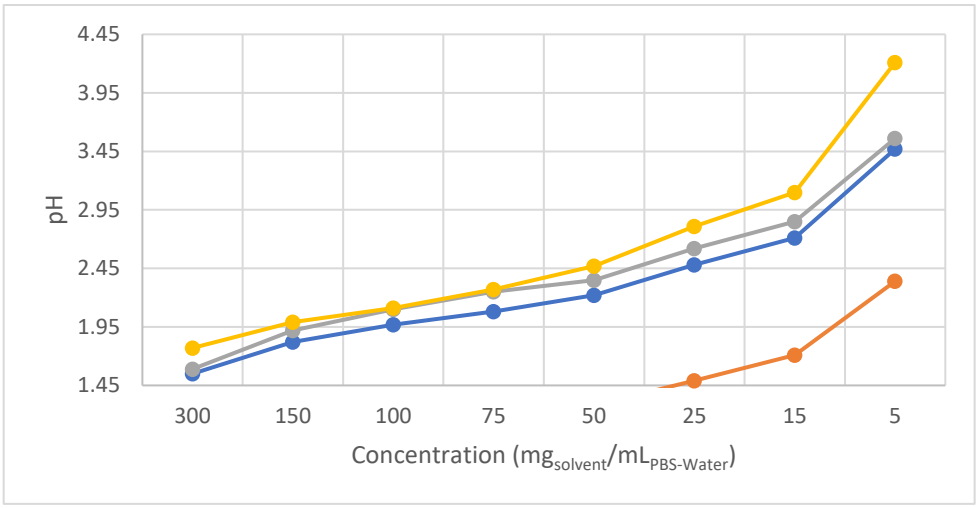


Figure C5-3. Evaluation of pH regarding pure NADES concentration in PSB water (mg/mL); a) overview; b) 7.3 < pH < 7.5; 1.45 < pH < 4.45.

According to Radošević *et al.*, pure ChCl:U exhibits low toxicity, as this solvent only demonstrates antibacterial activity against *E.coli* (37 ± 5 mm with ChCl:U and 10% of water), and no antibacterial activity against *Proteus mirabilis*, *Salmonella typhimurium*, *Pseudomonas aeriginosa*, and *Staphylococcus aureus*.[134] We agree with this statement from Tables C5-4 and C5-5. In our case study, whether pure or containing an extract, ChCl:U showed no antibacterial activities against *L. innocua*, and *B. cereus,* in solid broth from 150 mg$_{Solvent}$/mL, in liquid broth against *E. coli* (Tables C5-4 and C5-5). The difference in results may be due to the bacterial strains used.[135] To corroborate our data, ampicillin (antibiotic) was tested, and led to different DIZs (Table C5-4) depending on the strains. For instance, *B. cereus* (12.3 ± 1.7 mm) is more resistant to the antibiotic than *E. coli* or *L. innocua* (respectively 21.0 ± 2.0 and 28.7 ± 0.6 mm), whose bacterial resistance depends on the sample tested, in this specific case, the antibiotic. Furthermore, *B. cereus* seems to be more resistant to pure or extract-containing NADES than other strains (Table C5-5). ChCl:U and ChCl:U:W pure or containing an extract do not exhibit antibacterial activity with respect to their MIC (when%$_{inhibition}$ ≥ 90%) and their EC_{50} (when%$_{inhibition}$ = 50%) against *B. cereus* (Table C5-6). The percentage inhibition of pure NADES and NADES with extracts is from 0 to 30% for concentrations between 5 and 300 mg$_{Samples}$/mL. The fact that urea-based solvents are not toxic to bacteria can be attributed to the components. Indeed, ChCl and U are essential for the development of living cells.[111] A second study mentions that pure NADES such as ChCl:U can help maintain enzyme stability. This stability is attributed to the hydrogen bond networks, which NADES activities even though that

urea is used as a protein denaturing agent.[135,229] In the case of pure ChCl:U, no antibacterial activity is cited in the literature, indicating that pure NADES does not denature bacterial protein membranes. Our investigations suggest similar results with pure ChCl:U and ChCl:U:W on *B. cereus* and *L. innocua* (Tables C5-4 and C5-5). However, pure ChCl:U demonstrates antibacterial activity against *E. coli* at a concentration of 150 $mg_{Solvent}$/mL (Table C5-5). According to Table C5-4, pure or extract-containing ChCl:U does not exhibit any antibacterial activity. On the other hand, EC_{50} of ChCl:U containing extract is lower than the EC_{50} ChCl:U of pure NADES, respectively at 47.2 ± 11.5 $mg_{Extract}$/mL and 106.4 ± 8.8 $mg_{Solvent}$/mL (Table C5-5). EC_{50} values indicate antibacterial activity of the extracted bioactive compounds. However, according to Table C5-4, ChCl:U with extracts demonstrates no antibacterial activity (no DIZ). Table 5 indicates antibacterial activity (MIC at 150 $mg_{Samples}$/mL). The difference between these two tests can be explained by the fact that ChCl:U with extract and pure NADES do not necessarily kill bacteria but only inhibits their growth. They are bacteriostatic and not bactericidal.

Table C5-5. EC_{50} and Minimum Inhibitory Concentration (MIC) of pure NADES and NADES with extracts on different bacterial strains.

Samples	*Listeria innocua*				*Escherichia coli*				*Bacillus cereus*			
	EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)		EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)		EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)	
	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents
ChCl:CA	29.9 ± 3.0	24.1 ± 0.3	50	50	22.5 ± 0.2	20.3 ± 0.9	50	50	17.2 ± 1.3	17.4 ± 1.2	50	50
ChCl:OA	23.2 ± 3.2	18.8 ± 1.5	50	50	19.4 ± 1.7	17.2 ± 0.7	50	50	6.0 ± 0.3	5.8 ± 0.1	25	25
ChCl:MA	28.9 ± 1.0	30.7 ± 1.4	50	50	25.5 ± 1.1	24.3 ± 0.4	50	50	19.4 ± 0.8	20.3 ± 0.7	50	50
ChCl:LA	34.3 ± 9.9	35.8 ± 1.7	75	75	40.8 ± 0.7	36.9 ± 3.4	100	100	30.5 ± 0.8	36.5 ± 3.8	75	75
ChCl:U	47.2 ± 11.5	106.4 ± 8.8	150	150	-[a]	-[a]	-[b]	-[b]	-[a]	-[a]	-[b]	-[b]
ChCl:U:W	90.3 ± 1.1	130.5 ± 10.7	150	150	-[a]	-[a]	-[b]	-[b]	-[a]	-[a]	-[b]	-[b]

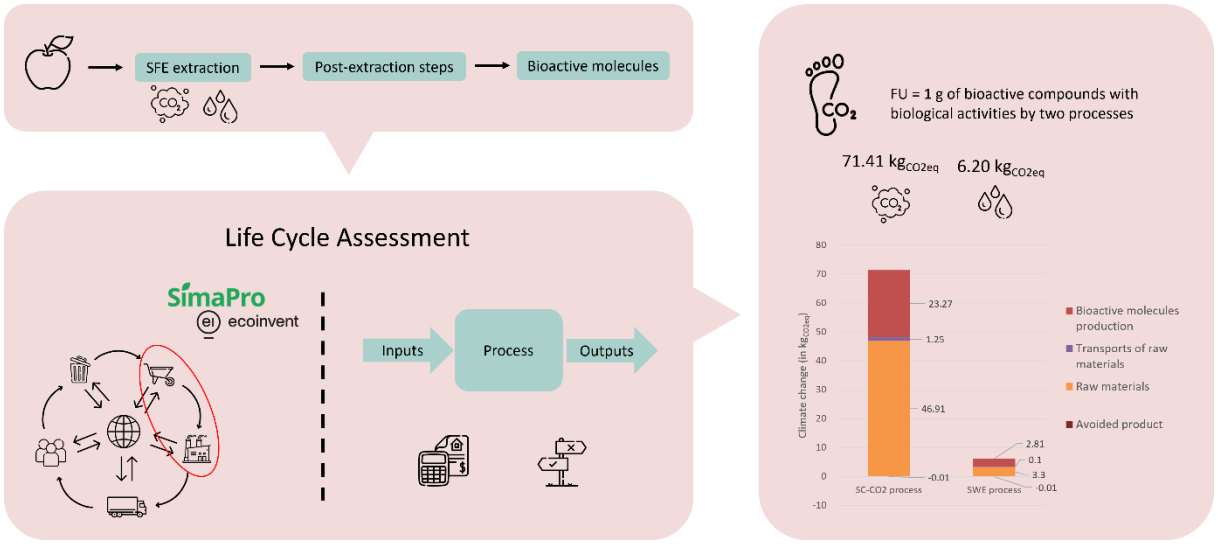
[a]Antibacterial effects were not observed (when 50% of inhibition); [b]Antibacterial effects were not observed (when ≥ 90% of inhibition).

IV.Conclusion

In conclusion, bioactive compounds from AP were successfully recovered an efficient green extraction system using six ChCl-based NADES with four organic acids (citric, oxalic, malic, and lactic acid) and two ureas (one of them was supplemented with 20% water). The highest content of Total Polyphenols was obtained with ChCl:U with extracts (13.15 ± 4.70 GAE mg/g extract). Nevertheless, the highest antioxidant (DPPH Radical Scavenging Capacity) and antibacterial activities were obtained for ChCl:OA with extract, respectively 35.59 ± 9.53 $mg_{Extract}$/mL (EC_{50}) and 25 $mg_{Extract}$/mL (MIC) on *B. cereus*. A correlation between DRSC, Total Anthocyanins Content (TAC) and viscosity was observed. At the lowest viscosities, the highest antioxidant properties and TAC were observed. Several parameters improve the solvent extraction capacity of: (1) the addition of water improves the extraction yield of biomolecules regarding the TAC and DRSC of ChCl:U and ChCl:U:W with extracts (respectively 16.63 ± 2.44 and 31.41 ± 11.13 M3GE µg/mL and 176.11 ± 16.82 and 111.91 ± 25.06 $mg_{Extracts}$/mL) due to decreased viscosity (increase of mass transfer); (2) acidic conditions allow more selective extraction than alkaline conditions, especially for anthocyanins (*e.g.* TAC from ChCl:OA extract is 64.81 ± 4.65 and from ChCl:U:W with extract is 31.41 ± 11.13 M3GE µg/mL). For acidic NADES, the biological properties of the extracts are mainly attributed to the solvents (urea-based solvents do not exhibit antibacterial activity against *B. cereus*). Work is currently in progress to eliminate the solvent and thus study the activities of pure bioactive molecules.

Chapter 6

Comparative Life Cycle Assessment of Extraction of Bioactive Compounds from Apple Pomace using Subcritical and Supercritical Fluids



Chapter 6 compares the carbon footprint of supercritical CO_2 and subcritical water extractions for the extraction of bioactive compounds from AP. This chapter is under review in *Cleaner Environmental Systems* (Elsevier) in 2024.

Apple pomace (AP), a by-product of the apple juice and cider industries, represents a significant waste challenge, generating approximately 5 million tons produced in 2021. Often disposed of in landfills, AP contributes to health and environmental risks. Despite its disposal, AP remains a valuable source of bioactive compounds, recognized for their biological properties. This study assesses the carbon footprint associated with extracting these bioactive compounds using innovative technologies, namely supercritical CO_2 (SC-CO_2) and subcritical water extraction (SWE). Utilizing SimaPro software and the ecoinvent database, the Life Cycle Assessments (LCA; cradle-to-gate) were conducted for extracting 1 g of bioactive compounds from AP. The findings reveal that the SC-CO_2 process emits 71.42 kg_{CO2eq}, while the SWE results in significantly lower emissions of 6.20 kg_{CO2eq}. These results highlight the environmental impact of different extraction technologies and emphasize the potential for more sustainable practices in valorizing AP.

I.Introduction

In 2022, global apple production exceeded 95.8 million tonnes, with approximately 25 – 30% of these apples processed into value-added products like apple juice, jams, and cider.[8,19] Remarkably, 65% of these products are derived from juice or cider production, resulting in a substantial by-product known as apple pomace (AP). Unfortunately, AP is predominantly underutilized and frequently relegated to landfills. The annual production of AP is estimated to range between 4.7 and 5.6 million tonnes. As a residual material post-apple pressing, AP poses health and environmental risks attributed to its high moisture content (80 – 85%), making it prone to microbial decomposition.[9] Addressing these concerns necessitates the essential valorization of this waste stream. AP comprises skin, flesh, seeds and stem. A well-known saying about the bioactive compounds in apples is *'An apple a day keeps the doctor away.*' The bioactive compounds in apples and AP mainly include polyphenols, organic acids, triterpenes, sterols and carbohydrates.[179] Phenolic compounds, for example, exhibit biological activities such as antioxidant, antidiabetic, antitumor and antimicrobial properties which have specific applications.[15,48,51,55,153] Traditionally, bioactive molecules are extracted using conventional techniques such as Soxhlet extraction. However, these classical extraction techniques require large amount of organic solvents, high temperatures, long extraction times and often do not respect the principles of *Green Chemistry.*[92,153] In contrast, the unconventional subcritical and supercritical fluid extraction (SFE) techniques, explored in this presented research, offer a promising alternative that is usually more sustainable and environmentally friendly, and complies with several principles of *Green Chemistry.*[70,153] These methods are considered environmentally friendly (cheap, non-toxic, available, safe solvents) and effective for high-quality extract production in the food industry.[78,87,92,153] For instance, Ferrentino *et al*. compared Soxhlet (ethanol) and supercritical CO_2 (SC-CO_2) for the extractions of AP compounds to study their antioxidant activity (Total Polyphenols Content and DPPH Radical Scavenging Capacity).[92] SC-CO_2 extraction enhances antioxidant activity up to 84%. Water is an easily accessible and non-hazardous solvent. However, the use of water is a viable option only for dissolving compounds, such as bioactive compounds, under subcritical conditions, taking advantage of changes in its dielectric constant and polarity depending on the temperature.[230] Ko *et al*. extracted bioactive compounds from *Orostachys japonicus* A. Berger using a subcritical water extraction (SWE) process.[87] Comparing SFE (SWE: 220 °C and 15 min) and conventional methods (ethanol: 60 °C and 2 h), the TPC values resulted in 39.9 ±

4.1 and 8.0 ± 2.6 mg GAE/g respectively. In both examples, SFE demonstrated a better preservation of bioactive compounds. In addition to preserving bioactive compounds, SFE are considered as promising techniques that use green solvents. Green solvents, also called as eco-solvents, reduce environmental impact because they come from alternative, typically bio-based, substances. Eco-solvents can be classified into several categories: ionic liquids, (natural) deep eutectic solvents, bio-based solvents, switchable solvents, water, SFE, and solvent-free. Some of these methods have been used for centuries. One of the oldest solvent-free procedures is olive oil extraction, which involves crushing and separating the oil from the olive pomace by a purely physical mechanism.[115]

In the current context of global warming, it is essential to respect the three pillars of Sustainable Development: social, environmental, and economic areas. The use of green solvents tends to adhere to both the principles of sustainable development and *Green Chemistry*. Academia and industry are increasingly looking for ways to recycle and/or valorize AP. For some years now, companies such as *SAMARA*, a North American brand, have been using apple waste to manufacture imitation leather.[10] AP can also be sold simply dried and ground. *HUBCYCLE*, a French company, sells various types of waste to other companies.[11] In this form, AP can be used as a source of sugars or nutrients.

Aix-Marseille University and *Symrise* recently collaborated on a thesis on the recovery of wax from AP for cosmetic purposes, aiming to use it as a bio-based cosmetic product and avoid the addition of petrochemical preservatives.[12] These recovery methods are currently at pre-industrial or industrial stages but are not yet fully developed. According to *SciFinder*, the valorization of AP continues to progress year after year. Numa *et al*. determined through economic analysis that the valorization of bioactive compounds from AP using SC-CO_2 extraction is viable.[12] However, assessing the environmental impacts of biomass extraction is crucial and complementary to economic analysis. Life Cycle Assessment (LCA), carried out in accordance with ISO 14040-44 standards, provides a comprehensive method for quantifying the environmental footprint of existing and future eco-designed products and processes.[231] LCA examines five life phases: extraction and production of raw materials, transportation, production and distribution of the product, use and end-of-life (Figure C6-1).

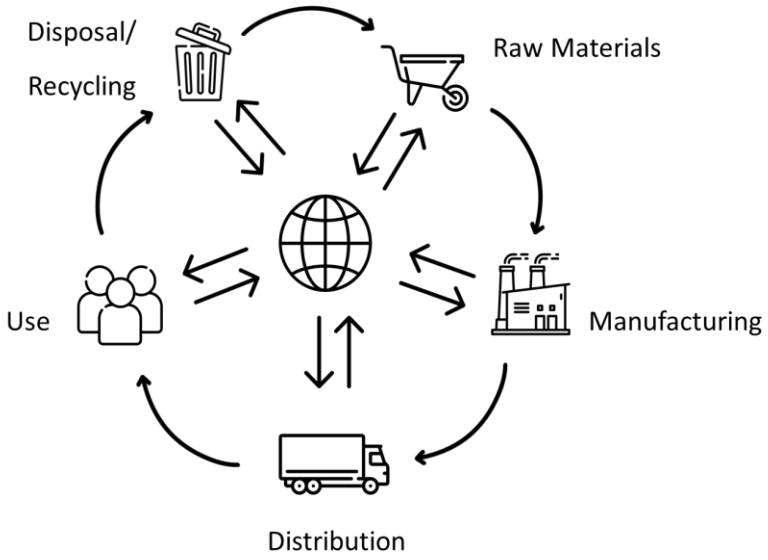


Figure C6-1. Scheme of Life Cycle with 5 phases.

This approach is particularly relevant for chemists focusing on biomass extracts and natural substances, as it meets the growing consumer demand for environmentally-friendly products. Espada *et al*. compared solvent-based extraction process with SC-CO_2 extraction, finding that that SC-CO_2 extraction was favourable for producing β-carotene from *Dunaliella salina*, with a carbon footprint of 270 kg_{CO2eq} versus 525 kg_{CO2eq} for solvent-based extraction.[232] However Carlqvist *et al*. found that SC-CO_2 extraction had a higher carbon footprint than hot water extraction, respectively 5.8 and 0.61 kg_{CO2eq}/1 kg of polyphenols from spruce bark.[233] Depending on the selected biomass, SFE may not always enable environmentally-friendly extraction mainly due to the high energy consumption.[233,234] It is therefore necessary to calculate the carbon footprint of each time use of SFE.

Furthermore, companies are increasingly employing LCA in the early stages of process development to evaluate environmental viability, especially in the food industry, which is a significant consumer of electricity and concerned about its carbon footprint.[235] While various studies have undertaken LCAs on the valorisation of AP or its constituent molecules, to our knowledge, none have specifically delved into the LCA of bioactive compounds extraction from AP using SFE.[236–242] The objectives of this scientific paper are to study the LCA of SWE and SC-CO_2 extraction methods, to compare their ecological impacts, and to determine their environmental sustainability. The LCA of two extraction methods was carried out under our specific extraction conditions bioactive compounds with biological activities.

II. Goal and Scope

The objective of this work was to evaluate and compare the environmental impact of extracting bioactive compounds from AP using SC-CO_2 extraction and SWE. The analysis aimed to enable the formulation of proposals for improvement. Bioactive compounds were extracted for their biological properties in this study. The functional unit (FU), defined as producing 1 g of bioactive compounds with biological activities from AP, was used for one of the two processes. In accordance with ISO 14040-44, the FU serves as a quantified description of a product system and a reference unit in an LCA.[237] The climate change impact indicator, measured in kg_{CO2eq}, was the unique indicator selected for this study. This widely used indicator facilitates comparison of results across different studies by measuring the impact of greenhouse gases associated with the product system.[243] The "environmental footprint v3.1" assessment method was chosen for impact calculations, available in the SimaPro software.

The scope of this article was defined as cradle-to-gate, encompassing the impacts of life phases including the extraction and production of raw materials (AP, chemicals, *etc*.), transportation of these materials, and the manufacturing of the product (extraction of bioactive compounds). The system boundaries are shown in Figure C6-2. Although the transportation phases are not depicted in Figure C6-2, they are included in the study. The phases of product use and end-of-life (gate-to-grave perimeter) were excluded from the LCAs as they were carried out for laboratory-scale processes and do not take into account for the sale or use of AP bioactive compounds. Consequently, the inclusion of a cradle-to-grave impact assessment would introduce excessive uncertainties and risk biasing the LCA.

To construct the LCAs, a clear and precise process was essential, ensuring that the data modeled in the software could be accurately collected. The initial steps in each process involved the recovery, drying, and grinding of AP (Figure C6-2). For the SC-CO_2 extraction, as illustrated in Figure C6-2 (green dot lines), the apple waste was extracted using SC-CO_2 and ethanol (EtOH; as a co-solvent). The resulting extract, containing both bioactive compounds and wax, was centrifuged to separate these components. Subsequently, the EtOH was replaced with water, and the extract was freeze-dried. In the SWE scenario, shown in Figure C6-2 (blue dot lines), the waste underwent extraction using the SWE technique. This extract was also centrifuged to separate the AP and the bioactive compounds solution. The bioactive compounds were then isolated from the rest of the extract using resin washing, followed by

the removal of EtOH, and freeze-drying of the bioactive compounds extract. In both cases, the solid waste (AP after extraction) was not considered in the study.

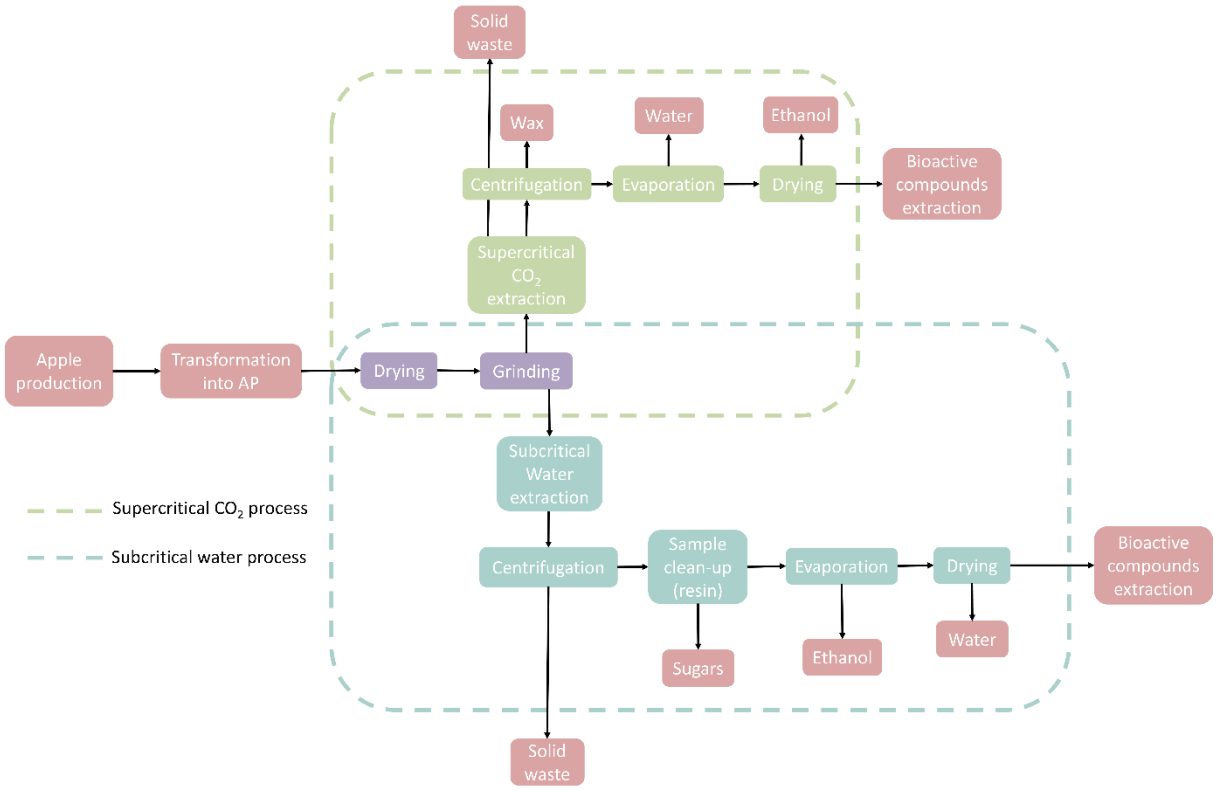


Figure C6-2. Scheme of system boundaries of both processes; in green dot lines SC-CO_2 extraction process; in blue dot lines SWE process.

III. Inventory analysis

The inventory analysis, also referred to as data recovery, represents the most extensive and complex phase of a Life Cycle Assessment (LCA). This phase involves a detailed input and output accounting, summarized in Table C6-2, for processes such as SC-CO_2 extraction. Transportation within these LCAs includes moving raw materials to the production or extraction site, specifically the EDYTEM lab. Due to the lack of specific supplier information, estimates were made to closely approximate the actual conditions of the study. For the SC-CO_2 extraction LCA, it was estimated that bottled CO_2 gas was transported from Air Liquide in Grenoble (38360, Sassenage, France) to the EDYTEM laboratory (Université Savoie Mont-Blanc, CNRS, 73370, Le Bourget-du-Lac, France), covering a distance of 85.1 km. The EtOH solvent was assumed to be produced at the Carlo Erba plant (27100, Val-de-Reuil, France),

located 670 km away. The power consumption of the SC-CO_2 device, which could not be measured directly, was estimated using the effective and maximum power of the SWE device as a reference (Table C6-1 and Equation C6-1). Based on the manufacturer's data, the maximum power of the SC-CO_2 device is estimated at 9,000 W, leading to an effective power calculation of 1,860 W as per Equation C6-1.

Equation C6-1. Calculation of power effective for SC-CO_2.

$$P_{effective_{SC-CO_2}} = \frac{P_{effective_{SCW}} \times P_{max_{SC-CO_2}}}{P_{max_{SCW}}}$$

Table C6-1. Summary of powers of SC-CO_2 and SWE equipments.

	P_{max} (W)	$P_{effective}$ (W)
SWE	1,500	300
SC-CO_2	9,000	*1,860*[a]

[a]Calculation of power effective for SC-CO_2 process in Appendix (Equation A-1).

French electricity is largely carbon-free, thanks to nuclear power plants.[244] The carbon footprint of processes, especially the electricity consumed, is influenced by the country in which they are operated. For instance, Carlqvist *et al*. compared the carbon footprint of three different extraction methods (hot water, ultrasound and SFE) as a function of the electricity source.[233] They found that the impact of the Nordic and Swedish electricity markets was similar, while the Central European Power association had a higher carbon footprint, particularly for ultrasound-assisted extraction. Thus, the carbon footprint of French electricity is significantly reduced due to the predominance of nuclear power generation.

Table C6-2. Life cycle inventory for the SC-CO_2 process.

For **50 mg** of bioactive compounds	Flow	Quantity	Unit/FU
Life cycle	Inputs		
Raw material	AP	100.00	g/FU
Transport	Transport AP	5.36	kgkm
Production	Elec freezer	0.20	kWh/FU
Production	Elec freeze-drying #1	14.56	kWh/FU
Production	Elec crush	0.03	kWh/FU
Production	Water crush	5.00	mL/FU
Raw material	Gas CO_2	0.95	kg/FU
Transport	Transport gas	239.22	kgkm
Production	Elec SC-CO_2 device	2.50	kWh/FU
Raw material	EtOH SC-CO_2	68.40	mL/FU
Transport	Transport EtOH	36.16	kgkm
Production	Elec centrifuge	0.99	kWh/FU
Raw material	EtOH centrifuge	100.00	mL/FU
Transport	Transport EtOH	52.86	kgkm
Production	Elec rotary evap	4.50	kWh/FU
Production	Water rotary evap	15.00	mL/FU
Production	Elec freezer	0.20	kWh/FU
Production	Elec freeze-drying #2	7.28	kWh/FU
	outputs		
Waste	Water freeze-drying #1	80.00	mL/FU
Avoided product	Wax	0.350	g/FU
Waste	Water freeze-drying #2	15.00	mL/FU

The inputs and outputs of the SWE are shown in Table C6-3. The transport corresponds to transport from raw materials sites to the production/extraction sites. Similar to SC-CO_2 process, several estimates were made for SWE (gas cylinder/EtOH sites, electricity, *etc.*). The resin used for SWE is Amberlyte XAD-16, a polystyrene-divinylbenzene. Thus, a mix of styrene-divinylbenzene polymers from the ecoinvent database was selected. The production site identified for Amberlyte XAD-16 is Illkirch (Bas-Rhin department, France; 462 km from EDYTEM lab, the experiment site).

Experiments with SWE were carried out in Turin, Italy. Nevertheless, for accurate comparison, we have considered that the extraction was also carried out at EDYTEM lab. The amount of N_2 in the process was estimated using the ideal gas law. At the beginning of extraction, 3 nitrogen flushes were performed at 1 bar and the pressure was maintained at 5 bar. The device was at room temperature, 21 °C, when the gas was injected. The volume of the vessel was 1 L; 200 mL of water and material (AP dry) were added. The remaining volume was estimated at 800 mL, 0.0008 m^3.

Equation C6-2. Ideal gas law: calculation of total weight of N_2(g) used in SWE.

$$PV = nRT$$

Where P is a pressure (Pa); V a volume (m^3); R is the ideal gas constant (8.314 $J.mol^{-1}.K^{-1}$); T is a temperature (K).

Table C6-3. Life cycle inventory for the SWE process.

For **800 mg** of bioactive compounds	Flow	Quantity	Unit/FU
Life cycle	Inputs		
Raw material	AP	125.00	g/FU
Transport	Transport AP	6.70	kgkm
Production	Elec freezer	0.20	kWh/FU
Production	Elec freeze-drying #1	18.20	kWh/FU
Production	Elec grinding	0.03	kWh/FU
Production	Water grinding	5.00	mL/FU
Raw material	Gas N_2	7.33[a]	kg/FU
Transport	Transport gas	158.74	kgkm
Production	Elec SWE device	0.117	kWh/FU
Raw material	Water SWE	200.00	mL/FU
Production	Elec centrifuge #1	0.48	kWh/FU
Raw material	EtOH resin	315.60	g/FU
Transport	Transport EtOH resin	211.45	kgkm
Raw material	Resin	0.74	g/FU
Transport	Transport resin	0.342	kgkm
Production	Elec bioblock #1	0.29	kWh/FU
Production	Elec Buchner filtration #1	0.01	kWh/FU
Production	Elec bioblock #2	0.11	kWh/FU
Raw material	EtOH solvent (80/20 EtOH/water)	252.48	g/FU
Transport	Transport EtOH	169.16	kgkm
Raw material	Water solvent (80/20 EtOH/water)	80.00	mL/FU
Production	Elec Buchner filtration #2	0.01	kWh/FU
Production	Elec bioblock #3	0.11	kWh/FU
Production	Elec Buchner filtration #3	0.01	kWh/FU
Raw material	EtOH resin	78.90	g/FU
Transport	Transport EtOH resin	52.86	kgkm
Production	Elec rotary evap	4.50	kWh/FU
Production	Elec freezer	0.20	kWh/FU
Production	Elec freeze-drying #2	9.71	kWh/FU
	outputs		
Waste	Water freeze-drying #1	100.00	mL/FU
Avoided product	Sugars	14.00	mg/FU
Waste	Water freeze-drying #2	80.00	mL/FU

[a]Calculation of quantity of N_2 used in SWE process in Appendix (Equation A-2).

IV.Impact assessment

Figure C6-3 illustrates the environmental impact assessment of the SC-CO_2 and SWE processes for the production of 1 g of bioactive compounds, revealing climate change footprints of 71.42 and 6.20 kg_{CO2eq}, respectively. For both techniques, the contribution to carbon footprint of transportation and avoided products are negligible. The figure highlights the variation in carbon footprints between these two techniques. The avoided products for the SC-CO_2 and SWE processes are wax and sugars, respectively. Under SWE extraction conditions, several compounds were extracted, including carbohydrates, undesirable compounds (5-hydroxymethylfurfural, furfural) and polyphenols. The Story apple variety is sweet, requiring post-treatment of SWE extracts with resin to remove sugars and avoid biological activities from sugars, especially for antibacterial tests, as sugars can promote bacterial growth.[170] Unpublished results indicated that the contribution of wax obtained by SC-CO_2 extraction process to TPC (antioxidant activity) was negligible, leading to the removal of the wax in this study.

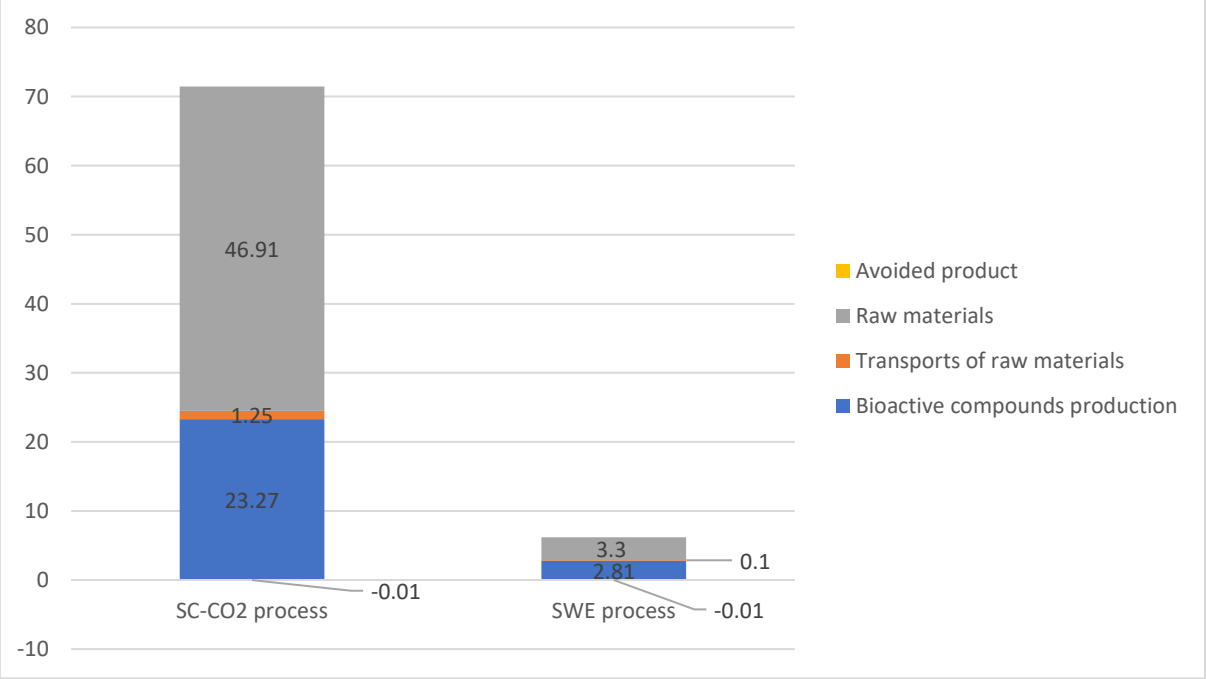


Figure C6-3. Comparison of environmental impacts in different phases of bioactive compounds production on climate change (in kg_{CO2eq}).

V.Discussion

The transportation of raw materials in both the SC-CO_2 process and SWE has a minimal impact on their overall carbon footprints. It contributes only 1.25 kg_{CO2eq}/g of bioactive compounds for the SC-CO_2 process and 0.10 kg_{CO2eq}/g for the SWE process. This finding is consistent with research by Humber *et al*., which indicates that transport has a low carbon footprint over distances of approximately 100 km.[152] In this study, most raw materials were sourced within this range, except for EtOH from Carlo Erba (670 km) and resin from Fisher Scientific (462 km). The production phase, particularly in the SC-CO_2 process, is more significant, accounting for 46.91 kg_{CO2eq} or 53.2% of its total carbon footprint (Figure C6-3). In Figure C6-4, this phase included the consumption of electricity and water, with freeze-drying steps alone consuming 72% of the total electricity.[234,245,246] According to one study, in the UK, the total energy consumption of all industries is between 17.7 and 19.3%, with a significant portion attributed to the drying step.[246] Freeze-drying is a preferred method for removing water while preserving heat-sensitive molecules.[12,69,92]

The co-product for SC-CO_2 process has a negligible beneficial impact on the LCA, reducing the footprint by only - 0.01 kg_{CO2eq}/g of bioactive compounds (Figure C6-3). In the SWE process, the primary input associated with energy consumption is EtOH representing 45.1% of carbon footprint (Figure C6-4). This requires the separation of bioactive compounds and by-products (sugars) using resin. The avoided product in this process, sugars, has a negligible beneficial impact on the LCA, reducing the footprint by only - 0.01 kg_{CO2eq}/g of bioactive compounds as SC-CO_2 process.

In Figure C6-5, all negligible inputs were removed. In terms of transport, electricity for small steps, freeze-drying, and rotary evaporation, the carbon footprint in the SC-CO_2 process was 13.8 times higher than that in the SWE process. The mass yield of bioactive compounds in the SWE process was 16 times higher than in the SC-CO_2 process. In addition, the gas used in the SC-CO_2 process had a greater impact. This variation can be explained not only by the mass yield but also by the type of gas used. The gas used in SC-CO_2 was CO_2, while in the SWE process, N_2 was used. In terms of climate change, CO_2 has a greater impact than N_2 on the same parameter.

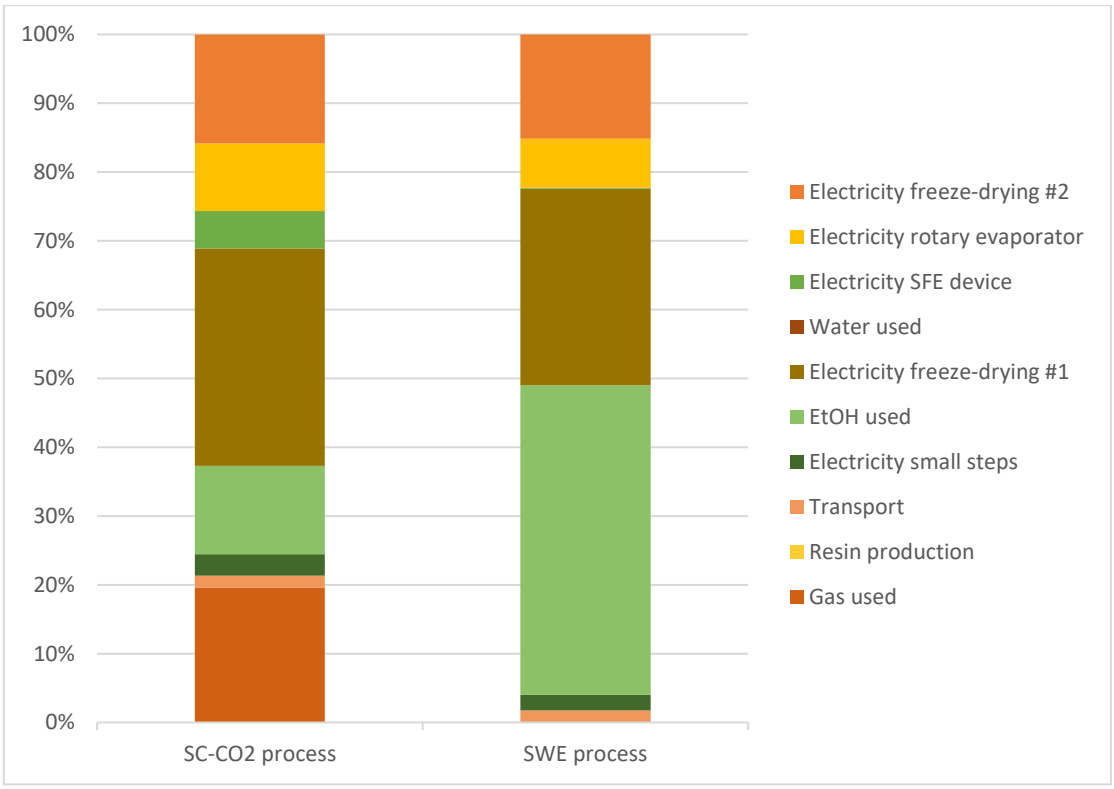


Figure C6-4. Environmental impacts depending on the different steps of bioactive compounds production on climate change.

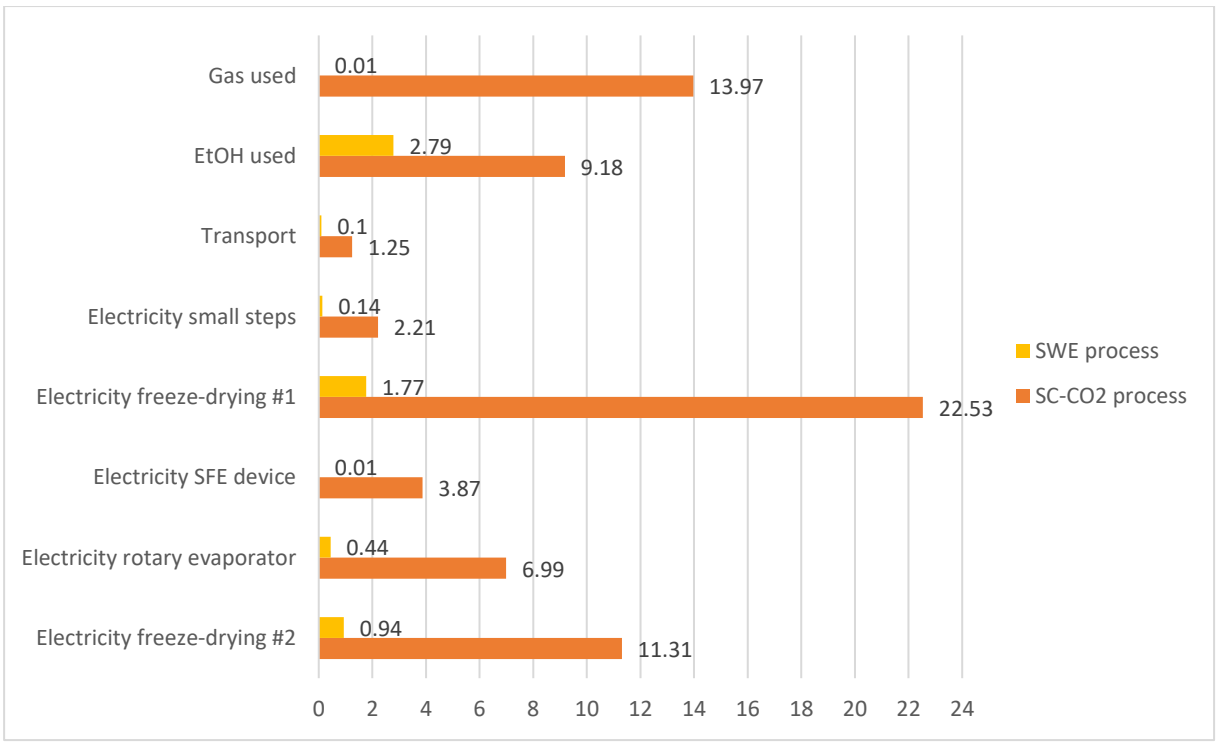


Figure C6-5. Comparison of environmental impacts between SWE and SC-CO_2 processes to produce 1 g of bioactive compounds production based on each step.

Comparing our results with those in the literature may prove difficult due to differences in the functional unit (FU) used in each study. Each research effort defines its own FU, making direct comparisons difficult. Gassara *et al*. conducted a study on the valorization of AP in Quebec, based on a total of 16,209 tons of waste generated in 2007, analyzed various scenarios including animal feed, landfill, incineration, enzyme production, and composting.[238] Enzyme production (906.81 t_{CO2eq}/year) and animal feed (963.98 t_{CO2eq}/year) were identified as the scenarios with the lowest carbon emissions while incineration led to the highest CO_2 equivalent emissions, with 16,420.14 t_{CO2eq}/year. Landfill and composting resulted in emissions of 1,841.57 t_{CO2eq} and 1,273.78 t_{CO2eq}, respectively. By comparison, if all AP in Quebec were recovered using the SC-CO_2 process under our study conditions, this would correspond to emissions of 11,600,000 t_{CO2eq}. In contrast, using the SWE would result in a carbon footprint of 803,966 t_{CO2eq}. This impact could be further reduced to 574,447 t_{CO2eq} by using wet AP. Although the goal was to approach the carbon footprint of incineration, the difference remains significant. Comparisons between supercritical fluid extraction (SFE) techniques and animal feed scenarios indicate that SFE can result in a higher environmental impact, primarily due to the significant electricity or heat consumption involved.[234] This observation is consistent with our results. However, several authors have carried out LCAs to determine the carbon footprint of bioactive compounds production with different extraction techniques (Table C6-4). The carbon footprints reported in these studies for the SC-CO_2 process range between 0.028 and 270 kg_{CO2eq}, the highest value being for the production of 1 g of β-carotene at 270 kg_{CO2eq}.[232] Although this molecule is apolar and should be easily extracted with SC-CO_2, Espada *et al*.'s high footprint can attributed to Spain's energy mix, where natural gas accounted for 29.5% of electricity in 2022.[23,232] Another explanation is that Espada *et al*. focused on ethe extraction of specific molecules, whereas Ferreira *et al*. targeted a family of molecules, resulting in lower carbon footprints of 0.028–0.097 kg_{CO2eq}/g of carotenoids.[92,247]

Similarly, De Marco *et al*. conducted SC-CO_2 extraction to obtain caffeine, which resulted in a low carbon footprint of 0.23 and 0.29 kg_{CO2eq}/g of dry caffeine.[248] Our case study shows a footprint of 71.41 kg_{CO2eq}/g of bioactive compounds with the SC-CO_2 extraction process, indicating that our extraction had a significant impact. The differences between our results and those in the literature can be attributed to several factors, such as the use of co-solvent (EtOH), mass yield, and the specific molecules extracted. Indeed, in our case study, non-polar molecules (wax) were eliminated due to their lack of biological activity, leading to a decrease

in mass yield and an increase in carbon footprint. If only the mass yield were considered, the carbon footprint would be lower. However, In the cases of De Marco *et al*. and Ferreira *et al*., the target molecules were mainly apolar.[247,248] The difference with Espada *et al*. can be explained primarily by the energy mix differences between France and Spain, where the main energy sources are nuclear and natural gas, respectively.[23,232,244]

The extraction yield may significantly impact climate change. To observe the impact, an attempt on *SimaPro* was made by modifying the mass yield obtained using the SC-CO_2 process to achieve 800 mg of bioactive compounds like with the SWE process. The result was approximately 6.20 kg_{CO2eq}/g of bioactive compounds, similar to the yield obtained *via* SWE. The primary aim of SC-CO_2 extraction is to recover apolar molecules such as wax.[70] Carlqvist *et al*. compared three different extraction methods and determined their carbon footprints if the yields were identical.[233] At the same yield, hot water extraction had a lower impact than ultrasound-assisted extraction and SFE, with respective values of 0.48, 5.9, and 6.3 kg_{CO2eq}/kg of polyphenols.

A prominent method for biogas production in Europe is anaerobic digestion, which processes various types of waste, including crop residues like AP, animal manure, municipal solid waste, and municipal wastewater. Anaerobic digestion is widely used for treating large volumes of waste. The International Energy Agency highlights Europe as the leading region employing this method.[23] AP is commonly processed for biogas production. Evangelisti *et al*. evaluated the carbon footprint of producing biogas from 35,574 tons of food waste per year in the London Borough, which was 2,000 t_{CO2eq}.[249] If 35,574 tons of AP were processed in an anaerobic digestion facility, the carbon footprints of the SC-CO_2 and SWE processes would be approximately 2,540,000,000 and 1,760,000,000 t_{CO2eq}, respectively. On the other hand, Gassara *et al*. show that treating 16,209 tons of AP by anaerobic digestion results in 911.28 t_{CO2eq}, indicating that anaerobic digestion is one of the least polluting solutions, similar to animal feed and enzyme production.[238]

Table C6-4. Comparison of carbon footprint between our case study and selected results from literature.

Biomass	Extraction	Carbon footprint (kg_{CO2eq})	FU	Ref
AP	$SC\text{-}CO_2$	71.41	1 g of bioactive compounds	-[a]
	SWE	6.20		
	SWE	4.43		
	Anaerobic digestion[b]	2.000.000	Total production of food waste in London Borough of 35,574/year	249
	animal feed	963,980	Total production of AP in Québec in 2007 of 16,209 tons	238
	landfill	1,841,570		
	incineration	16,420,140		
	enzyme production	906,810		
	composting	1,273,780		
Lingonberry pomace	pH-based process	1.67	1 kg of protein ingredient	237
		2.5		
Saccharina latissima		2.05		
Ulva fenestrata		2.9		
Dunaliella salina	$SC\text{-}CO_2$	270	1 g of β-carotene	232
Tetraselmis suecica	Solvent-based extraction	56.7	1 kg of *T. suecica*	250
		0.65 (3.25)	1 g of bioactive compounds (5 g)	
Wastewater-grown microalgae	$SC\text{-}CO_2$	0.028 – 0.097 (28 – 97)	1 g of carotenoids (1 kg)	247
Spruce bark	$SC\text{-}CO_2$	0.0058 (5.8)	1 g of polyphenols (1 kg)	233
	UAE	8,000 (8.0)	1 g of polyphenols (1 mg)	251
Chicory grounds	UAE	5.8	0.55 L of chicory grounds extraction exhibiting 220 µmol TE antioxidant capacity	252
Coffee beans	$SC\text{-}CO_2$	0.29 (3.29)	1 g of dry caffeine (11.4 g)	248
		0.23 (2.66)		
Acerola pomace	UAE	50%[c]	1 g of polyphenols (1,400 mg)	253
Jambolan pomace		52%[c]		
Moringa oleifera leaves	Water extraction	0.00175 (1.75)	1 g of polyphenols (1 kg)	254
Beet seeds	UAE	88%[d]	16.6 mmol TEAC for the AA of the extract	244
Red wine pomace	PLE	36 – 172	1 g of polyphenols (1 kg)	255
	Solvent-based extraction	28 – 60		

[a]Data from our case study; [b]In the case of 100% of food waste is AP; [c]Compared to conventional extraction (100%); [d]Compared to microwave-assisted extraction (100%); AP: Apple Pomace; PLE: Pressurized Liquid Extraction; $SC\text{-}CO_2$: Supercritical CO_2; SWE: Subcritical Water Extraction; TE: Trolox Equivalent; UAE: Ultrasound-Assisted Extraction.

Several impact indicators exist, including eutrophication, acidification, and water use (Table C6-5). However, Climate Change is widely recognized and frequently used in studies. Additionally, the units of these indicators can vary depending on the method or software used. For instance, in our case, the Land Use indicator is measured in Pt (points that combine multiple environmental impacts into a single score), whereas in Carlqvist *et al.*'s study, it is measured in m^2a (square meter per year).[233] In our case study, the SWE process demonstrated a lower impact across all impact assessments (Table C6-5)

Table C6-5. Indicators from "Environmental Footprint" methods classified by level of robustness and recommendation for SC-CO_2 and SWE processes.

Indicators	Unit	Impact assessment	
		SC-CO_2 process	SWE process
Acidification	mol_{H+eq}	3.07E-01	3.46E-02
Climate change	kg_{CO2eq}	7.14E+01	6.20E+00
Particulate matter	disease incidence	3.54E-06	3.47E-07
Marine eutrophication	kg_{Neq}	2.07E-01	4.66E-02
Freshwater eutrophication	kg_{Peq}	1.52E-02	1.33E-03
Terrestrial eutrophication	mol_{Neq}	7.64E-01	1.07E-01
Human toxicity, cancer effect	CTUh	2.88E-07	2.56E-08
Human toxicity, non-cancer effect	CTUh	1.63E-06	2.02E-07
Ionizing radiation	$kBq_{U-235eq}$	3.05E+02	2.14E+01
Land use	Points	9.55E+02	2.12E+02
Ozone depletion	$kg_{CFC11eq}$	2.10E-06	1.92E-07
Photochemical ozone formation	$kg_{NMVOCeq}$	2.17E-01	2.07E-02
Resource use, fossils	MJ	6.95E+03	5.03E+02
Water use	m^3 depriv.	1.00E+02	2.46E+01

CFC11: Chlorofluorocarbon; CTUh: Comparative Toxic Unit for humans; NMVOC: Non methane volatile organic carbon compounds; U-235: Uranium 235.

This analysis underscores the importance of considering the entire life cycle of a product or process when evaluating its environmental impact. The findings highlight the potential for significant reductions in carbon footprint through process optimization and the selection of more sustainable extraction methods. As the demand for environmentally friendly products and processes continues to grow, studies like this play a crucial role in guiding industry practices towards more sustainable solutions.

VI. Future prospects and limitations

Several improvements can be made to both extraction methods to reduce their carbon footprint. De Marco *et al*. calculated a new carbon footprint for the extraction of caffeine using SC-CO_2 extraction process and compared it to the base case, finding that the base and improved scenarios were 3.29 and 2.66 kg_{CO2eq}/11.4 g of caffeine from coffee beans, respectively (Table C6-4).[248] To decrease the footprint, the authors suggested reducing the amount of fertilizers and using green electricity such as photovoltaic panels. The coffee bean yield should remain similar with 10 – 20% less fertilizer. In our case study, changing the type of electricity is not necessary because French electricity is mainly derived from nuclear energy, which is considered carbon-free.[244] However, every step of the bioactive molecule extraction processes requires electricity. For both SWE and SC-CO_2 extractions, freeze-drying steps significantly impacted the carbon footprint (Figure C6-4). While these steps are essential for SC-CO_2 extraction, the first freeze-drying step can be removed for the SWE process. Technically, AP was dried for transport between the EDYTEM and DSTF labs, but SWE was considered performed in the EDYTEM lab. Therefore, eliminating the first drying step and keeping the AP moist could significantly reduce the carbon footprint from 6.20 to 4.43 kg_{CO2eq}/g of bioactive compounds. This also means it would not be necessary to freeze and grind the AP, potentially further reducing the carbon footprint.

Like the first freeze-drying step, the use of EtOH has a significant impact (Figure C6-4). Several studies have found that the solvent plays a crucial role in the carbon footprint.[233,251] Barjoveanu *et al*. found that EtOH had a significant impact on the process carbon footprint but they managed to reduce it by recovering and reusing the solvent.[251] To further reduce or eliminate the EtOH footprint, membrane filtration methods such as ultrafiltration and nanofiltration can be employed. These methods are carried out using aqueous solutions, eliminating the need for organic solvents like EtOH. However, due to insufficient data on the carbon footprint associated with membrane use, definitive conclusions about their potential benefits cannot be drawn at this time. Alternatively, MeOH can replace EtOH. For instance, Zaib *et al*. conducted an LCA comparing the synthesis of compounds using different solvents.[105] They found that the lowest carbon footprints for the synthesis of 200 g of acetophenone were attributed to MeOH (0.590 kg_{CO2eq}), EtOH (0.967 kg_{CO2eq}), and DES (1.82 kg_{CO2eq}). In contrast, the carbon footprints for DCM and ethyl acetate were 8.23 and 3.60 kg_{CO2eq}, respectively. In our case study, while solvent impact will still be present, it could be reduced by using MeOH,

for example. However, this solvent has a health score of 7 which is problematic for it use.[256] EtOH is preferable for this score. Another solution is to use water instead of EtOH as a co-solvent for SC-CO_2 extraction, which offers two major benefits: reducing the carbon footprint associated with EtOH and eliminating the carbon footprint of the first freeze-drying step by keeping AP wet. However, new extraction tests would be required, and the moisture content would need careful control.

The extraction yield significantly impacts climate change. An attempt was made to modify the yield obtained using the SC-CO_2 process to achieve 800 mg of bioactive compounds, resulting in a carbon footprint of approximately 6.20 kg_{CO2eq}/g of bioactive compounds, similar to that obtained via SWE. The primary aim of SC-CO_2 extraction is to recover apolar molecules such as wax.[70] Ferrentino *et al*. and De La Peña Armada *et al*. extracted bioactive compounds from AP using SC-CO_2 with higher mass yields than in our case under similar conditions.[92,97] Their LCA could differ significantly from ours due to variations in biomass composition, which is influenced by factors such as weather, soil conditions, and plant variety.[21,111] These are largely uncontrollable parameters. As mentioned, the target molecules in the studies by De Marco *et al*. and Ferreira *et al*. were mainly apolar, whereas in our study, apolar molecules (wax) were removed due to their lack of biological activity, leading to a decrease in mass yield and an increase in carbon footprint.[247,248] If only the mass yield were considered, the carbon footprint would be lower.

Numa *et al*. extracted bioactive compounds from AP using SC-CO_2 extraction under similar operating conditions to ours and found this extraction to be economically viable through an economic analysis.[12] However, further economic analysis is required to assess the viability of our process. As mentioned, results can vary significantly with similar biomass due to unpredictable factors such as soil conditions, weather, and plant variety.

At last, the LCA data for extraction from biomass or waste found in the literature are rarely presented in a consistent manner (*e.g.*, calculated per gram of a target molecule, per batch extracted, *etc*.). Standardizing these data would be valuable to enable the generalization of results concerning the raw materials studied and the processes employed, facilitating easier comparison of systems and highlighting the main trends and areas for further investigation. In this study, we aimed to achieve such standardization and identify the best ways to compare our findings with existing literature (Table C6-4). This approach allows for a clearer

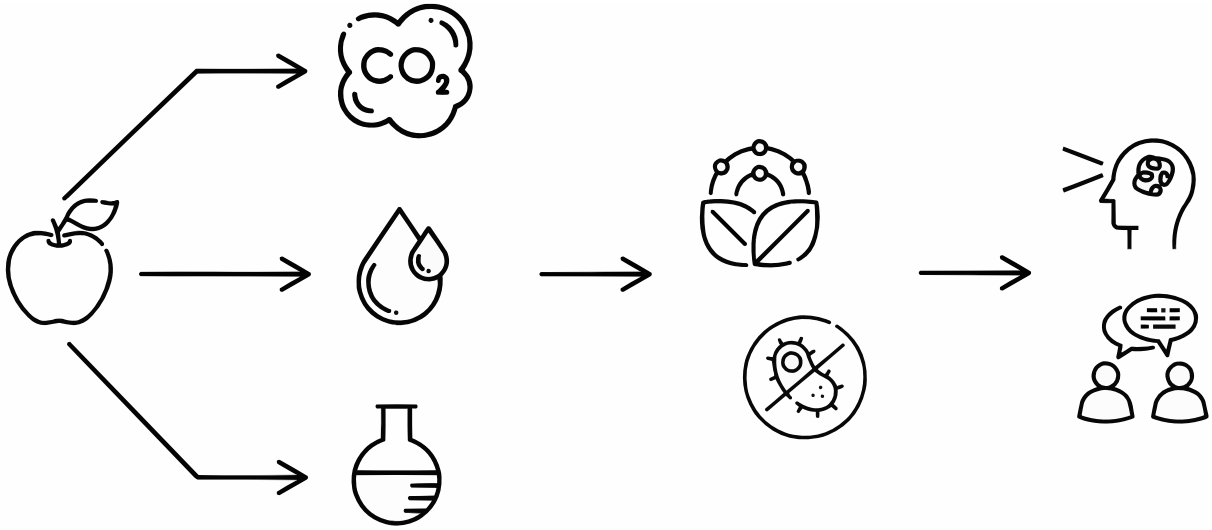
understanding of the environmental impacts associated with different extraction methods and aids in identifying the most sustainable practices.

VII.Conclusion

Several companies and researchers are currently working on the valorization of AP. Life Cycle Assessments (LCA) have been conducted on various methods for valorizing this waste. However, to our knowledge, none has yet addressed the valorization of biomolecules using green technologies, particularly SC-CO_2 and SWE methods. Nonetheless, several researchers are investigating the extraction of biomolecules from AP and even other types of waste for their biological activities, such as antioxidant, antimicrobial and anti-inflammatory properties. This study has highlighted the significant environmental implications of extracting bioactive compounds from AP using SC-CO_2 and SWE technologies. The LCA results indicate that the SWE process exhibits a substantially lower carbon footprint (6.20 kg_{CO2eq} per 1 g of bioactive compounds) compared to the SC-CO_2 process (71.42 kg_{CO2eq} per 1 g). There is still considerable potential for further reducing the environmental impact. The mass yield of bioactive compounds from the SWE process was 16 times greater than that of the SC-CO_2 process, partially explaining the lower carbon footprint of the SWE process. These findings suggest that current methods of bioactive molecule extraction at laboratory scale, and potentially at semi-industrial and industrial scales, may not be as environmentally friendly as previously assumed. By using wet AP, the carbon footprint of the SWE process could be further reduced by eliminating the initial freeze-drying and grinding steps, potentially lowering the footprint to below 4.43 kg_{CO2eq}. Therefore, it is recommended to explore alternative extraction techniques, such as the use of deep eutectic solvents, which could offer more eco-friendly alternatives compared to sub- and supercritical fluids. Both processes need to minimize or eliminate the use of EtOH, potentially replacing it with water, MeOH, or employing membrane techniques. The final carbon footprints observed in this study are consistent with those reported in the literature. However, even though the carbon footprint from our study is comparable to those found in the literature, this does not necessarily indicate that our extraction methods are favorable for scale-up and industrial applications. As suggestion, others valuable molecules could be extracted from AP such as carbohydrate polymers (pectin, lignin, cellulose, *etc.*), which occur in greater abundance than bioactive compounds and impacting the yield.

In conclusion, this research significantly contributes to our understanding of the environmental impacts associated with bioactive compounds extraction methods. It underscores the importance of continuing to develop more sustainable and environmentally friendly extraction processes. The results of this study will guide future research and industrial practice, steering towards more environmentally friendly and efficient solutions.

General conclusion and Perspectives



In this part of the manuscript, a general conclusion about the observation and results are presented. According to the Total Polyphenols Content (TPC) and the DPPH Radical Scavenging Capacity (DRSC), the better green technique for bioactive compounds is subcritical water extraction (SWE). The SC-CO_2 and Natural Deep Eutectic Solvents (NADES) extractions followed respectively their antioxidant properties. However, the antibacterial activity of extracts obtained by SWE is similar to extracts obtained by acid-based NADES. The antibacterial activity from NADES and their extracts were mainly attributed to the acidity of NADES. Future experiments were discussed to improve the study of AP with green extractions.

Human beings increase their consumption over years impacting environment and biodiversity. In 2009, Rockström and 28 scientists identified factors for the regulation of stability et resilience of Earth, called *planetary boundaries*. There are 9 planetary boundaries, novel entities, stratospheric ozone depletion, atmospheric aerosol loading, ocean acidification, biogeochemical flows (nitrogen and phosphorus), freshwater change (freshwater use and green water), land-system change, biosphere integrity (genetic and functional) and climate change (CO_2 concentration and radiative forcing). By 2009, only 3 planetary boundaries had been crossed, and by 2023, 6 had been breached. To limit our consumption, it is necessary to find illimited resources such as waste, moving away from linear economy to embrace circular economy (CE). According to FAO, 14% of food were lost between harvest and retail levels and 17% of food is wasted between retail and consumers levels in 2019.

The CE concept has gained popularity worldwide over the last decade. It is based on seven pillars in three areas, the main objective being the prevention of waste generation: (1) supply by economic actors (extraction, production and sustainable supply chain; eco-design; industrial and territorial ecology; functional economy), (2) consumer demand and behavior (product life extension; responsible consumption) and (3) waste management (recycling and valorization of materials and organic matter) (Introduction, Figure I-1).

In our case study, the waste is apple pomace (AP). This waste was selected due to its quantity and the fact that apples are produced in Savoie and Piedmont. The biological applications for the AP extracts are antioxidant and antibacterial activities. Green solvents (or eco-solvent) were selected for their low carbon footprint on the environment (Chapter 1). Several new solvents have been and continue to be developed with an easy transfer from fossil-based organic solvent to green solvent and be less expensive (Chapter 1, Figure C1-2).

Supercritical CO_2 (SC-CO_2) is considered as green solvents due to CO_2, which is a non-toxic, non-flammable, inexpensive gas and available in large amount. SC-CO_2 extraction was the first eco-solvent used for the molecules extraction from AP. A Design of Experiment (DoE) was conducted in order to maximize the mass yield of extracts which ranging between 0 and 1.45%. The results for the optimal conditions are 78 °C, 287 bar and 12 $g_{CO2}.min^{-1}$, 5%$_{EtOH}$, 1 h with AP particles size of 500 nm. To preserve bioactive compounds, the temperature was reduced to 50 °C. Ultrasound (US) pretreatment was used to improve the mass yield and the biological activities of extracts. The mass yield and the Total Polyphenols Content (TPC) increased respectively by 12.90 and 10.4% (Chapter 4, Figures C4-2 and C4-7). Crude and US

pretreatment extracts exhibited less than 50% of DPPH at 50 $mg_{Extracts}/mL_{MeOH}$, respectively 38.16 ± 3.63 and 45.78 ± 3.68%$_{inhibition}$. The lower EC_{50} indicates higher antioxidant activity of extracts meaning that smaller quantity is needed to inhibit 50% of DPPH. The EC_{50} values of both extracts obtained by SC-CO_2 were under 50% leading to the incapacity to measure EC_{50}. However, extracts obtained by the supercritical gas contained between 1.04 and 1.13 mg Trolox® Equivalent (TE)/$mg_{Extracts}$ (Chapter 4, Table C4-5). The antibacterial activity of extracts obtained by SC-CO_2 was not observed on Petri dish, and DMSO exhibits antibacterial property according to the microplate tests (solvent effect). To conclude about this eco-solvent, the mass yield and antioxidant activity were improved attributed to the US pretreatment. Other pretreatments and antibacterial assays should be tested and another solvent should be evaluated to avoid solvent effect. For pretreatment, enzymatic pretreatment can be tested with cellulase and/or pectinase.

The second green solvent was water. The safety of water is similar to CO_2. Water is cheap, abundant, non-toxic, *etc*. However, its extraction capacity at room temperature and pressure is low for polyphenols. Subcritical water extraction (SWE) was selected to tune water. Furthermore, SWE is a complementary technique to SC-CO_2 extraction. A DoE was conducted to maximize the mass yield of esxtracts with a range between 40 and 60%. The results for the optimal conditions are 157 °C, 5 bar_{N2} and 1:8 (AP:Water, *w/v*), 23.4 min with AP particles size of 500 nm. However, the temperature was reduced to 140 °C because AP was burned above 150 °C according to preliminary extraction tests. The same pretreatment as SC-CO_2 was used to improve biological activities of extracts in different conditions of pretreatment. The TPC value of *US pretreatment* extract was higher by 25.5% compared to *without pretreatment* extract (Chapter 4, Figure C4-9). Nevertheless, the antioxidant activity measured by DPPH Radical Scavenging Capacity (DRSC) was lower by 13.8% at 15 $\mu g_{Extracts}/mL_{MeOH}$ (Chapter 4, Figure C4-10). US pretreatment extracted more polyphenols but degraded extracts. Despite the damage of antioxidant activity by the US, antibacterial activity from US pretreatment was higher, between 2 – 6% depending on strains. As perspective for SWE studies, the suggestions include changing the mathematical model for DoE, using another US system, pretreating with enzymes, *etc*.

According to Figure C1-2 (Chapter 1), the tested green solvents were water (SWE), supercritical and subcritical fluids (SC-CO_2 and SWE) and switchable solvents (SC-CO_2 and SWE). To complete the list of eco-solvents from Figure C1-2 (Chapter 1), NAtural Deep Eutectic Solvent

(NADES) was chosen. NADES belongs to several categories: Deep Eutectic Solvent, bio-based solvents, switchable solvent (depending on the extraction methods), and ionic liquid (promising physicochemical properties like NADES). A total of 6 NADES were studied, including 4 organic-acids and 2 urea as hydrogen bond donor (HBD) and choline chloride (ChCl) as hydrogen bond acceptor (HBA) were used to synthesize. All NADES were diluted with 20% of water (*w/w*) expect one (ChCl:U). The extraction conditions were 45 min, 50 °C, 1:10 (AP:NADES, *w/w*) with AP particle size of 500 nm. All extracts exhibited antioxidant (TPC and DRSC) and antibacterial activities. Organic-based NADESs demonstrated strong and effective biological properties compared to urea-based ones. The Total Anthocyanidins Content (TAC) was measured due to the coloration of extracts. For acidic NADESs, the extracts were red while alkaline ones were yellow-green. TAC values showed that organic-based solvents extracted more pigments than urea-based solvents. A correlation between TAC, EC_{50} and viscosity values was observed suggesting that anthocyanidins exhibited the antioxidant properties of extracts and low viscosity helps for their extraction (Chapter 5, Table C5-3). All NADESs demonstrated antibacterial activity against all the strains. However, the antibacterial property was mainly attributed to the solvent itself (Chapter 5, Table C5-5). To observe only the extracted molecules, separation between NADES and bioactive molecules is necessary. For future NADES studied, the combination of technologies should be tested. For instance, instead of using classic organic solvents (hexane, ethanol, *etc*.) with US, microwave, SFE, NADES can easily replace conventional solvents.

With all this information, comparison between the eco-solvents is possible. According to Figure GC-1 and Table GC-1, SWE extracted more polyphenols and exhibited higher antioxidant activity (DRSC) compared to the other solvents. The tested NADESs were the lowest green solvents to extract polyphenols according to TPC and DRSC values. As mentioned above, solvents NADES demonstrated antioxidant activity, sometime higher than extracts (ChCl:OA). In conclusion, the more effective extraction is SWE regarding the antioxidant activities. Extracts obtained by SWE showed higher TPC and DRSC values (Table GC-1).

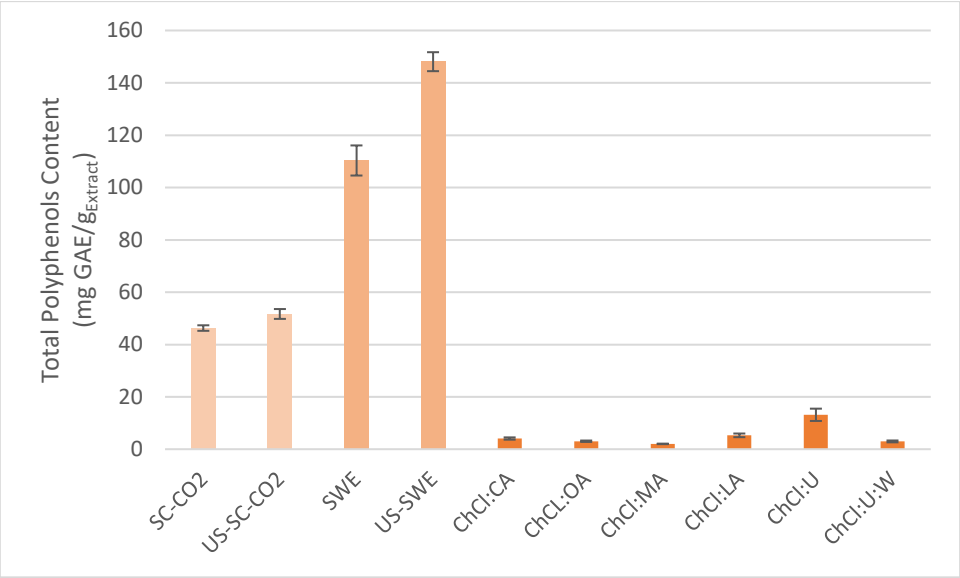


Figure GC-1. Total Polyphenols Content of AP extracts in mg GAE/$mg_{Extract}$.

Table GC-1. Summary of EC_{50} values of DPPH Radical Scavenging Capacity and Total Polyphenols Contents values (antioxidant activity) of AP extracts.

Samples	DRSC EC_{50} ($\mu g_{Samples}/mL_{MeOH}$)		TPC (mg GAE/$g_{Extracts}$)	
	Extracts	Solvents	Extracts	Solvents
$SC\text{-}CO_2$	-[a]	-[b]	46.30 ± 2.11	-[b]
$US\text{-}SC\text{-}CO_2$	-[a]	-[b]	51.68 ± 3.77	-[b]
SWE	6.6 ± 0.7	-[b]	110.33 ± 11.5	-[b]
$US\text{-}SC\text{-}CO_2$	7.6 ± 1.8	-[b]	148.08 ± 7.25	-[b]
ChCl:CA	425,280 ± 51,220	441,340 ± 25,370	4.09 ± 0.87	2.00 ± 0.56
ChCl:OA	35,590 ± 9,530	21,550 ± 1,400	3.05 ± 0.53	2.33 ± 0.16
ChCl:MA	268,090 ± 26,350	656,920 ± 72,850	2.04 ± 0.19	1.20 ± 0.11
ChCl:LA	308,060 ± 56,380	1,930,440 ± 140,290	5.30 ± 1.40	1.67 ± 0.47
ChCl:U	176,710 ± 16,820	-[b]	13.15 ± 4.70	1.36 ± 0.34
ChCl:U:W	111,910 ± 25,060	-[b]	2.98 ± 0.72	1.32 ± 0.13

[a]Antioxidant activity observed under $50\%_{inhibition}$; [b]Solvent effect not observed.

The values of Minimum Inhibitory Concentration (MIC) and EC_{50} against *Listeria innocua*, *Escherichia coli* and *Bacillus cereus* are summarized in Table GC-2. The MIC and EC_{50} of extracts obtained by SC-CO_2 were not observed due to the solvent effect from DMSO. For extracts obtained by NADES and SWE, MIC and EC_{50} values were observed. Only for ChCl:U and ChCl:U:W, MIC and EC_{50} values were not measured due to the lack of antibacterial property of extracts and solvents. Against *L. innocua* and *B. cereus*, extracts obtained by SWE exhibited higher antibacterial activity than extracts obtained by NADES. Against *E. coli*, ChCl:OA demonstrated higher antibacterial property mainly attributable to the solvent. To conclude the antibacterial activity of AP extracts, extracts obtained by SWE and NADES exhibited similar MIC and EC_{50}. However, extracts obtained by SWE being dried do not exhibit solvent effect compared to NADES extracts. Thus, for extracts obtained by SWE, only the activity from bioactive molecules was observed.

The thesis focused on the valorization of AP using *Green Technologies* such as eco-solvents. To answer to that question, Life Cycle Assessments (LCA) of bioactive compounds extraction from AP using SC-CO_2 and SWE were conducted thanks to the expertise of Carla Marty, a Master's student intern supervised during the PhD thesis. The carbon footprint of SC-CO_2 and SWE processes were respectively 71.42 and 6.20 kg_{CO2}/g of bioactive compounds for a cradle-to-gate analysis. These findings suggest that current extraction methods at laboratory scale, and potentially at semi-industrial and industrial scales, may not be as environmentally friendly as assumed for molecules from AP. These green techniques may not be suitable for this biomass. However, literature suggests that SC-CO_2 extraction for AP is economically viable. For NADES extraction, the LCA was not conducted due to the lack of information about the carbon footprint of NADES components and the mass yield of molecules leading to overly significant approximations.

Table GC-2. Summary of EC_{50} and MIC (antibacterial activity) of AP extracts.

Samples	*Listeria innocua*				*Escherichia coli*				*Bacillus cereus*			
	EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)		EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)		EC_{50} (mg_{Sample}/mL)		MIC (mg_{Sample}/mL)	
	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents	Extracts	Solvents
SC-CO_2	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
US-SC-CO_2	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
SWE	22.0 ± 1.2	-[b]	50	-[b]	15.9 ± 3.1	-[b]	50	-[b]	11.3 ± 4.7	-[b]	50	-[b]
US-SWE	19.3 ± 4.3	-[b]	50	-[b]	11.8 ± 2.3	-[b]	50	-[b]	14.6 ± 2.6	-[b]	50	-[b]
ChCl:CA	29.9 ± 3.0	24.1 ± 0.3	50	50	22.5 ± 0.2	20.3 ± 0.9	50	50	17.2 ± 1.3	17.4 ± 1.2	50	50
ChCl:OA	23.2 ± 3.2	18.8 ± 1.5	50	50	19.4 ± 1.7	17.2 ± 0.7	50	50	6.0 ± 0.3	5.8 ± 0.1	25	25
ChCl:MA	28.9 ± 1.0	30.7 ± 1.4	50	50	25.5 ± 1.1	24.3 ± 0.4	50	50	19.4 ± 0.8	20.3 ± 0.7	50	50
ChCl:LA	34.3 ± 9.9	35.8 ± 1.7	75	75	40.8 ± 0.7	36.9 ± 3.4	100	100	30.5 ± 0.8	36.5 ± 3.8	75	75
ChCl:U	47.2 ± 11.5	106.4 ± 8.8	150	150	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]

ChCl:U:W	90.3 ± 1.1	130.5 ± 10.7	150	150	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]	-[c]

[a] Solvent effect observed; [b] Solvent effect not observed; [c] Antibacterial activity observed (< 90%)

To conclude this manuscript, several improvements can be made. For both SFE techniques, another mathematical model should be tested as the lack-of-fit demonstrates that Central Composite Rotatable Design is not adequate. Furthermore, DoE should consider biological activity to determine optimal conditions. To increase polyphenols extraction with SWE, another ratio (AP:Water) should be tested. For both SFE, and especially for SWE, another pretreatment should be performed such as enzymatic pretreatment. US pretreatment decreases the antioxidant activity for SWE (DRSC). For NADES extraction, the absence of mass yield, or the biological property of solvent necessitates the separation NADES and polyphenols. To avoid additional post-extraction steps, ready-to-use NADES should be considered. These solvents should be more attractive to compagnies. The next step for LCA is to calculate the carbon footprint of NADES extraction to complete the comparison between eco-solvents. To further reduce SWE carbon footprint, AP should not be dried before extraction. The last but not the least, solvent-free extraction should be tested. AP contains 75 – 85% of moisture and the content water into biomass can be used as solvent.

Appendix

Theoretical bacterial growth kinetics and determination of growth phase: associated with Chapter 2 (*Materials and Methods***), Chapter 4 (***Antioxidant and Antibacterial Activities of Bioactive Compounds Extracted from Apple Pomace with Subcritical and Supercritical Fluids***) and Chapter 5 (***Extraction of Polyphenols and Anthocyanins from Apple Pomace with Natural Deep Eutectic Solvents: Evaluation of their Antioxidant and Antibacterial Activities***).**

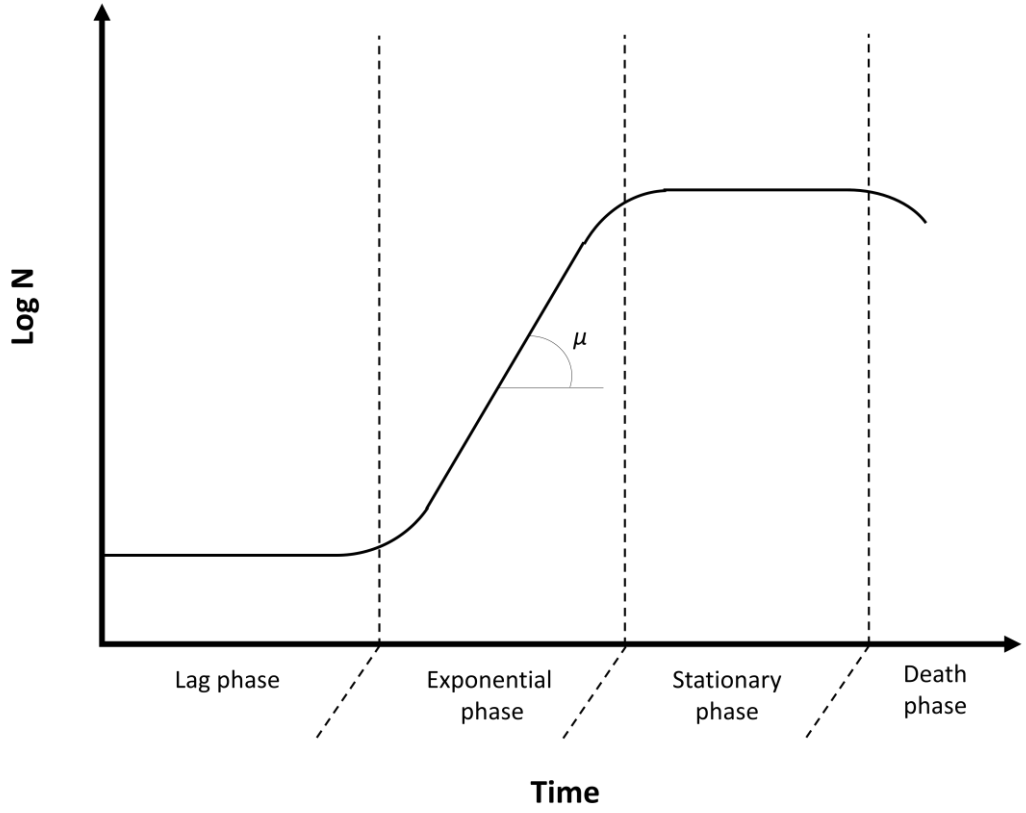


Figure A-1. Theoretical curve bacterial growth.

The **lag phase** is when bacteria are adapting to their new environment. During this phase, they synthesize the necessary molecules for growth.

In the **exponential** (log) **phase**, bacteria have adapted and begin to grow and divide rapidly depending on their optimal conditions such as temperature, oxygen, and pressure.

In the **stationary phase**, the growth rate slows down because nutrients become scarce. The number of new cells created is roughly equal to the number of cells dying, leading to a stable population.

During the **death phase**, unfavorable conditions like nutrient depletion and toxic waste accumulation cause the death rate of bacteria to exceed the growth rate, resulting in a decline in the population.

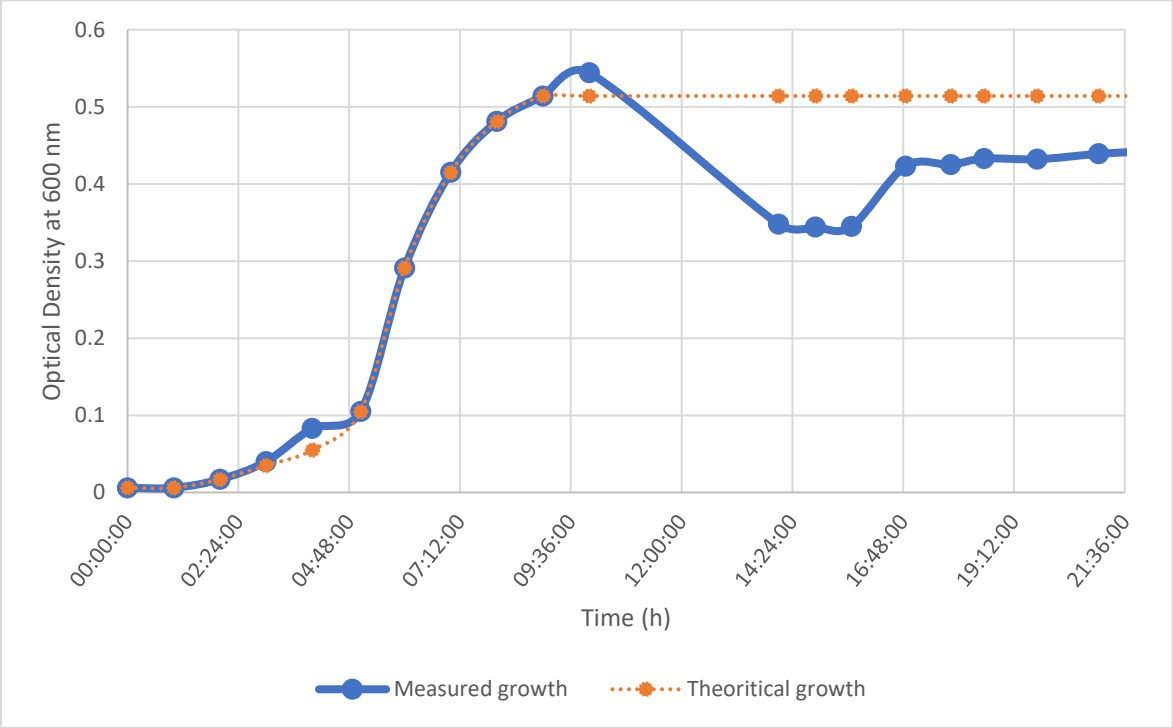


Figure A-2. Growth kinetic of *Escherichia coli*.

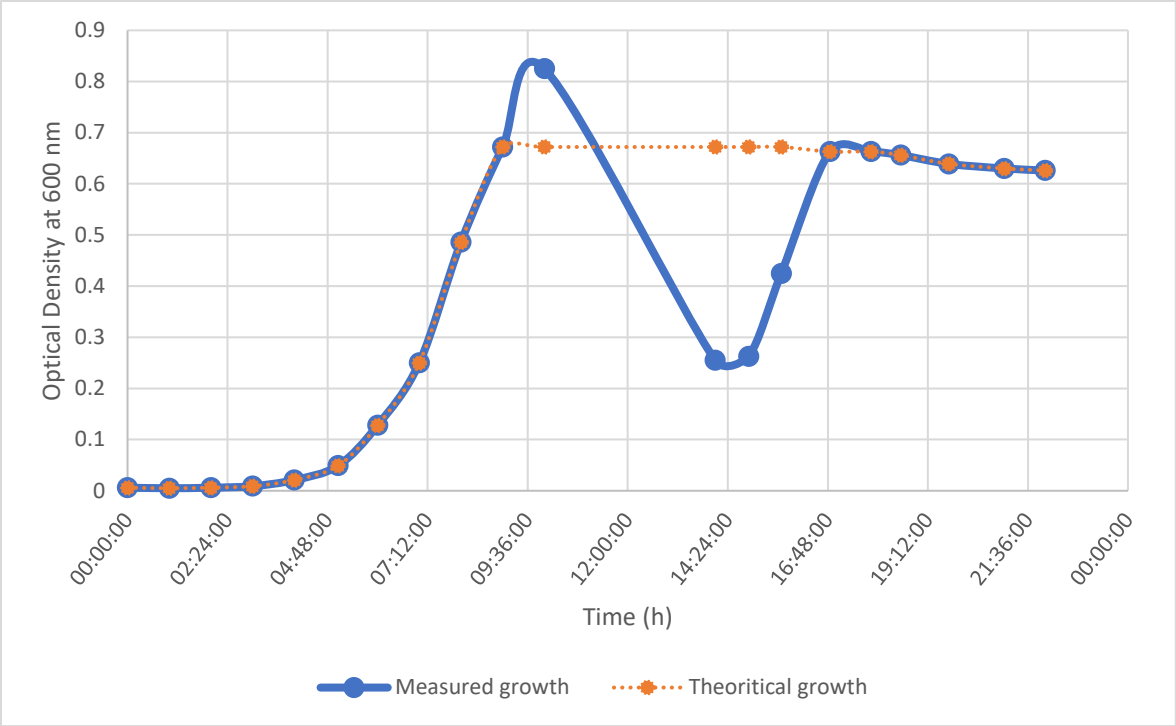


Figure A-3. Growth kinetic of *Bacillus cereus*.

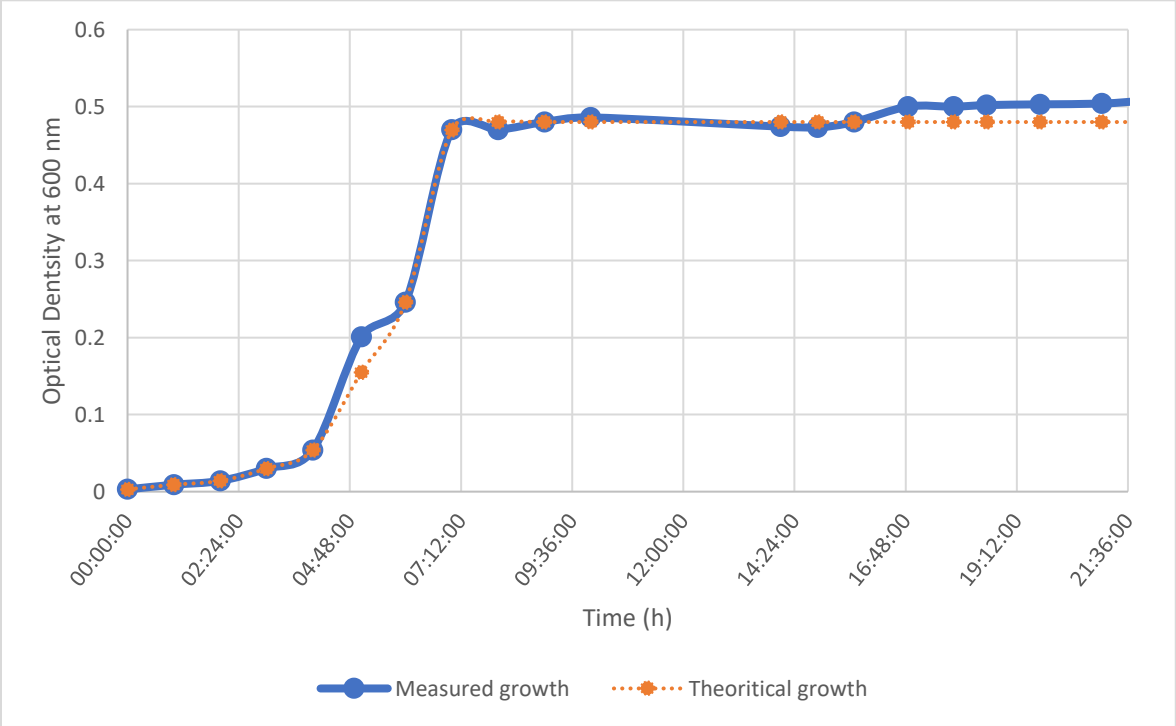


Figure A-4. Growth kinetic of *Listeria innocua*.

Table A-1. Summary of Exponential phase of each strain used in this study.

Strain	Start (h)	Finish (h)
Escherichia coli	5	7
Bacillus cereus	7	8
Listeria innocua	5	7

Responses Surfaces from the Design of Experiment using SC-CO_2 extraction technique: associated with Chapter 3 (*Optimal Conditions of Subcritical and Supercritical Fluids Extraction for Bioactive Compounds from Apple Pomace***).**

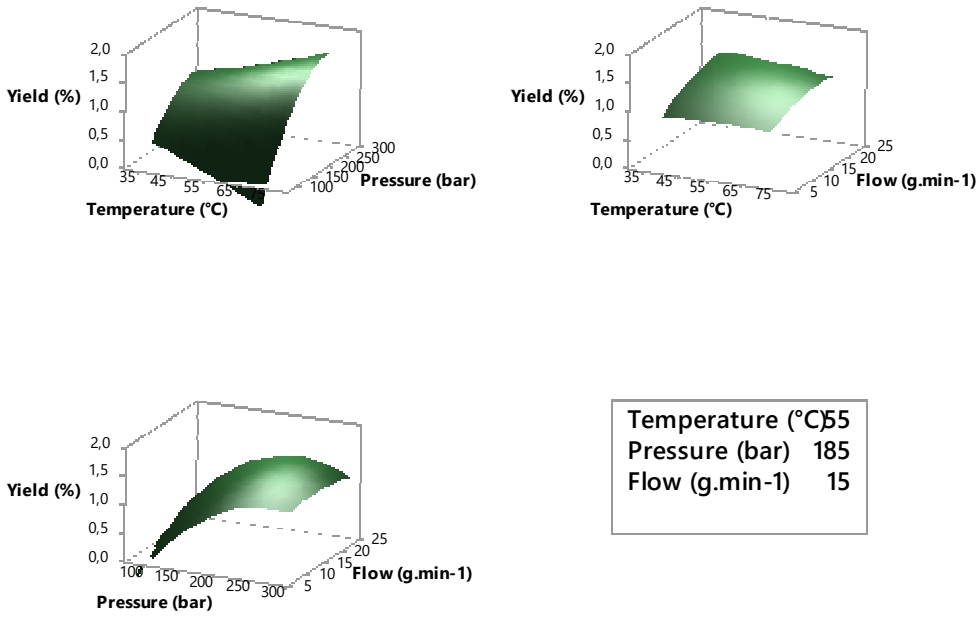


Figure A-5. Response surfaces of interactions of temperature/pressure (left top) at 15 g.min^{-1}; temperature/flow (right top) at 185 bar; and pressure/flow (left bottom) at 55 °C for yield extraction with SC-CO_2 extraction.

Responses Surfaces from the Design of Experiment using SWE technique: associated with Chapter 3 *Optimal Conditions of Subcritical and Supercritical Fluids Extraction for Bioactive Compounds from Apple Pomace*.

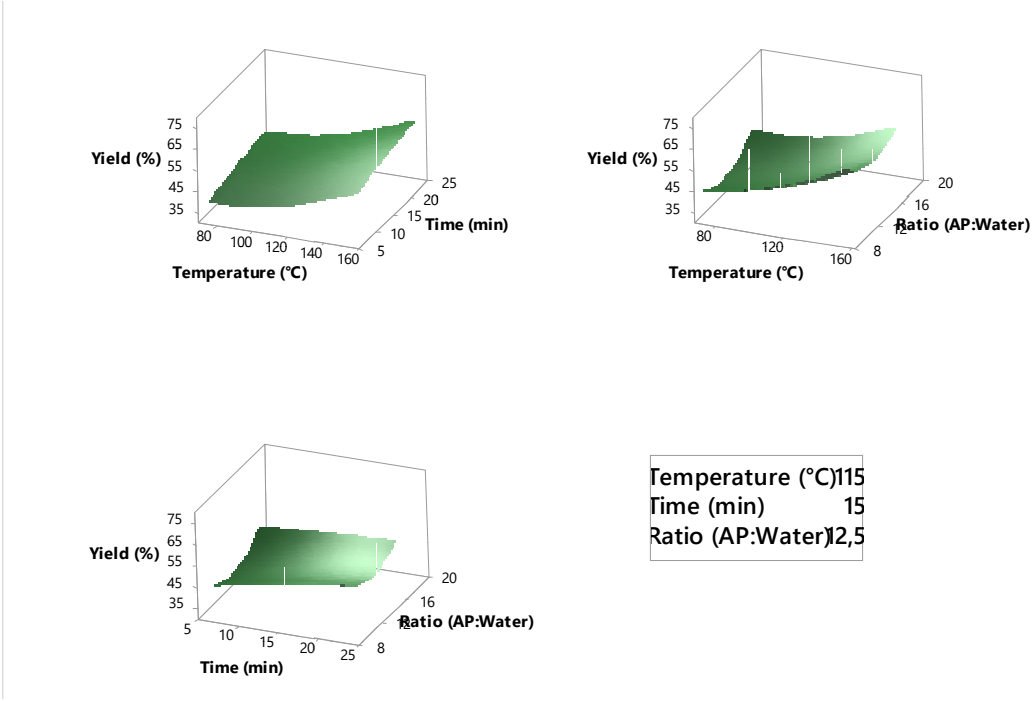
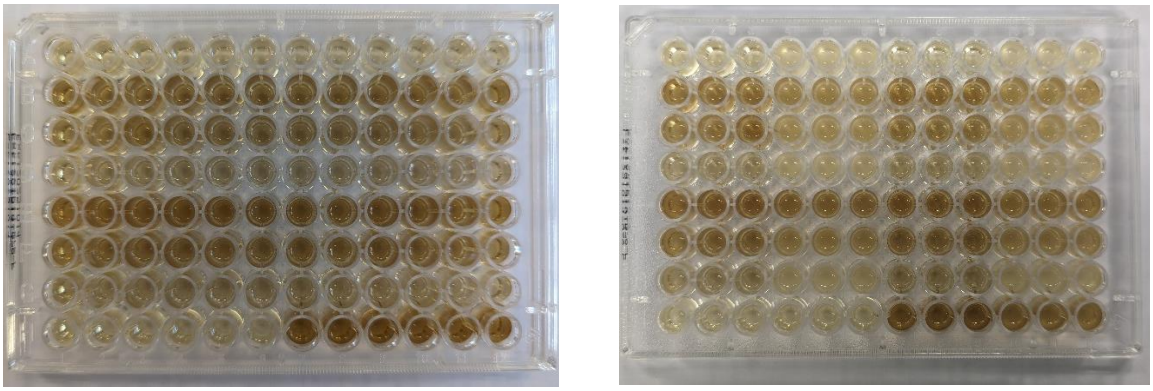


Figure A-6. Response surfaces of interactions of temperature/time (left top) at a ratio of 1:8 (AP:Water); temperature/ratio (right top) at 15 min; and time/flow (left bottom) at 115 °C for yield extraction with SCW extraction.

Antibacterial tests on microplate: associated with Chapter 4 (*Antioxidant and Antibacterial Activities of Bioactive Compounds Extracted from Apple Pomace with Subcritical and Supercritical Fluids***) and Chapter 5 (***Extraction of Polyphenols and Anthocyanins from Apple Pomace with Natural Deep Eutectic Solvents: Evaluation of their Antioxidant and Antibacterial Activities***).**



Before incubation overnight at 30 °C After incubation overnight at 30 °C

Figure A-7. Example of results in microplate; extracts obtained by SWE against *Escherichia coli*.

DRSC – Antioxidant test: associated wit Chapter 5 (*Extraction of Polyphenols and Anthocyanins from Apple Pomace with Natural Deep Eutectic Solvents: Evaluation of their Antioxidant and Antibacterial Activities***).**

By trying to remove NADES (Amberlyte XAD-16), it was possible to measure the EC_{50} of extracts even if there remains a small amount of solvent (Table S1). At this concentration, the solvent had no impact on antioxidant activity. The EC_{50} of extracts is substantially lower, resulting in a better antioxidant property after the sample clen-up. However, post-extractions steps may damage (caused by light, air *etc*.) the samples or quantity of NADES may be not exactly the same in each sample.

Table A-2. Antioxidant activity from extracts after removing solvents.

NADES	DRSC EC_{50} ($mg_{Extract}$/mL)	
	Before sample clean-up	After sample clean-up
ChCl:CA	425.28 ± 51.22	0.1085 ± 0.0095
ChCl:OA	35.59 ± 9.53	0.0815 ± 0.0104
ChCl:MA	268.09 ± 26.35	0.0849 ± 0.0044
ChCl:LA	308.06 ± 56.38	0.1910 ± 0.0077

Calculation of power effective for SC-CO_2 process: associated with Chapter 6 (*Comparative Life Cycle Assessment of Extraction of Bioactive Compounds from Apple Pomace using Subcritical and Supercritical Fluids***).**

Equation A-1. Calculation of power effective for SC-CO_2 process.

$$P_{effective_{SC-CO_2}} = \frac{P_{effective_{SCW}} \times P_{max_{SC-CO_2}}}{P_{max_{SCW}}}$$

$$P_{effective_{SC-CO_2}} = \frac{300 \times 9{,}000}{1{,}500}$$

$$\boxed{P_{effective_{SC-CO_2}} = 1{,}860\ W}$$

Calculation of quantity of N_2 used in SWE process: associated with Chapter 6 (*Comparative Life Cycle Assessment of Extraction of Bioactive Compounds from Apple Pomace using Subcritical and Supercritical Fluids***).**

Equations A-2. Calculation of quantity of N_2 used in SWE process.

$$n = \frac{PV}{RT}$$

Moles of N_2(g) per flush:

$$n_{flush} = \frac{100{,}000 \times 0.0008}{8.314 \times (21 + 273.15)}$$

$$n_{flush} = 0.0327\ mol$$

$$n_{flush\ total} = 0.0327 \times 3$$

$$n_{flush\ total} = 0.0981\ mol$$

Moles of N_2(g) per extraction:

$$n_{extraction} = \frac{500{,}000 \times 0.0008}{8.314 \times (21 + 273.15)}$$

$$n_{flush} = 0.1636\ mol$$

Total moles of N_2(g) per extraction:

$$n_{total} = n_{flush\ total} + n_{extraction}$$

$$n_{total} = 0.0981 + 0.1636$$

$$n_{total} = 0.2617\ mol$$

Total weight of N_2(g) per extract:

$$m = M(N_2) \times n$$

$$m = 28 \times 0.2617$$

$$\boxed{m = 7.33\ g}$$

Résumé en français – Summary of Manuscript in French

Selon la Commission européenne, plus de 2,609 millions de tonnes de déchets ont été produits par les habitants de l'Union européenne (UE) en 2020, dont environ 88 millions de tonnes de pertes alimentaires.[1,2] Le Conseil de l'UE vise à réduire de moitié le gaspillage alimentaire d'ici 2030. Pour atteindre cette réduction des déchets, le Conseil européen a lancé le *Pacte Vert Européen*. Ce pacte promeut une chaîne d'approvisionnement raccourcie de la production à la consommation, appelée de la « *ferme à la fourchette* ». Ce pacte détaille également des objectifs et un plan d'actions en faveur de l'économie circulaire (EC).[1]

Le concept d'économie circulaire a gagné en popularité dans le monde entier au cours de la dernière décennie. Il repose sur sept piliers dans trois domaines, dont l'objectif principal est de prévenir la génération de déchets dans le cadre d'une approche globale : (1) l'approvisionnement par les acteurs économiques (extraction, production et chaîne d'approvisionnement durable ; écoconception ; écologie industrielle et territoriale ; économie fonctionnelle), (2) la demande et le comportement des consommateurs (extension de la durée de vie des produits ; consommation responsable) et (3) la gestion des déchets (recyclage et valorisation des matériaux et de la matière organique) (Figure R-1).[3]

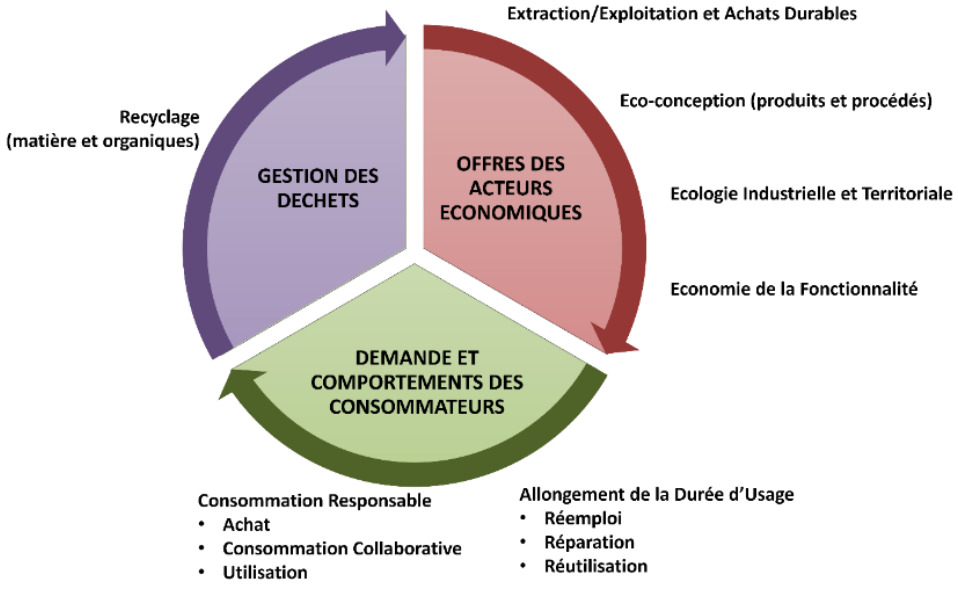


Figure R-1. Schéma de l'Economie Circulaire – ADEME.[3]

Conformément au développement durable, l'EC a un impact positif sur l'environnement et la société, offre de nouvelles opportunités en termes de comportement des consommateurs et conduit à un besoin d'innovation dans tous les domaines. L'EC est de plus en plus mise en avant dans le domaine de la chimie, par exemple pour le recyclage des batteries de voiture ou pour la production cosmétique. L'EC est au cœur du projet de valorisation. La biomasse choisie fait partie de la gestion des déchets et les processus d'extraction testés doivent être écoconçus.

Au début de ce travail, plusieurs critères ont été établis pour sélectionner une biomasse à étudier sur 3 ans, en tant que déchet à valoriser dans ce contexte de l'EC. Le premier critère est la disponibilité des déchets sélectionnés dans les territoires de Savoie et du Piémont, car ce travail de thèse s'inscrit dans le cadre d'une collaboration européenne, entre deux universités de l'Alliance *UNITA Universitas Montium*, l'*Université Savoie Mont-Blanc* et l'*Università di Torino, basées sur ces deux territoires ciblés*.[4] Le deuxième critère est la grande quantité de déchets disponible dans ces deux territoires afin d'être en adéquation avec l'EC.. Le dernier critère est la nécessité de trouver des molécules à haute valeur ajoutée dans la biomasse choisie. Pour le premier critère, un inventaire du secteur agriculture et agroalimentaire et de production de chaque région a été dressé. Pour la Savoie, les produits sélectionnés étaient les pommes, le vin et les crozets (type de pâtes), et pour le Piémont, les produits étaient le vin, le chocolat, le riz, les pâtes et la pomme. La production de pommes au Piémont n'est pas très connue internationalement, pourtant la production est plus élevée qu'en Savoie, environ 19%. En éliminant les produits qui ne correspondaient pas entre les deux régions, plusieurs déchets ont été sélectionnés : les déchets de fabrication de pâtes, de vignes et de pommes. Pour le critère de grande quantité (en poids), les pâtes ne produisaient pas suffisamment de déchets et n'ont pas été sélectionnés pour ce projet. Les deux derniers aliments produisent une quantité significative de déchets non valorisés dans les deux régions et contiennent des molécules à haute valeur ajoutée. Les déchets de vin n'ont pas été sélectionnés car plusieurs projets ont été réalisés et continuent d'être étudiés par les deux laboratoires, en particulier le projet *VITIVALO* à l'*Université Savoie Mont-Blanc*.[5] Avec toutes les informations recueillies, les déchets de pomme ont été choisis, appelé marc de pommes ou déchets de pommes pressées (*Apple pomace*). La production de pommes en Savoie et au Piémont est de 12 000 tonnes (en 2020) et 225 000 tonnes (en 2022), respectivement.[6,7] Une partie de cette production est transformée en produits à valeur ajoutée, tels que des tartes,

du cidre et du jus de pomme. Entre 16,25 et 19,5% de la production est transformée en jus de pomme ou cidre.[8] La production estimée de déchets de pommes est comprise entre 585 et 702 tonnes en Savoie et entre 10 968 et 13 162 tonnes au Piémont (Figure R-2).

Le moyen le plus courant de traiter ces déchets dans le monde entier est de les enfouir dans le sol. Cependant, cela peut poser de graves problèmes sanitaires et environnementaux.[9] Dans l'UE, une autre méthode est utilisée : la méthanisation. Les déchets de pommes sont partiellement recyclés avec cette solution. Le procédé génère de l'électricité et du biogaz. Selon *Philippe Bernot*, PDG de *Source du Verger*, il n'est plus possible en France de nourrir le bétail avec les déchets de pommes à cause de restrictions réglementaires et les exigences de sécurité sanitaire dans le pays.

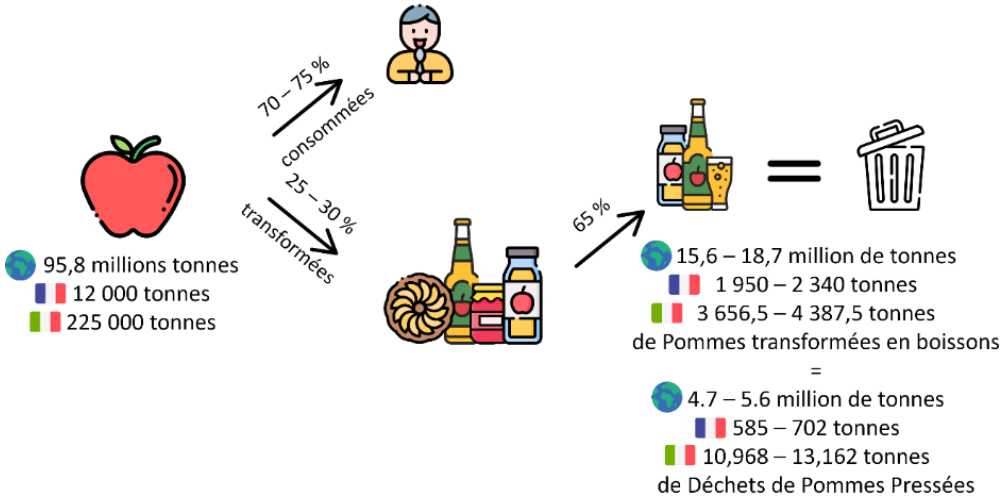


Figure R-2. Récapitulatif de la production mondiale, savoyarde et piémontaise de pommes et de déchets de pommes pressées.

Les déchets doivent pouvoir être tracés par exemple. La méthanisation est un processus qui peut traiter facilement plusieurs tonnes de déchets (selon la capacité de la structure) en produisant du biométhane. Cette méthode valorise le carbone et l'hydrogène de la matière organique mais pas les molécules à valeur ajoutée présentes dans les différentes biomasses. Le monde académique et l'industrie cherchent de plus en plus des moyens de recycler et/ou de valoriser les déchets de pommes. Depuis quelques années, des entreprises comme *SAMARA*, une marque nord-américaine, utilisent les déchets de pommes pour fabriquer du cuir imitation.[10] Les déchets de pommes peuvent être vendus simplement séchés et broyés. *HUBCYCLE* est une entreprise française qui vend divers types de déchets à des entreprises sous cette forme lyophilisée.[11] Sous cette forme, les déchets de pommes peuvent être utilisés

comme source de sucres ou de nutriments. L'*Université Aix-Marseille* et *Symrise* (entreprise en production de saveurs et parfums) ont récemment mené à bien une thèse sur la récupération de la cire présente dans les déchets de pommes à des fins cosmétiques pour éventuellement l'utiliser comme produits cosmétiques biosourcés, évitant l'ajout de conservateurs pétrochimiques.[12] Ces méthodes de récupération sont actuellement préindustrielles ou industrielles, mais ne sont pas pleinement développer.

Cependant, les polyphénols présents dans les pommes ont des propriétés biologiques très intéressantes. Ces biomolécules ont des activités antioxydantes, antidiabétiques, anticancéreuses, anti-inflammatoires et bien d'autres. Dans ce projet, intitulé *VAL'Apple*, les propriétés antibactériennes des phytomolécules des déchets de pommes ont été extraites avec des techniques innovantes et se voulant respectueuses de l'environnement. Une fois la biomasse sélectionnée, les techniques d'extraction ont été choisies en fonction de l'innovation et de la chimie verte (réduction des solvants, *etc*.). Plusieurs procédés d'extraction ont été identifiés, notamment les ultrasons, les micro-ondes et les fluides supercritiques entre autres. Les choix se sont portés sur les éco-solvants et notamment les fluides subcritiques (eau) et supercritique (CO_2) et des solvants bio-sourcés (Solvant Eutectique Profond Naturel) car chaque type de solvants verts offre une extraction sélective.

Ce manuscrit est divisé en 6 chapitres. Le premier chapitre est une revue de l'état de l'art des extractions utilisant les éco-solvants mentionnés ci-dessus extrayant des molécules d'intérêt pour leurs activités biologiques, notamment pour leurs propriétés antioxydantes et antibactériennes sélectionnées. Les données ont été mises à jour et une section sur les éco-solvants (solvants verts) a été ajoutée par rapport à l'article accepté en 2024. Le deuxième chapitre est consacré aux matériels et méthodes utilisés au cours de ces 3 années de doctorat. Les 3ème et 4ème chapitres se concentrent sur les extractions au CO_2 supercritique et à l'eau subcritique, avec l'optimisation des conditions d'extraction, l'amélioration des rendements massiques et des activités biologiques. Le chapitre 5 traite des activités biologiques des extraits en utilisant les Solvant Eutectique Profond Naturel (NADES) comme éco-solvant. En tant que dernier chapitre, une évaluation du cycle de vie a été réalisée pour déterminer la faisabilité de la récupération chimique de ces extraits et son impact environnemental. Enfin, une conclusion générale est donnée à la fin du manuscrit. Dans le cadre de la partie en langue française, un résumé de chaque chapitre a été écrit et/ou traduit excepté le 2ème chapitre portant sur les méthodes employées pendant 3 ans.

Au cours de la dernière décennie, le concept d'Economie Circulaire (EC) a gagné en popularité dans le monde entier. Comme mentionné précédemment, il repose sur sept piliers répartis en trois domaines dont l'objectif principal est de prévenir la génération de déchets dans le cadre d'une approche globale (Figure R-1).[3]

Les produits naturels peuvent être extraits de différentes manières : par extraction conventionnelle (ex. extraction Soxhlet, macération, *etc*.) ou par des procédés d'éco-extraction (ex. CO_2 supercritique, eau subcritique, extraction assistée par ultrasons, *etc*.) suivant les principes de la *Chimie Verte*.[16] Ces procédés représentent une grande opportunité d'évolution face au contexte environnemental et permettent d'abandonner ou au moins de réduire l'utilisation des procédés conventionnels pouvant être technologiquement obsolètes (coûts environnementaux et financiers élevés).

La pomme est l'un des fruits les plus produits dans le monde.[18] Selon l'*Organisation des Nations Unies* pour l'alimentation et l'agriculture (FAO), plus de 95,8 millions de tonnes ont été produites dans le monde en 2022.[19] Le plus grand producteur est la Chine, représentant depuis de nombreuses années environ la moitié de la production mondiale totale (Figure R-3). En 2022, la Turquie, les États-Unis et la Pologne sont d'autres grands producteurs, représentant respectivement 5,0%, 4,6% et 4,4% de la production mondiale. Selon Kammerer *et al.*, 25 à 30% de la production de pommes est transformée en produits à valeur ajoutée, principalement en jus.[8] Les déchets de pommes sont un résidu obtenu après pressage des pommes. Les déchets représentent 20 à 35% du poids frais (FW ; *Fresh Weight*).[20] Il se compose de 94,5% de FW de chair et de peau, de 4,1% de FW de graines et de 1,1% de FW de pédoncules.[21] La teneur en humidité est d'environ 75 à 80%.[9,21] Cette quantité non négligeable représente un déchet pouvant avec des répercussions dangereuses pour l'Homme et l'environnement s'il n'est pas correctement traité. Selon Bhushan *et al.*, la teneur en humidité favorise la décomposition microbienne, ce qui entraîne une fermentation imprévisible des déchets de pommes, et la matière organique hautement biodégradable entraîne des problèmes environnementaux et sanitaires.[9]

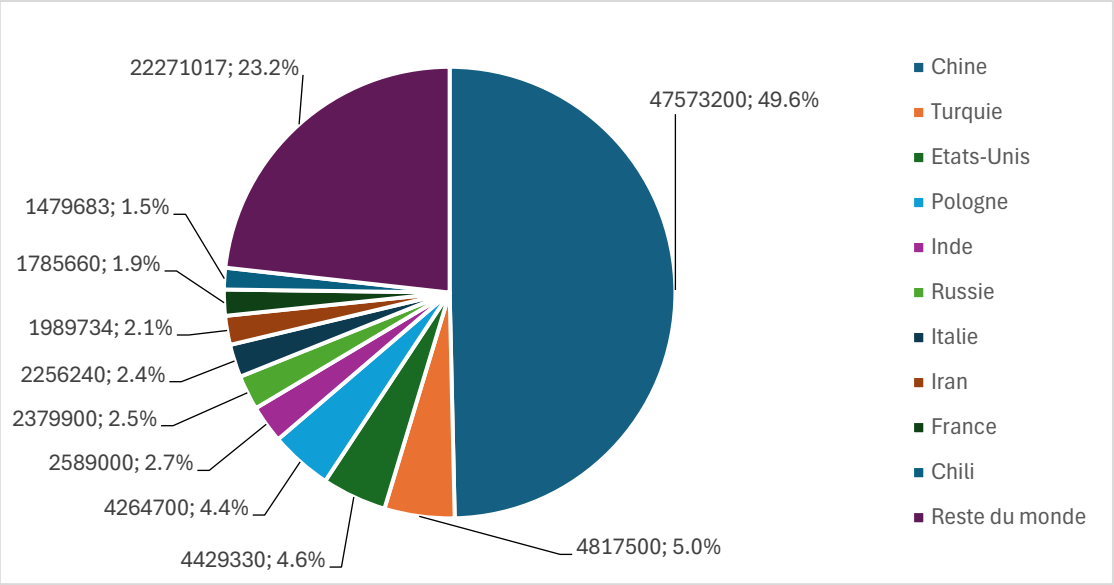


Figure R-3. Principaux producteurs de pommes dans le monde en 2022 (quantité en tonnes ;%).[19]

La littérature identifie plusieurs applications potentielles des déchets de pommes. Celles-ci comprennent l'alimentation animale, la source de carburant, le substrat pour la production d'éthanol par fermentation, le matériau de compostage et la matière première pour la production de biogaz dans les processus de traitement des déchets.[21] Plus récemment, la poudre de déchets de pommes a été utilisée pour créer du cuir vegan comme mentionné auparavant.[10,176] Une méthode importante pour la production de biogaz est la méthanation, applicable à divers types de déchets, y compris les résidus de culture comme les déchets de pommes, le fumier animal, les déchets solides municipaux et les eaux usées municipales. L'*Agence Internationale de l'Energie* (AIE) met en avant l'Europe comme principale région utilisant la méthanation. Cette prévalence est largement due aux politiques européennes favorables et aux incitations financières, faisant de l'Europe le plus grand utilisateur de cette technologie.[23]

Les déchets de pommes ne sont pas une exception dans ce contexte, car ils sont souvent traités dans des installations de production de gaz. Cependant, il est important de noter que les déchets de pommes contiennent des molécules de grande valeur, telles que les polyphénols, qui ont des activités biologiques significatives et des effets positifs sur la santé de l'Homme et des animaux.[24–26] Les principaux composés phénoliques dans les pommes comprennent des dérivés de quercétine, des dihydrochalcones, des mono-, di- et oligomères de flavanols, ainsi que des esters d'acide caféique et *p*-coumarique.[27,28] Les propriétés antioxydantes de ces

polyphénols ont été largement étudiées et documentées.[29,30] Le 1er chapitre de manuscrit de thèse est un état de l'art sur l'utilisation des éco-solvants en ne se concentrant pas sur l'activité antioxydante, mais sur d'autres propriétés biologiques telles que les activités antibactériennes, antifongiques et antivirales (activités antimicrobiologiques). Les Fluides Supercritiques (et subcritiques) reposent sur des propriétés physiques, sur la pression et la température. Les phases liquide et gazeuse deviennent une phase homogène au-dessus du point critique. Alors le solvant atteint son point supercritique (Figure R-4).

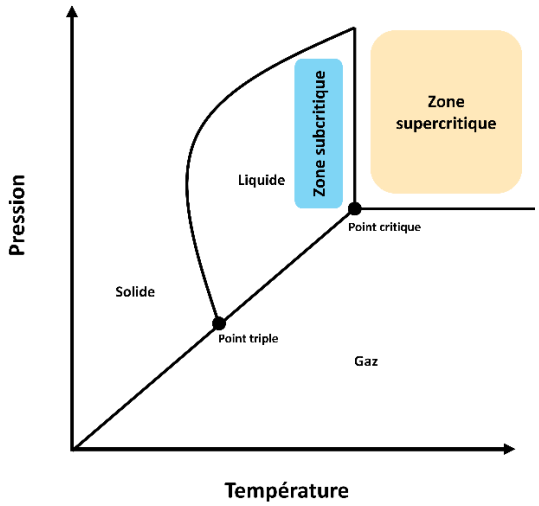


Figure R-4. Schéma de digramme de phase ; zone bleue : zone subcritique (ex. eau subcritique) ; zone jaune : zone supercritique (ex. CO_2 supercritique).

Depuis quelques années, l'Extraction par Fluides Subcritiques et Supercritiques (en anglais, *supercritical and subcritical fluids extraction* ; SFE) est une technique prometteuse pour extraire des molécules. Peu de processus industriels utilisent la SFE. De plus, la SFE permet d'extraire les composés phénoliques dans des conditions douces par rapport aux extractions conventionnelles. Les phytochimiques sont extraits à basse température (45 – 60 °C), ou à haute température (> 100 °C) mais pour une courte durée d'extraction (10 – 30 min).[86,153] Les techniques vertes peuvent protéger les polyphénols de l'air (O_2) et/ou de la lumière.[92,153,162] Les méthodes classiques utilisent une température élevée (ex. extractions par reflux ou Soxhlet) et/ou une longue durée d'extraction (ex. la macération), ce qui conduit à la dégradation plus rapide des polyphénols.[92,153] Le CO_2 supercritique (SC-CO_2) est représenté dans la zone jaune (Figure R-4). L'état de la matière du SC-CO_2 est atteint lorsque la température critique et la pression critique sont atteintes, au-dessus de 31,1 °C et 73 bar. Au-

dessus du point critique, le CO_2 a des propriétés intéressantes : la viscosité réagit comme un gaz (0,02 – 0,12 mPa.s à 40 °C) et la densité comme un liquide (700 – 1 100 $kg.m^{-3}$ selon la pression et la température).[70,163] Le CO_2 est un excellent solvant en raison de ses propriétés et de sa sécurité d'utilisation. Le solvant est non toxique, non inflammable, respecte la norme GRAS (« Generally Recognized As Safe » par la *Food and Drug Administration*), bon marché et est disponible. Le CO_2 est une molécule apolaire, et par affinité, ce solvant extrait les composés non-polaires tels que la cire, les lipides, l'huile, *etc*.[12,70,72–74] Comme mentionné dans les Chapitres 1 et 3, pour améliorer les extractions de molécules polaires comme les polyphénols, il est nécessaire d'utiliser un co-solvant tel que l'eau ou l'éthanol (EtOH).[153] Lorsque la concentration en EtOH dépasse 30%, le CO_2 devient saturé en EtOH et un ajout de co-solvant change drastiquement le point critique (Tableau C3-2).[96] Par conséquent, il est conseillé de ne pas utiliser plus que cette quantité. L'eau subcritique est une technique complémentaire au SC-CO_2.[70] Afin d'extraire différentes familles de molécules, l'extraction à l'eau subcritique (SWE) a été utilisée pour comparer la méthode d'éco-extraction au SC-CO_2 et observer les différences. L'eau subcritique est de l'eau liquide au-dessus de son point d'ébullition et pressurisée mais ces propriétés physicochimiques sont modifiées (Figure R-4, zone bleue). La température et la pression critiques sont respectivement de 374,15 °C et 221 bar.[70] Sous SWE, la diminution de la constante diélectrique permet l'extraction de plus de molécules par rapport à l'eau sous pression atmosphérique.[79,86–88,166] L'eau est un excellent solvant en raison de ses propriétés et de sa sécurité, tout comme le CO_2. Le solvant est non toxique, non inflammable, respecte la norme GRAS, bon marché et disponible en grande quantité. De plus, l'eau est une molécule polaire. Le but de la SWE est d'extraire des molécules polaires telles que les polyphénols, les glucides, les polysaccharides, *etc*.[70,83,86,87,98]

Le tableau R-1 présente quelques résultats issus de la littérature sur les extraits des déchets de pommes avec des activités antimicrobiennes avec le type d'extraction utilisé.

Tableau R-1. Exemples sélectionnés des activités antimicrobiennes (Tableau issu du Chapitre 1)

Types d'extraction	Quantité utilisée	Microorganisme testé	Référence
Enzymatique	Oven-dried: 1 – 3 mm (DIZ) > 100 mg/mL (CMI) Lyophilisé: 1 – 3 mm (DIZ) > 100 mg/mL (CMI)	*Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *MRSA*, *Escherichia coli*	[60]
Eau chauffée	11,50 ± 0,53 mm (DIZ)	*Escherichia coli*	[62]
	9,05 ± 0,71 mm (DIZ)	*Pseudomonas aeruginosa*	
	12,75 ± 0,71 mm (DIZ)	*Staphylococcus aureus*	
	14,00 ± 1,07 mm (DIZ)	*Enterococcus faeclis*	
Méthanol	710,9 ± 1,6 µg/mL (EC_{50})	HSV-1	[26]
Acétone	576,7 ± 17,2 µg/mL (EC_{50})		
Méthanol	629,6 ± 50,7 µg/mL (EC_{50})	HSV-2	
Acétone	450,7 ± 40,8 µg/mL (EC_{50})		
Extraction Soxhlet (Chloroforme), ultrason/MeOH (70%)	100 µg/mL	*Neosartorya fischeri*, *Botrytis* sp., *Petriella setifera*	[69]

DIZ: Diamètre de la zone d'inhibition; CMI: Concentration minimale d'inhibition; EC_{50}: Concentration efficace médiane; HSV: virus de l'herpes

Peu d'articles ont été publiés sur l'activité antimicrobiennes des déchets de pommes, et en particulier pour les extraits obtenus à l'aide des fluides subcritiques et supercritiques (Table R-1). Cela peut être dû au manque d'équipement ou de moyens pour les SFE et pour les tests microbiologiques pouvant limiter les deux utilisations dans un même procédé. Cependant, la récupération des déchets de pommes, un déchet significatif en quantité et disponible chaque année (4,7 à 5,6 millions de tonnes de déchets de pommes en 2022), reste une possibilité prometteuse dans un contexte d'EC et de valorisation des déchets. Comme rapporté dans le Chapitre 1, même si l'activité antioxydante des extraits des déchets de pommes par fluides subcritiques et supercritiques a été largement étudiée, les applications impliquant des activités antimicrobiennes issues de l'extraction des déchets de pommes doivent être explorées.

Les méthodes d'éco-extraction pour les composés bioactifs et les applications potentielles pour leur valorisation ont été étudiées dans le cadre de cette thèse. Les éléments de la biomasse étudiée changent en raison de paramètres contrôlables et incontrôlables, des conditions météorologiques et du sol, de la variété de la biomasse, *etc*.[21,111] Des plans d'expériences ont été réalisés afin d'extraire efficacement les composés bioactifs en utilisant le CO_2 supercritique (SC-CO_2) et l'eau subcritique (SWE). Plusieurs modèles mathématiques ont été rapportés dans la littérature, notamment le *Broken plus Intact Cell model*, le plan d'expériences composite centré (*Central Composite Rotatable Design* ; CCRD), le plan d'expériences de *Box-Behnken*, entre autres.[12,76,81] Dans nos études de cas, le CCRD a été utilisé pour les deux techniques. Pour chaque SFE, un total d'au moins 60 expériences a été réalisé et l'optimisation des conditions a été appliquée avec succès pour l'extraction des biomolécules.

Pour l'extraction par SC-CO_2, les conditions optimales sont de 287,7 bar, 71,8 °C et 12,7 $g_{CO2}.min^{-1}$. Dans ces conditions, la cire, l'huile et les polyphénols ont été extraits mais n'ont pas été différenciés. Dans le plan d'expériences de l'extraction par SC-CO_2, les conditions optimales ont été atteintes en fonction du maximum de rendement massique (%) incluant la masse de toutes les biomolécules extraites. Les auteurs de la littérature n'ont jamais distingué tous ces éléments. Pour éviter la dégradation des composés bioactifs, les extractions par SC-CO_2 sont fixées à 50 °C, 287,7 bar et 12,7 $g_{CO2}.min^{-1}$. Pour améliorer le rendement massique, les prétraitements ont été discutés. Plusieurs prétraitements existent, tels que l'enzymatique, les ultrasons (US), les micro-ondes, les NADES, *etc*. Le prétraitement enzymatique (avec cellulase et pectinase) a été envisagé (quelques expériences ont été réalisées) mais a été écarté en raison des contraintes de temps. Le prétraitement des US a été choisi pour plusieurs raisons. Tout d'abord le laboratoire *EDYTEM* est expert dans le domaine des ultrasons et deuxièmement plusieurs auteurs ont rapporté que les US étaient efficaces pour l'augmentation des rendements massiques et des activités biologiques.[187,188,190–195] Plusieurs systèmes US existent : cuphorn, sonde, bain, réacteur à sifflet, etc.[111,191,197,198] Chaque système a des avantages et des inconvénients (Table C4-3). Les conditions des prétraitements par ultrasons étaient de 10 min, 20 kHz et 50% d'amplitude en utilisant un système cup-horn fait maison. Le système cup-horn a été choisi pour son efficacité et sa capacité, avec notre réacteur construit au laboratoire ayant un volume de 1 L. Comme discuté ci-dessus, l'un des objectifs de l'utilisation du prétraitement US était d'améliorer le rendement massique des composés

bioactifs à partir des déchets de pommes avec l'extraction SC-CO_2. Le rendement massique était de 1,12 ± 0,07% sans prétraitement. Une augmentation de 12,90% a été observée avec le prétraitement US (Figure C4-2). L'amélioration du rendement massique peut être causée par plusieurs facteurs induits par les US. Pendant le prétraitement, la température est localement plus élevée en raison du flux acoustique et de la cavitation.[203] L'augmentation de la température semble être bénéfique pour l'amélioration du rendement massique. Un autre effet du prétraitement US est la rupture des parois cellulaires de la biomasse. Liu *et al*. ont analysé des graines d'*Iberis amara* par des images de microscopie électronique à balayage (SEM) après différents types d'extraction.[187] La biomasse traitée par prétraitement US était poreuse par rapport aux graines non traitées. La morphologie a changé en raison des bulles de cavitation induites par les US. La nouvelle porosité des graines permet au SC-CO_2 de pénétrer plus facilement dans la biomasse traitée, conduisant à une amélioration du rendement massique.[204] De plus, le traitement US réduit l'énergie de liaison entre les molécules (par exemple les polyphénols) et la biomasse végétale. Les US provoquent la désorption des molécules absorbées sur la matrice végétale en raison de l'effet de micro-agitation.[187] L'objectif d'amélioration du rendement massique a été atteint. L'activité antioxydante ($\%_{inhibition}$) des extraits de prétraitement US a augmenté par rapport aux extraits bruts, respectivement 45,78 ± 3,68 et 38,16 ± 3,63% d'inhibition (Figure C4-6 et Table C4-5). L'augmentation était d'environ 16,6% entre ces deux extraits. Les extraits de prétraitement US ont montré 51,68 ± 3,77 mg GAE (acide gallique équivalent)/$g_{extrait}$ pour l'essai en teneur en polyphénols totale (en anglais *Total Polyphenols Content* ; TPC). Les valeurs de TPC des extraits bruts étaient de 46,30 ± 2,11 mg GAE/$g_{extrait}$. La variation entre ces deux extraits était de 5,38 mg GAE/$g_{extrait}$, représentant une augmentation de 10,4% (Figure C4-7 et Table C4-5). L'amélioration de l'activité antioxydante peut être causée par plusieurs facteurs induits par le prétraitement US. Pendant le prétraitement, la température est localement plus élevée en raison du flux acoustique et de la cavitation. L'augmentation de la température semble être bénéfique pour l'amélioration de l'activité antioxydante. Barba *et al*. ont observé que la température peut avoir un effet positif sur l'extraction des polyphénols.[196] Par exemple, à 20 °C avec des US (sonde, 24 kHz dans l'eau), le TPC des extraits de mûres était inférieur à celui à 50 °C. Au contraire, Egüés *et al.* ont déterminé que des températures élevées ont des effets positifs et négatifs sur l'activité antioxydante des polyphénols à partir de déchets de pommes utilisant des US (sonde, 20 kHz).[206] À haute température (65 et 90 °C), le TPC était

plus élevé, mais la capacité de piégeage des radicaux DPPH (en anglais *DPPH Radical Scavenging Capacity* ; DRSC) était plus faible. L'activité antioxydante était endommagée par des températures plus élevées. Selon Egüés *et al*., le TPC aurait pu être influencé par les sucres extraits. Comme pour l'augmentation du rendement massique, le prétraitement US a provoqué la rupture de la paroi cellulaire de la biomasse. La conséquence de cet effet est l'augmentation de la surface de contact, augmentant les interactions entre les molécules et le solvant. Dans cette étude de cas, l'amélioration de l'activité antioxydante était corrélée à l'amélioration du rendement massique. L'augmentation de l'activité antioxydante a été atteinte selon nos résultats. Le tableau R-2 résume l'activité antioxydante des extraits obtenus par SC-CO_2. En ce qui concerne l'activité antibactérienne, aucune activité n'a été observée sur boîte de Pétri contre les bactéries *Listeria innocua* (*L. innocua*), *Escherichia coli* (*E. coli*) et *Bacillus cereus* (*B. cereus*).

Tableau R-2. Résumé des activités antioxydantes des extraits par SC-CO_2.

Echantillons	DRSC		TPC (mg GAE/$g_{extract}$)
	Trolox® eq. (µg TE/$mg_{extract}$)	$\%_{inhibition}$[a]	
brut	1 036,87 ± 92,71	38,16 ± 3,63	46,30 ± 2,11
Traité par US	1 231,29 ± 93,85	45,78 ± 3,68	51,68 ± 3,77

[a]avec une concentration de 50 $mg_{Extrait}$/mL

Pour l'extraction par SWE, les conditions optimales sont de 157 °C, 23,4 min et 1:8 (AP:Eau; *p/v*). Dans ces conditions, plusieurs composés ont été extraits, y compris les glucides, les composés indésirables (furfural et 5-hydroxyméthylfurfural ; FFR et 5-HMF) et les polyphénols. Les composés 5-HMF et FFR sont considérés comme indésirables pour plusieurs raisons : leur toxicité, leur formation lors de traitements thermiques, leur impact négatif sur la qualité des produits alimentaires. Les applications des extraits sont antioxydantes et antibactériennes. Pour cette raison, les extraits de SWE ont été traités post-extraction avec de la résine pour éliminer les sucres afin d'éviter les activités biologiques de ces derniers, surtout pour les tests antibactériens. Les sucres peuvent favoriser la croissance des bactéries. Selon le Table C3-12, le temps d'extraction et la température doivent être plus longs. Néanmoins, le temps d'extraction ne doit pas dépasser 30 min selon Ibrahim *et al*.[98] La température doit également

être augmentée mais au-dessus de 140 °C, dans notre cas les déchets de pommes sont brûlés.[83] Afin d'extraire plus de polyphénols, le ratio AP:Eau devrait être différent, tel que 1:30 à 1:100 pourraient être testés. Les conditions finales d'extraction seront de 140 °C, 1:8 (AP:Eau, *p/v*) pendant 23,4 min. Les conditions de prétraitement étaient de 10 min, 20 kHz et 500 W avec le prétraitement US. Les essais d'activité antioxydante ont été décrits comme TPC et EC_{50} à partir de la DRSC. L'EC_{50} ou CE_{50} en français est la concentration efficace médiane à laquelle 50% du DPPH est inhibé. Plus la valeur de l'EC_{50} est petite, plus l'extrait est antioxydant. Les valeurs de TPC avec et sans prétraitement US étaient respectivement de 148,08 ± 7,25 et 110,33 ± 11,55 mg GAE/$g_{extrait}$, soit une augmentation de 25,5% (Figure C4-9). Selon les résultats du TPC, il y avait une extraction plus élevée de polyphénols avec le prétraitement US. La variation entre ces deux extraits peut s'expliquer par le fait que les US génèrent des bulles de cavitation conduisant à la rupture des parois cellulaires des déchets de pommes. Les images SEM de la peau de raisin *Siah-Sardasht* avant et après SWE et le prétraitement US. Les images ont révélé que le prétraitement US a endommagé la biomasse comme prévu, mais l'effet de la technique SWE a davantage détérioré la matrice végétale. [213] Ces dommages conduisent à l'amélioration de l'extraction des biomolécules en augmentant l'interaction soluté/solvant. Le deuxième type de mesure de l'activité antioxydante était l'utilisation de DPPH, et les valeurs sont décrites par le pourcentage de DPPH inhibé (%inhibition) ou par EC_{50} ($\mu g_{Extrait}/mL_{MeOH}$). Les valeurs EC_{50} des différents traitements étaient de 6,6 ± 0,7 et 7,6 ± 1,8 $\mu g_{Extrait}/mL_{MeOH}$ pour les extraits sans prétraitement et les extraits de prétraitement US respectivement (Table C4-6). Selon la figure C4-9, les valeurs de $\%_{inhibition}$ des extraits sans prétraitement et de prétraitement US étaient respectivement de 70,43 ± 9,69 et 60,68 ± 4,87% d'inhibition à la même concentration d'extraits (15 $\mu g_{Extrait}/mL_{MeOH}$). L'extrait avec la plus grande activité antioxydante était l'extrait sans prétraitement. La variation entre ces deux extraits a diminué de 13,8%. L'augmentation de l'activité antioxydante n'a pas été atteinte selon nos résultats. Le tableau R-3 résume l'activité antioxydante des extraits de déchets de pommes obtenus par SWE.

Tableau R-3. Résumé des activités antioxydantes des extraits par SWE.

Samples	DRSC		TPC
	EC_{50} ($\mu g_{Extract}/mL_{MeOH}$)	$\%_{inhibition}$[a]	(mg GAE/$g_{extract}$)
Non-traité par US	6.6 ± 0.7	70.43 ± 9.69	110.33 ± 11.5
Traité par US	7.6 ± 1.8	60.68 ± 4.87	148.08 ± 7.25

[a]avec une concentration de 15 $mg_{Extrait}/mL$

Selon le Table C4-7, la Concentration Minimale Inhibitrice (CMI) de tous les extraits obtenus par SWE de déchets de pommes est de 50 $mg_{Extrait}/mL_{eau}$. Lorsque l'EC_{50} est calculé, des variations sont observées et les extraits sont discriminés les uns par rapport aux autres. Des études antérieures ont indiqué que les bactéries à Gram positif (par exemple *L. innocua* et *B. cereus*) sont généralement plus sensibles que les bactéries à Gram négatif (par exemple *E. coli*) en raison des différences de structure de la paroi cellulaire. Les bactéries à Gram négatif possèdent une membrane supplémentaire de lipopolysaccharides, offrant une couche de protection supplémentaire à la paroi cellulaire.[134,137] Nos résultats sont cohérents avec la littérature. Les extraits présentent un EC_{50} inférieur contre *L. innocua* et *B. cereus*. Par exemple, la valeur de l'EC_{50} des extraits sans prétraitement contre *E. coli* est de 22,0 ± 1,2 $mg_{Extrait}/mL_{eau}$ par rapport aux mêmes extraits avec des valeurs EC_{50} de 15,9 ± 3,1 et 11,3 ± 4,7 $mg_{Extrait}/mL_{eau}$ respectivement contre *L. innocua* et *B. cereus*. Pour la même inhibition (50%), une concentration plus élevée d'extraits est nécessaire pour être efficace contre *E. coli*. Une autre façon de présenter l'inhibition des extraits contre les bactéries est le pourcentage d'inhibition à une concentration fixe.

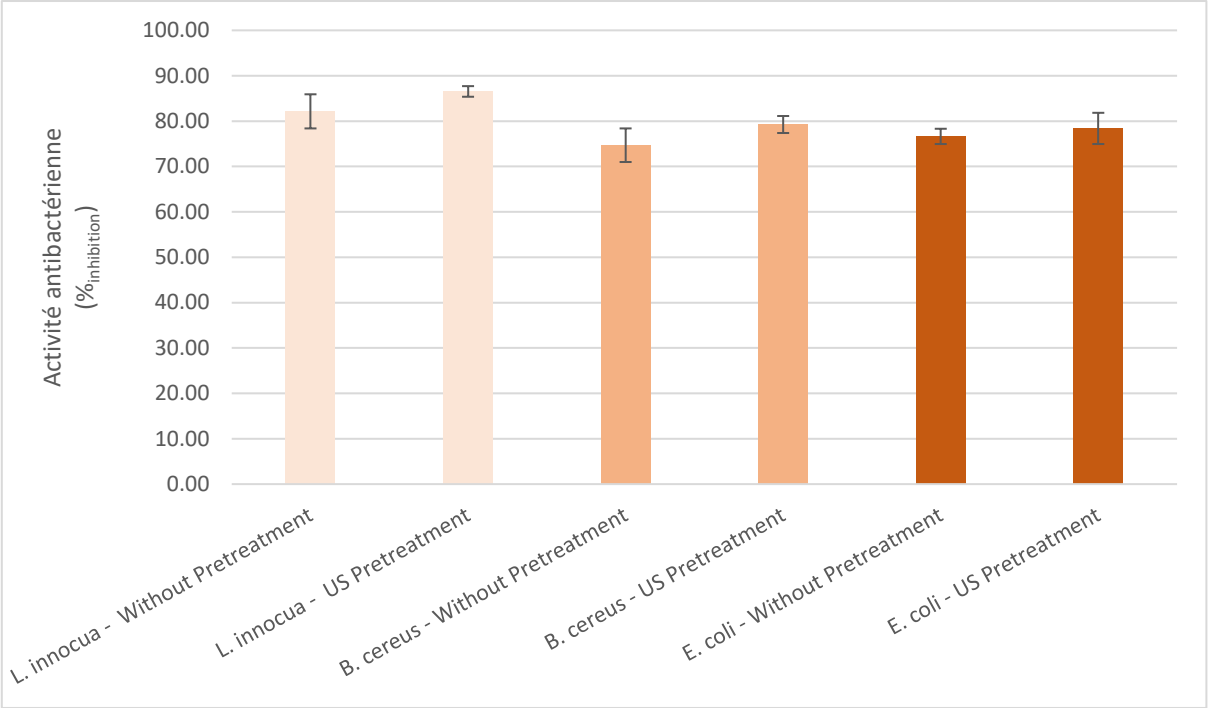


Figure R-5. Activité antibactérienne des extraits des déchets de pommes obtenu par SWE (à 50 $mg_{Extrait}/mL_{eau}$).

Ce chapitre 5 visait à évaluer les propriétés antioxydantes et antibactériennes des extraits de déchets de pommes en utilisant des solvants naturels à eutectique profond (NADES). Au cours des dernières décennies, les fluides subcritiques et supercritiques ont été étudiés pour des extractions écologiques, basées sur des paramètres physiques tels que la pression et la température. Un procédé bien connu est le procédé DIAMANT®, utilisé pour éliminer le trichloroanisole, la molécule responsable du goût de bouchon dans le vin.[158] Cependant, les fluides subcritiques et supercritiques nécessitent des dépenses importantes pour leur acquisition et leur maintenance.[111] L'utilisation des solvants eutectiques profonds naturels (NADES) représentent une technique prometteuse, composée de composés biosourcés. Les NADES offrent des avantages tels que l'accessibilité financière, le caractère renouvelable, la faible toxicité, la biodégradabilité, la sélectivité d'extraction et la faible pression de vapeur. Cependant, des défis subsistent, notamment en ce qui concerne la viscosité et la séparation post-extraction des produits phytochimiques et des NADES. Les NADES se composent d'un composé accepteur de liaison hydrogène (HBA) biosourcé et d'un composé donneur de liaison hydrogène (HBD) biosourcé. Notamment, le point de fusion des NADES est inférieur à celui de leurs composés HBA et HBD constitutifs. Par exemple, le NADES ChCl:Urée (1:2) a un point de

fusion de 12 °C, tandis que le chlorure de choline (ChCl) fond à 302 °C et l'urée à environ 134 °C.[129] Selon la formulation spécifique des NADES, cette technologie permet une extraction sélective et réduit le besoin d'étapes de réaction supplémentaires. Six NADES, à base de chlorure de choline (ChCl), ont été utilisés comme solvants respectueux de l'environnement. Parmi ceux-ci, quatre ont été combinés avec des acides organiques (acide citrique, lactique, oxalique et malique), tandis que les deux restants étaient avec de l'urée, pour l'extraction de composés bioactifs. ChCl:urée s'est avéré être le solvant avec le plus haut TPC, mesurant 13,15 ± 4,70 mg GAE/$g_{extrait}$. En ce qui concerne l'activité antioxydante et le contenu total en anthocyanidines (TAC), ChCl:acide oxalique a enregistré les valeurs les plus élevées à 35,59 ± 9,53 mg d'extrait/mL et 64,81 ± 4,65 équivalent malvidine-3-glucose µg/mL, respectivement (Figure R-5).

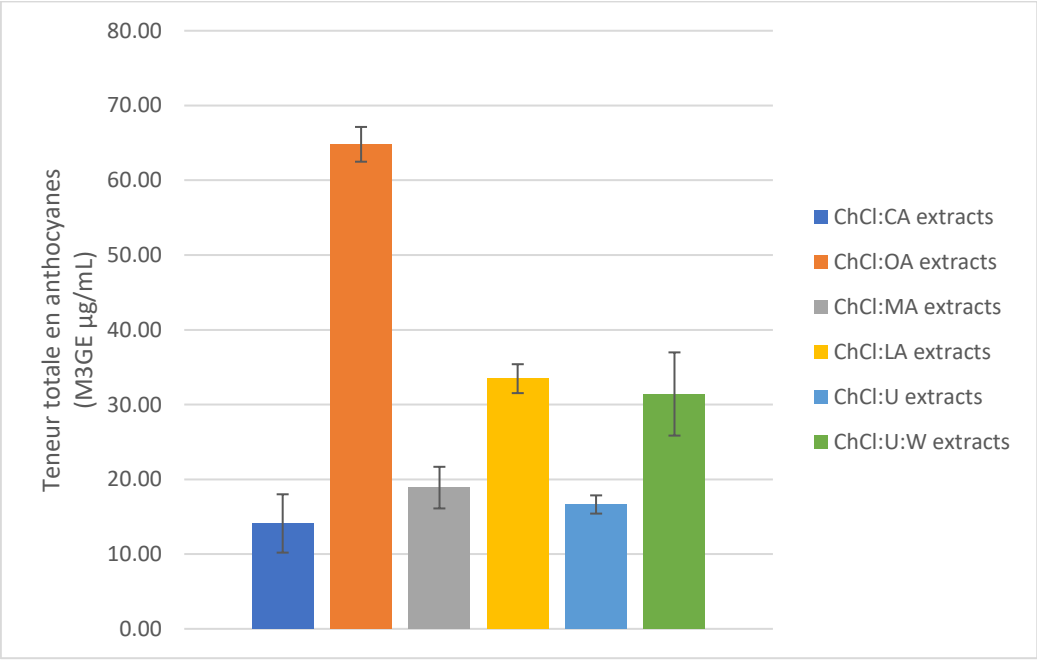


Figure R-6. Teneur totale en anthocyanes des extraits en malvidine-3-glucoside équivalent µg/mL.

Le pH des solvants a joué un rôle crucial dans l'extraction sélective ; un pH acide a facilité l'extraction des anthocyanidines, tandis qu'un pH basique conduit à un manque de sélectivité par rapport aux conditions acides. L'extraction des anthocyanidines a été corrélée à la fois avec l'activité antioxydante des extraits et la viscosité des solvants (Table C5-3). De plus, l'activité antibactérienne des extraits a été évaluée contre *B. cereus*, *L. innocua* et *E. coli*. Tous les extraits ont démontré des propriétés antibactériennes contre les souches testées, les extraits

de ChCl:acide oxalique présentant des concentrations minimales inhibitrices particulièrement faibles (en mg d'extrait/mL) et des valeurs EC_{50} (en mg d'extrait/mL) également basses. La raison pour laquelle les NADESs comprenant des acides exhibent des activités antibactériennes est à cause du pH. Tous les NADESs employés ont un pH inférieur à 5 à toutes les concentrations testées (Figure C5-3).

Le chapitre 6 a mis en évidence les implications environnementales significatives de l'extraction des composés bioactifs des déchets de pommes à l'aide des technologies CO_2 supercritique (SC-CO_2) et l'eau subcritique. L'évaluation du cycle de vie (ACV) réalisée conformément aux normes ISO 14040-44, offre une méthode complète pour quantifier l'empreinte environnementale à la fois des produits/procédés existants et futurs conçus selon des critères écologiques.[231] L'Analyse du Cycle de Vie examine cinq phases du cycle de vie : l'extraction et la production des matières premières, le transport, la production et la distribution du produit, l'utilisation et la fin de vie (Figure R-6). Dans le cas de notre étude, le périmètre des ACVs est du Berceau-à-l'Usine. Ce périmètre évalue les impacts environnementaux d'un produit depuis l'extraction des matières premières (berceau) jusqu'à la fabrique du produit (Usine). Ce choix est justifié par le fait que nous ne connaissons pas les clients potentiels et l'utilisation des composés bioactifs par les clients. Pour éviter de fausser les résultats, le périmètre a été défini de tel sorte à prendre en compte que les phases directement abordées dans le cadre du projet *VAL'Apple*.

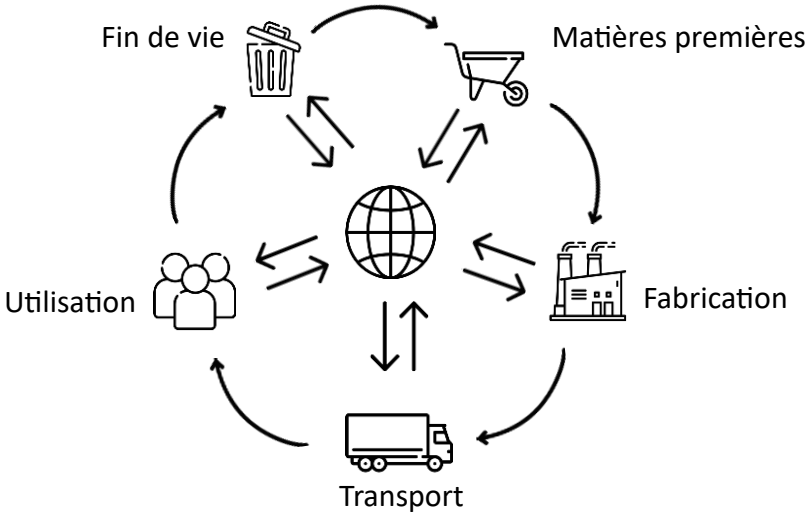
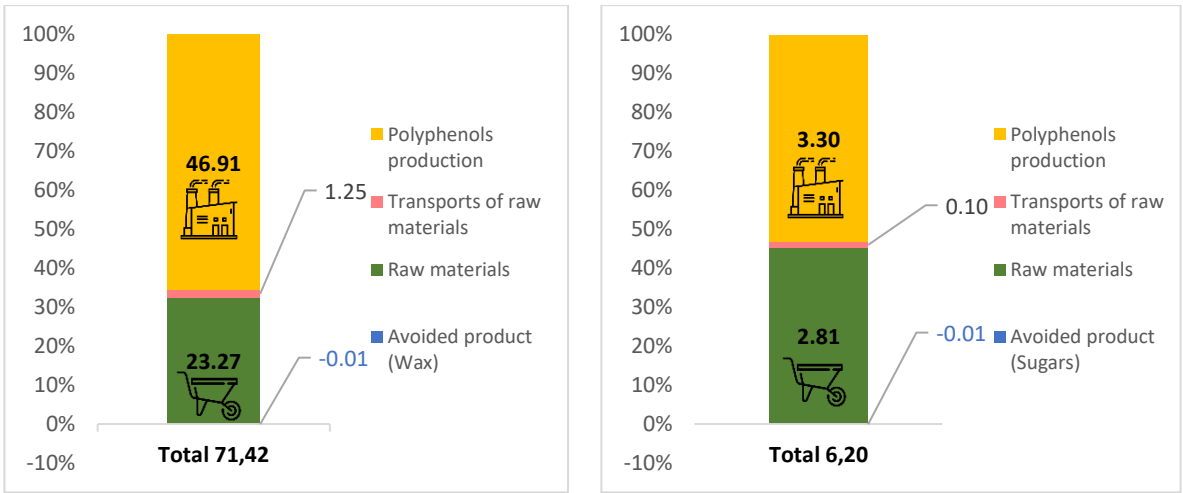


Figure R-7. Schéma du Cycle de Vie avec 5 phases.

Les ACVs indiquent que, bien que le l'extraction eau subcritique (SWE) présente une empreinte carbone nettement inférieure (6,20 kg_{CO2eq} par 1 g de composés bioactifs) par rapport au procédé SC-CO_2 (71,42 kg_{CO2eq} par 1 g), il existe encore un potentiel considérable pour réduire davantage l'impact environnemental, en particulier dans le SWE (Figure R-7). Dans le cas de l'étude et pour des raisons techniques, les déchets de pommes ont été séchés et broyés augmentant l'empreinte carbone du procédé inutilement. A titre comparatif, une étude québécoise a calculé l'empreinte carbone de différentes voies de valorisation des déchets de pommes (16 209 tonnes) en un an : nourriture pour le bétail, enfouissement, incinération, production d'enzyme, et compostage respectivement 964, 1 842, 16 420, 907 et 1 2374 t_{CO2eq}.[238] Pour la même quantité de déchets de pommes valorisée par les SFE étudiées, l'empreinte carbone de l'extraction SC-CO_2 est de 11 600 000 t_{CO2eq} et celui du SWE est de 803 966 t_{CO2eq}. Ces résultats des deux ACVs suggèrent que les méthodes actuelles d'extraction des polyphénols à l'échelle du laboratoire, et potentiellement à des échelles semi-industrielles et industrielles, pourraient ne pas être aussi respectueuses de l'environnement en utilisant les SFE. Par conséquent, il est recommandé d'explorer des techniques d'extraction alternatives, telles que l'utilisation de NADES, qui pourraient offrir des alternatives plus respectueuses de l'environnement par rapport aux fluides subcritiques et supercritiques.



Impact environnemental du SC-CO_2 Impact environnemental du SWE

Figure R-8. Impacts environnementaux des différentes phases sur la production de polyphénols avec les deux procédés sur le changement climatique (en kg_{CO2eq})

Avec l'aide de Carla Marty, stagiaire encadrée dans le cadre de ce doctorat, l'évaluation de l'empreinte carbone des extractions utilisant des NADES n'a pas pu se faire car la base de données de *SimaPro* et les recherches de données chiffrées n'ont pas pu aboutir à des valeurs cohérentes. De plus comme mentionné dans le Chapitre 5, la séparation des biomolécules et des NADES étant difficile, le rendement aurait été faussé et par conséquent l'évaluation de l'empreinte carbone également. En outre, d'autres molécules d'intérêt pourraient être extraites des déchets de pommes, telles que les polymères de glucides (pectine, lignine, cellulose, etc.), qui sont présents en plus grande abondance que les polyphénols.

En conclusion, cette recherche contribue de manière significative à notre compréhension des impacts environnementaux associés aux méthodes d'extraction des polyphénols et souligne l'importance de continuer à développer des processus d'extraction plus durables et respectueux de l'environnement. Les résultats de cette étude guideront les futures recherches et pratiques industrielles et orienteront le secteur vers des solutions plus respectueuses de l'environnement et plus efficaces.

Pour conclure ce manuscrit de thèse, avec toutes ces informations, il est possible de comparer les éco-solvants. Selon le Table GC-1, l'extraction par eau subcritique (SWE) a permis d'extraire plus de polyphénols et a montré une activité antioxydante (DRSC) plus élevée par rapport aux autres solvants. Les solvants eutectiques profonds naturels (NADES) ont été les solvants verts les moins efficaces pour l'extraction des polyphénols selon le TPC et le DRSC. Comme mentionné ci-dessus, certains solvants NADES ont démontré une activité antioxydante, parfois supérieure à celle des extraits (ChCl:OA). En conclusion, l'extraction la plus efficace en termes d'activités antioxydantes est l'eau subcritique (SWE), qui a montré des valeurs plus élevées de TPC et de DRSC (Table GC-1).

La concentration minimale inhibitrice (CMI) et l'EC_{50} contre *L. innocua*, *E. coli* et *B. cereus* des extraits SC-CO_2 n'ont pas été observées en raison de l'effet du solvant DMSO. Pour les extraits obtenus par NADES et SWE, les valeurs de CMI et d'EC_{50} ont été observées. Seulement pour ChCl:U et ChCl:U:W, les valeurs de CMI et d'EC_{50} n'ont pas été mesurées en raison de l'absence de propriétés antibactériennes des extraits et des solvants. Contre *L. innocua* et *B. cereus*, les extraits SWE ont montré une activité antibactérienne plus élevée que les extraits obtenus par NADES. Contre *E. coli*, le ChCl:OA a démontré une propriété antibactérienne plus élevée principalement attribuable au solvant. Pour conclure sur l'activité antibactérienne des extraits de déchets de pommes, les extraits obtenus par NADES et SWE ont montré des valeurs

équivalentes de CMI et d'EC_{50}. Cependant, les extraits obtenus par SWE étant lyophilisés, ils ne présentent pas d'effet solvant comparé aux extraits NADES. Ainsi, pour les extraits SWE, seule l'activité des biomolécules a été observée.

Pour conclure ce manuscrit, plusieurs améliorations peuvent être apportées. Pour les deux techniques SFE, un autre modèle mathématique devrait être testé car le *Lack-of-Fit* démontre que le plan composite central rotatif n'est pas adéquat. De plus, la DoE devrait prendre en compte les activités biologiques pour déterminer les conditions optimales. Afin d'augmenter l'extraction des polyphénols avec la SWE, un autre ratio (AP:Eau) devrait être testé. Pour les deux SFE, et particulièrement pour la SWE, un autre prétraitement devrait être effectué, tel qu'un prétraitement enzymatique. Le prétraitement aux ultrasons diminue l'activité antioxydante pour les extraits obtenus par SWE (DRSC). Pour éviter des étapes post-extraction supplémentaires, des *NADES prêts à l'emploi* devraient être envisagés. Ces solvants devraient être plus attractifs pour les entreprises. La prochaine étape pour l'ACV est de calculer l'empreinte carbone de l'extraction par NADES afin de compléter la comparaison entre éco-solvants. Pour réduire davantage l'empreinte carbone de la SWE, l'AP ne devrait pas être séché avant extraction. Enfin, l'extraction sans solvant devrait être testée. L'AP contient 75 à 85% d'humidité et la teneur en eau de la biomasse peut être utilisée comme solvant.

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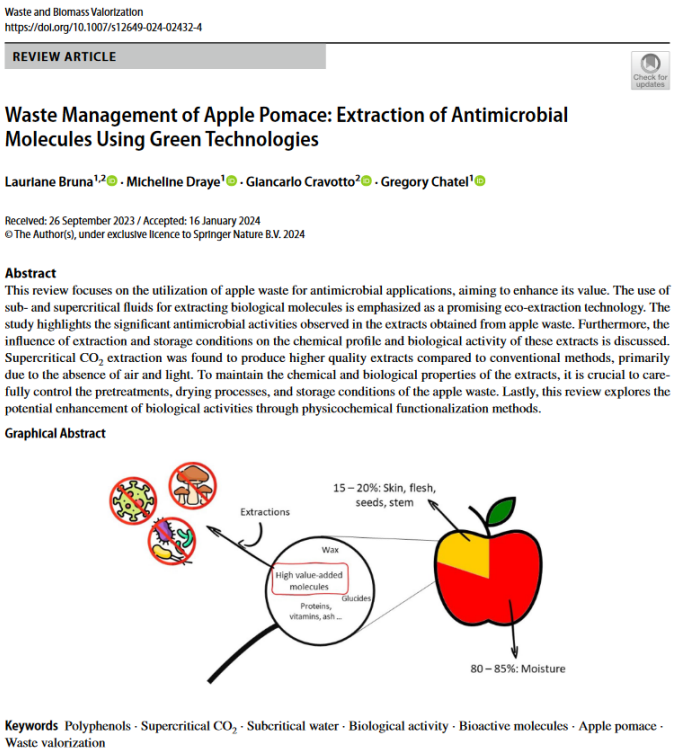
Publications and Conferences

Peer-reviewed journals publications

[1] **Bruna, L.**, Draye, M., Cravotto, G., Chatel, G., *Waste Management of Apple Pomace: Extraction of Antimicrobial Molecules Using Green Technologies*. Waste and Biomass Valorization (Springer) **2024**, 15 (8), 4541-4555. https://doi.org/10.1007/s12649-024-02432-4



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REVIEW ARTICLE

Waste Management of Apple Pomace: Extraction of Antimicrobial Molecules Using Green Technologies

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Abstract
This review focuses on the utilization of apple waste for antimicrobial applications, aiming to enhance its value. The use of sub- and supercritical fluids for extracting biological molecules is emphasized as a promising eco-extraction technology. The study highlights the significant antimicrobial activities observed in the extracts obtained from apple waste. Furthermore, the influence of extraction and storage conditions on the chemical profile and biological activity of these extracts is discussed. Supercritical CO_2 extraction was found to produce higher quality extracts compared to conventional methods, primarily due to the absence of air and light. To maintain the chemical and biological properties of the extracts, it is crucial to carefully control the pretreatments, drying processes, and storage conditions of the apple waste. Lastly, this review explores the potential enhancement of biological activities through physicochemical functionalization methods.

Graphical Abstract

Keywords Polyphenols · Supercritical CO_2 · Subcritical water · Biological activity · Bioactive molecules · Apple pomace · Waste valorization

Manuscripts submitted

[2] **Bruna, L.**, Draye, M., Cravotto, G., Chatel, G., *Comparative Life Cycle Assessment of Extraction of Bioactive Compounds from Apple Pomace using Subcritical and Supercritical Fluids*. Cleaner Environmental Systems (Elsevier), under review.

[1] **Bruna, L.**, Draye, M., Lyautey, E., Cravotto, G., Chatel, G., *Extraction of Polyphenols and Anthocyanidins with Natural Deep Eutectic Solvents from Apple Pomace: Evaluation of their Antioxidant and Antibacterial Activities.* Journal of Food Science and Technology (Springer), submitted.

Manuscripts in preparation

[2] **Bruna, L.**, Draye, M., Lyautey, E., Cravotto, G., Chatel, G., *In-situ sugars to 5-HMF reaction using Subcritical Water*, in progress for Food Bioscience (Elsevier).

[1] **Bruna, L.**, Draye, M., Lyautey, E., Fanget, F., Cravotto, G., Chatel, G., *Recovery of polyphenols from Apple Pomace with Supercritical CO_2 and their Biological Evaluations*, in progress for Journal of Supercritical Fluids (Elsevier).

Conferences

October 2024	**Bruna, L.,** Draye, Lyautey, E., Grillo, G., Cravotto, G., Chatel, G., *Green Extraction of Natural Products (Compiègne, France)*, Supercritical Fluids Extraction of Bioactive Molecules from Apple Pomace, **ORAL.**
June 2024	**Bruna, L.,** Draye, M., Lyautey, E., Fourreaux, C., Grillo, G., Cravotto, G., Chatel, G., *Journée de Printemps 2024 de la Section AURA de la Société Chimique de France (Grenoble, France)*, Extraction of Polyphenols and Anthocyanins with Natural Deep Eutectic Solvents from Apple Pomace: Evaluation of Antioxidant and Antibacterial Activities, **POSTER.**
June 2023	**Bruna, L.,** Draye, M., Rivoalen, C., Grillo, G., Cravotto, G., Chatel, G., *Tersys Implanteus 2nd Summer School (Avignon, France),* Biological Activities from Apple Pomace extracts obtained with NADES, **POSTER/SHORT ORAL.**
June 2023	**Bruna, L.,** Draye, M., Rivoalen, C., Grillo, G., Cravotto, G., Chatel, G., *Journée de Printemps 2023 de la Section AURA de la Société Chimique de France (Lyon, France),* Biological Activities from Apple Pomace extracts obtained with NADES, **ORAL.**
October 2022	**Bruna, L.,** Draye, Lyautey, E., Grillo, G., Cravotto, G., Chatel, G., *Green Extraction of Natural Products (Porec, Croatia)*, Supercritical Fluids Extraction of Bioactive Molecules from Apple Pomace, **ORAL.**
June 2022	**Bruna, L.,** Draye, M., Cravotto, G., Chatel, G., *Journée de Printemps 2022 de la Section AURA de la Société Chimique de France (Le Bourget-du-Lac, France)*, Méthode d'extraction innovante de Molécules Bioactives issues des Résidus Solides de Pommes, **POSTER**.
October 2021	**Bruna, L.,** Chatel, G., Cravotto, G., *UNITA WP4 Circular Economy Workshop (Zaragoza, Spain)*, Development of Enabling Technologies for Regional Biomass Valorization in a Context of Circular Economy, **ORAL**.

Résumé :
En 2022, plus de 95.8 millions de tonnes de pommes ont été produites dans le monde. Une partie significative de ce fruit est transformée en boissons (jus et cidre) générant une quantité importante de déchet de pommes. La production de ce résidu est estimée entre 4,7 et 5,6 millions de tonnes. Si ce déchet n'est pas correctement très traité, il peut engendrer des répercussions environnementales et sanitaires dû à la quantité d'humidité contenue (75 – 85%). Malheureusement, l'enfouissement est la solution la plus privilégiée dans le monde. Seule l'Union Européenne propose une alternative à ce traitement : la méthanisation. Cependant, les molécules biologiquement actives présentes dans ces déchets ne sont pas valorisées par cette voie de traitement. C'est dans ce contexte que le projet VAL'Apple propose d'explorer différentes solutions en utilisant des procédés innovants pour la valorisation de ces composés bioactifs. Ces technologies *vertes* sont l'utilisation du CO_2 supercritique, l'eau subcritque et les solvants à eutectiques profonds naturels. L'application étudiée dans ce projet est des applications antioxydantes et antibactériennes. Cette thèse a porté sur l'optimisation des paramètres d'extraction des fluides pressurisés et l'évaluation des propriétés biologiques des extraits. L'étude a également exploré l'impact de pré-traitements par ultrasons sur les rendements d'extraction et les activités biologiques. Les extraits obtenus *via* les solvants eutectiques profonds ont fait l'objet d'une caractérisation approfondie des propriétés ainsi que les solvants utilisés. Enfin, une analyse du cycle de vie a été menée afin d'évaluer l'empreinte environnementale des différents procédés d'extraction. Les résultats obtenus soulignent l'importance du choix du procédé d'extraction sur la préservation des composés bioactifs et sur l'impact environnemental global. Les solvants eutectiques profonds et les pré-traitements ultrasoniques apparaissent comme des pistes prometteuses pour de futurs travaux.

Mots-clés : déchets de pommes ; économie circulaire ; activité antibactérienne ; CO_2 supercritique ; eau subcritique ; solvant eutectique profond ; extraction végétale.

Abstract:
In 2022, over 95.8 million tons of apples were produced worldwide. A significant portion of this fruit is processed into beverages (juice and cider), generating a substantial amount of apple waste. The production of this residue is estimated to be between 4.7 and 5.6 million tons. If this waste is not properly treated, it can lead to environmental and health impacts due to its high moisture content (75-85%). Unfortunately, landfilling is the most common disposal method worldwide. Only the European Union offers an alternative to this treatment: methanation. However, the biologically active molecules present in this waste are not valorized through this treatment process. It is within this context that the VAL'Apple project proposes to explore different solutions using innovative processes to valorize these bioactive compounds. These green technologies include the use of supercritical CO2, subcritical water, and natural deep eutectic solvents. The applications studied in this project are antioxidant and antibacterial applications. This thesis focused on optimizing the extraction parameters of pressurized fluids and evaluating the biological properties of the extracts. The study also explored the impact of ultrasound pre-treatments on extraction yields and biological activities. The extracts obtained via deep eutectic solvents were subjected to in-depth characterization of both the properties of the extracts and the solvents used. Finally, a life cycle analysis was conducted to assess the environmental impact of the different extraction processes. The results obtained highlight the importance of the choice of extraction process on the preservation of bioactive compounds and on the overall environmental impact. Deep eutectic solvents and ultrasound pre-treatments appear as promising avenues for future work.

Keywords: apple pomace; circular economy; antibacterial activity; supercritical CO_2; subcritical water; deep eutectic solvent; plant extraction.