UNIVERSITÀ DEGLI STUDI DI MILANO
BICOCCA

SCUOLA DI DOTTORATO

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Department of Biotechnology and Biosciences

PhD program in
Converging Technologies for Biomolecular System
XXXVI Cycle

ECO-FRIENDLY EXTRACTION AND METABOLOMIC ANALYSIS FOR THE RECOVERY OF HEALTHY METABOLITES FROM DIFFERENT AGRI-FOOD BY-PRODUCTS

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ACADEMIC YEAR 2022/2023

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Abbreviations

AAPH = 2,2-azobis(2-amidinopropane) dihydrochloride

ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

ACN = acetonitrile

ANOVA = Analysis of variance

AOC = antioxidant capacity

AUC = area under the curve

BBB = blood-brain barrier

BPI = full mass base peak chromatograms

CCD = Central Composite experimental Design

CDNB = 1-chloro-2,4-dinitrobenzene

CE = circular economy

CF = caffeine

CID = collision-induced dissociation

COPD = chronic obstructive pulmonary disease

COX-1 = cyclooxygenase-1

COX-2 = cyclooxygenase-2

CSE = conventional extraction methods

CTX = cetuximab

CVDs = cardiovascular diseases

DDA = data-dependent scanning

DM = dry matter

DPPH = 2,2-diphenyl-1-picrylhydrazyl

DoE = design of experiments

ESI = electrospray ionization coupled liquid chromatography

EtOH = ethanol

EXT = extract

FAO = Food and Agriculture Organisation of the United Nations

FS = femminello siracusano

FZB = femminello zagara bianca

FW = food waste

GAE = gallic acid equivalent

GC = gas chromatography

GLS 8 = glucohirsutin

GLS 9 = glucoarabin

GLS10 = glucocamelinin

GLS11 = homoglucocamelinin

GLUT1 = dependent glucose transporter

GRAS = generally recognized as safe

GLSs = glucosinolates

GST = glutathione S-transferase

HPLC = high performance liquid chromatography

IHD = ischaemic heart disease

ISO = international organization for standardization

LC = liquid chromatography

LPH = lactase floridzinn hydrolase

MAE = microwave-assisted extraction

ME = maceration extraction

MeOH = methanol

MRM = multiple reaction monitoring

MS = mass spectrometry

MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NADES = natural deep eutectic solvents

NCDs = non-communicable diseases

NMR = nuclear magnetic resonance

NO = nitric oxide

$O_2\bullet^-$ = superoxide radical

ORAC = oxygen radical absorbance capacity

Opt 1 = optimized 1

Opt 2 = optimized 2

OVAT = one variable at a time

PA = probable activity

PAMPA = parallel artificial membrane permeability

PBL = polar brain lipid

PBS = phosphate buffered saline

PC = *Camelina sativa* pressed cake

PC1 = principal component 1

PC2= principal component 2

PCA = principal component analysis

PGI = protected geographical indication

PHWE = pressurized hot water extraction

PI = probable inactivity

PLE = pressurized liquid extraction

PLS = partial least squares regression

QC = qualitative control

RD = respiratory diseases

RNS = reactive nitrogen species

ROS = reactive oxygen species

RSM = response surface methodology

SE = soxhlet extraction

SFE = supercritical fluid extraction

SDG = sustainable development

SLE = solid-liquid extraction

SMILES = simplified molecular input line entry system

SNC = central nervous system

SNP = sodium nitroprusside

SOD = superoxide dismutase

SPE = solid-phase extraction

TB = theobromine

TE = trolox equivalents

TEAC = trolox equivalent antioxidant capacity

TIC = total ion current

TPC = total phenolic content

TPSA = topological polar surface area

UHPLC-HRMS = ultra-high-performance liquid chromatography high-resolution mass spectrometry

UNEP = food waste index report

UPLC = ultra-performance liquid chromatography

USAE = ultrasound-assisted extraction

Summary

Non-communicable diseases (NCDs) are a group of chronic diseases that are responsible for 70% of deaths worldwide. Unfortunately, with an aging population, these diseases are increasing and are expected to be the leading cause of death by 2030, with many health and economic impacts. Diet and lifestyle are among the most influential factors in the development of NCDs, so it is important to find molecules that can be taken to prevent the development of these diseases.

Since ancient times, nature has been an important source of bioactive compounds that could improve human health through preventive and/or curative actions. Nowadays, agro-industrial waste is also starting to be considered a good source for the recovery of bioactive molecules. The disposal of these wastes causes a high costs and environmental impact. Currently, different waste management methods have been developed and used for the production of energy, animal feed or as a source of active metabolites with beneficial properties. Some wastes from the food chain may have a higher metabolite content or present different analytes than the edible portion normally used. For this reason, it is important to analyze the metabolic profile of plants and by-products to assess their qualitative composition and identify potential bioactivities. For this purpose, numerous analytical techniques are available that can, with target and untarget analysis, be used for a metabolomics study.

The extraction of bioactive compounds has changed significantly over time. Unconventional techniques such as ultrasonic extraction (USAE), pressurised fluid extraction (PLE), supercritical fluid extraction (SFE) and microwave extraction (MAE) are becoming increasingly common. These have certain advantages, such as increased extraction yield and efficiency, reduced extraction cost and time, reduced environmental impact and the use of less toxic solvents that are unsafe for food use in favour of safe solvents such as water and ethanol.

The aim of this PhD project was therefore to search for active metabolites for the treatment and/or prevention of NCDs. Three different food by-products were investigated: cocoa bean shells, *Camelina sativa* seeds by-product and lemon by-products (peel, albedo, seeds and pulp). Following an initial LC-MS analysis to assess

the phytochemical profile, green extraction methods (USAE and PLE) were optimized by experimental design to recover methylxanthines from cocoa bean shells, glucosinolates from *C. sativa* seeds by-product, and polyphenols from lemon waste. The optimized extract was then tested in silico and by in vitro assays to assess its potential bioactivities. Finally, a simulation of gastrointestinal digestion using the international INFOGEST model was performed to assess the bioaccessibility of the analytes after oral intake. The results obtained highlighted the possibility of obtaining active metabolites with potential therapeutic properties from agro-industrial by-products.

This project has demonstrated how the study of metabolomics applied to agro-food waste allows the identification of potentially bioactive metabolites against NCDs. It has also shown that the optimization of the extraction process is crucial to improving its efficiency while making it more sustainable by reducing the use of organic solvents, time, and environmental impact.

Chapter 1

Introduction

1.1 Non-communicable diseases (NCDs)

Non-communicable diseases represent one of the major challenges that our society need to face during this century. Demographic, environmental, and economic changes are causing a great the rise in the incidence of these diseases in the population (Habib & Saha, 2010). Current data show that in 2019 NCDs accounted for ten of the leading causes of death, responsible for about 70 percent of deaths worldwide. In countries such as Italy or Spain, they account for 10 percent of premature deaths, but reach up to 20 percent in developing countries such as Africa and the Eastern Mediterranean (Borsoi et al., 2023; Budreviciute et al., 2020).

Non-communicable diseases, also known as chronic diseases, are conditions characterized by long duration and slow progression. These diseases are non-infectious and are the leading cause of disability worldwide, regardless of gender, age or geographical location. NCDs include many pathological conditions, but the most common are (Budreviciute et al., 2020; Nediani & Dinu, 2022):

- Cardiovascular diseases (CVDs): are a group of diseases related not only to the heart, such as ischaemic heart disease (IHD), stroke, congenital heart disease, coronary heart disease, cerebrovascular disease, peripheral arterial disease and rheumatic heart disease, but also to blood vessels, with hypertension and diseases related to cerebral, carotid and peripheral circulation. Although CVD affects both sexes equally, men have a higher incidence than women. According to the American Heart Association, there are seven key health factors and behaviours that contribute to an increased risk of heart disease and stroke: diet, smoking, overweight/obesity, physical inactivity, uncontrolled blood pressure, high cholesterol and high blood sugar (Budreviciute et al., 2020; Mc Namara et al., 2019).
- Neurodegenerative diseases (Maiese, 2020): Neurodegenerative diseases are a significant component of non-communicable diseases and include more than 600 disease entities that can progressively lead to nervous system dysfunction Neurodegenerative diseases, both acute and chronic, lead to disability and death in a significant number of people, which may approach more than 30 million people worldwide. The role of environmental and occupational exposures to

neurotoxic substances in the pathogenesis of neurodegenerative diseases is not fully understood, but many chemicals can affect the nervous system and some of them require metabolic activation to induce their toxic effects (Migliore & Coppedè, 2002).

- Cancer: the second leading cause of death in the world, is the result of uncontrolled cell proliferation and can affect any organ or tissue. It affects both sexes and is linked to genetic factors, but also and above all to environmental exposure to carcinogenic factors of a biological, chemical or physical nature. Smoking and poor diet are among the external factors that have the greatest impact on the development of cancer (Blackadar, 2016).
- Diabetes mellitus: Diabetes has attracted worldwide attention due to its high prevalence and incidence. It is not only a chronic disease but also a life-threatening condition. It can also lead to other serious conditions such as heart disease, kidney failure, and eye damage. Although diabetes is partly hereditary, lifestyle factors such as obesity, high sugar consumption and lack of physical activity can contribute significantly to the progression of diabetes (Alam et al., 2014; Esposito et al., 2014).
- Respiratory diseases (RD): include a wide range of diseases of the airways and other structures of the lungs. Most of the morbidity and mortality of MRCs increases with age. MRCs include chronic obstructive pulmonary disease (COPD), occupational lung disease, asthma and respiratory allergies, sleep apnoea and pulmonary hypertension. Genetic and environmental factors are risk factors for MRCs (Budreviciute et al., 2020).

The etiology of non-communicable diseases is complex because it is related to many factors, both endogenous and exogenous. Endogenous factors include non-modifiable conditions such as age, gender, and genetic aspects, while exogenous factors refer to modifiable environmental and behavioural factors such as lifestyle, sedentary behaviour, diet/nutrition, smoking, and alcohol (Budreviciute et al., 2020; Ezzati & Riboli, 2013). The ageing of the world's population is closely linked to the increase in chronic diseases, due to the body's reduced plasticity, which makes it less effective in counteracting the factors that can cause them (Ruthsatz & Candeias, 2020). It is therefore important to act

on several exogenous factors to prevent the development of NCDs and promote healthy ageing. Numerous studies have shown that the healthy behaviours people adopt throughout their lives have a significant impact on their risk of developing NCDs. Disease processes are seen as the body's response to environmental aggression. Nutrition and diet have important role in the prevention and /or development of chronic diseases (Martinon et al., 2021; Slawson et al., 2013; Tokunaga et al., 2012). The growth trend of this group of diseases has shown a higher incidence in developing countries, partly related to a drastic change in dietary patterns. There are many differences between the diets of populations living in rural areas and those living in urban areas; in particular, modernisation appears to be leading to a dietary pattern rich in saturated fats, sugars or carbohydrates and refined foods, while reducing the intake of fibre and vitamins (Habib & Saha, 2010). These changes appear to promote metabolic processes in the body that can increase oxidative stress, inflammatory metabolic disorders, and more, thereby increasing the development of pathological conditions. Oxidative stress is one of the main mechanisms involved in the initiation and progression of NCDs pathology, particularly in cardiovascular and neurological diseases and cancer (Seyedsadjadi & Grant, 2021; Hariri & Ghiasvand, 2016). Oxidative stress is associated with the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are normally generated by cellular metabolism. Under physiological conditions, cells have defence mechanisms capable of neutralising these radicals by electron transfer (Borsoi et al., 2023). However, due to exogenous or endogenous factors, the homeostasis of oxidoreductive processes can be altered, resulting in the accumulation of radicals that our body cannot counteract with its own self-protection system. In such cases, ROS and RNS that are not neutralised can damage biomolecules, especially DNA, causing mutations of various types (Borsoi et al., 2023). The abandonment of a reducing cellular environment in favour of an oxidising one also leads to the emergence of inflammatory processes that activate cytokine cascades and inflammation in the body, contributing to the deterioration of health and the emergence of chronic diseases (Phillips et al., 2019). Research into the dietary factors that influence these conditions has become paramount to better define effective strategies to reduce the risk of developing them (Grosso, 2018).

1.2 The role of phytochemicals in human wellness

The prevalence of NCDs in the population and their increase, partly as a result of increased life expectancy over the last century, calls for significant interventions to prevent their occurrence and progression in the population. Today, nutrition plays a key role in maintaining human well-being; dietary habits and trends in food production and consumption have health, environmental, and societal impacts (Cencic & Chingwaru, 2010). There is a growing demand for molecules with antioxidant, anti-inflammatory, chemopreventive, and neuropreventive properties that can be used in foods to improve health. The exploration of new sources and ingredients for inclusion in the diet or for the manufacture of nutraceuticals and/or dietary supplements is of great importance. Nutraceuticals are defined as foods or parts of foods that provide health benefits, including the prevention and/or treatment of disease states (Chandra et al., 2022). Thus, nutraceuticals are foods that are traditionally consumed during a meal or diet, as opposed to dietary supplements, which may also be consumed in the form of capsules, pills, tablets or liquids. Dietary supplements are added to the diet to increase the total daily intake of one or more compounds (Cencic & Chingwaru, 2010). The search for active molecules for human well-being has always been directed towards nature. The earliest historical evidence of the use of natural products for human health dates back to 2600 BC, where the use of cypress and myrrh oil for coughs, colds and inflammation were found, also the Egyptian civilisation used more than 700 herbal medicines (Atanasov et al., 2015; D. A. Dias et al., 2012). Moreover, most of the medicines we use today are of natural or plant origin, such as many antibiotics, morphine or acetylsalicylic acid.

Plants, are organisms characterised by an active metabolism capable of producing many molecules, which vary from species to species, with interesting beneficial properties (Kroymann, 2011; Veiga et al., 2020). The metabolism of plant organisms is very complex and can be divided into primary and secondary metabolism. Primary metabolism is the set of reactions common to all living organisms that lead to the synthesis of molecules essential for survival, such as: carbohydrates, lipids, proteins and nucleic acids. In the case of plants, photosynthesis also belongs to this class. Secondary metabolism, on the other hand, refers to the biosynthetic pathways that lead to the production of accessory chemical molecules called secondary metabolites. They are not

essential for plant survival, but they provide important defence mechanisms against predators and can facilitate reproductive processes, for example by attracting pollinating insects. In contrast to primary metabolites, secondary metabolites have a very wide and diverse distribution in the plant world and can characterise a single species or several species belonging to the same or different families; they are therefore of taxonomic importance as well as of value in terms of bioactivity. These molecules have often been used by man to produce drugs or other bioactive products. They are generally divided into 4 main groups: phenolic compounds, terpenoids, steroids, and finally nitrogenous compounds, among which the alkaloids are particularly important (Maugini et al.,2014; Rieseberg et al., 2023) Specifically, in this thesis we focused on three classes of compounds: methylxanthines, glucosinolates and polyphenols.

- Methylxanthines

methylxanthines are a group of phytochemical compounds belonging to the class of alkaloids. Specifically, methylxanthines are derived from xanthine, a purine base, which involves the presence of a nitrogen atom in their heterocyclic structure, so they belong to the purine alkaloid group (Bartella et al., 2019; Ejuh et al., 2020). The most common methylxanthines are caffeine, theobromine and theophylline, which are widely available in the human diet due to their abundance in nature, especially in various species used for food or drink (J. Schuster & Mitchell, 2019). Caffeine is particularly abundant in tea leaves, coffee and cocoa beans, beans and cola nuts. Theophylline is abundant in tea leaves and theobromine in cocoa beans and beans (Bartella et al., 2019).

The biosynthesis of purine alkaloids involves fewer than 4 enzymes and starts with the synthesis of caffeine. Most of the other purine alkaloids found in the plant world are intermediates of processes associated with the biosynthesis and catabolism of caffeine. Figure 1 shows a diagram of the synthesis of methylxanthines. In particular, the biosynthesis of caffeine is triggered by the conversion of xanthosine to 7-methylxanthosine by the enzyme 7-methylxanthosine synthetase. Subsequently, a nucleosidase catalyses the hydrolysis of 7-methylxanthosine into 7-methylxanthosine. From the latter, further methylation produces caffeine and theobromine. The methyl groups in purine alkaloids derive from S-adenosyl-L-methionine and, depending on the molecule, are added by SAM-dependent N-methyltransferase enzymes, either at position

1, 3, 7, 9 or to an oxygen atom at position 2. The main xanthine skeleton is derived from puric nucleotides synthesised in plants via de novo or salvage pathways (Ashihara et al., 2013).

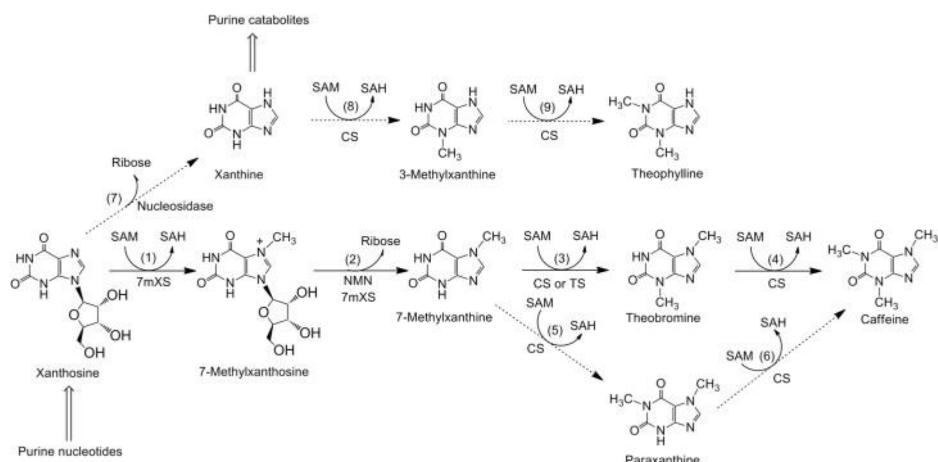

Figure 1: biosynthesis of methylxanthines in vegetable organisms (From Ashihara et al., 2013).

Their widespread use in the plant world is linked to their pesticidal properties, being toxic to insects and many pathogens (Ashihara et al., 2013). Studies of the effects of these molecules on human health have revealed interesting anti-inflammatory, vasodilator, immunostimulant and respiratory tract benefits in asthma, chronic bronchitis, emphysema and COPD. They also have important neuroprotective effects (Ejuh et al., 2020). The effectiveness of methylxanthines, such as caffeine and theobromine, in preventing Alzheimer's and Parkinson's disease has been demonstrated in several studies (Hong et al., 2020; Kim et al., 2019; Larsson & Orsini, 2018). A first suggested mechanism is the ability of these molecules to act as adenosine antagonists, resulting in stimulation of the central nervous system and increased mental performance and alertness. Further studies have demonstrated their modulatory effect in preventing the accumulation of misfolded proteins, neuroinflammation and oxidative stress (Janitschke et al., 2021).

- Glucosinolates

Glucosinolates (GLSs) are a group of secondary metabolites produced only by the order Brassicales, the type and abundance of which varies greatly between species and

growing conditions (Agerbirk & Olsen, 2012; Clarke, 2010). GLSs are found in the roots, seeds, leaves and stem of the plant, with the youngest tissues containing the highest levels. GLSs have a similar basic structure, consisting of D-thioglucose group linked to a sulfonated aldoxime group and a variable side chain derived from amino acids (Mitreiter & Gigolashvili, 2021) (Figure 2).

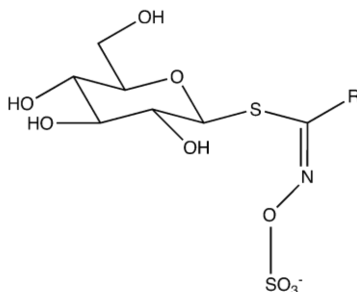

Figure 2 general structure of GLSs

They differ mainly for the structure of the aglycone R-chain, which may contain alkenyl, indolyl, hydroxyl, carbonyl, or diverse thiofunctions (Wathelet et al., 2004), and are classified into three groups based on the structure of their amino acid precursors:

- Aliphatic GLSs, derived from methionine, isoleucine, leucine or valine
- Aromatic GLSs, derived from phenylalanine or tyrosine
- Indole GLSs, derived from tryptophan

Starting from the respective amino acid, the biosynthesis of GLSs can thus be described in three steps (Figure 3). First, the carbon chain of the amino acid, except tryptophan, can be lengthened through a series of reactions that can be repeated up to a maximum of six cycles. This is followed by the synthesis of the central structure of the glucosinolate. Finally, the molecules thus obtained can have some modifications (methylation, oxidation, desaturation etc.) in their side chairs (Mitreiter & Gigolashvili, 2021).

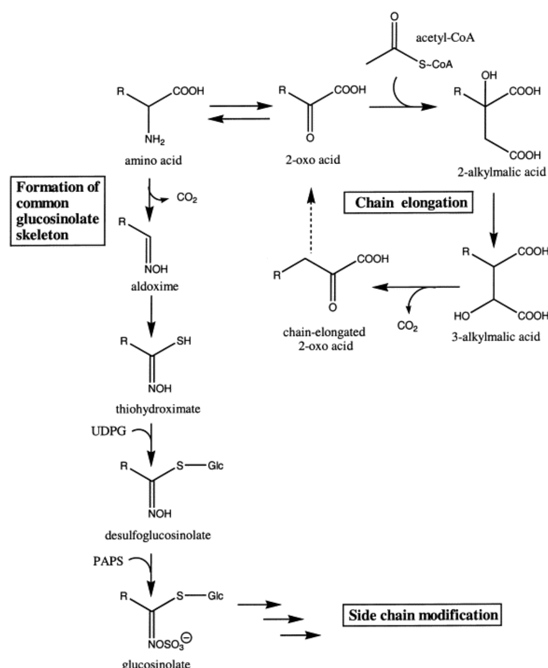

Figure 3: biosynthesis of glucosinolates from amino acids (From Graser et al., 2001).

Glucosinolates are believed to play an important role in plant responses to extreme or environmental stimuli and play an important role in plant defence against insects and pathogenic organisms through degradation by the enzyme myrosinase. Under physiological conditions, GLSs are stored in the vacule3 while myrosinase is located in the cytosol or the vesicles of the endoplasmic reticulum. Following cell damage caused by external agents such as insect or herbivores, GLSs are released and hydrolysed by myrosinase. The resulting aglycones can spontaneously rearrange into different toxic degradation derivatives (isothiocyanates, nitriles and thiocyanates) depending on the chemical conditions, in particular pH and the presence of specific proteins in the plant that prevent herbivores from feeding (Wittstock & Burow, 2010). The biosynthesis of GLSs is therefore regulated by numerous environmental and endogenous factors such as sugar availability, herbivore damage, insect attack, wounds, mineral nutrients, salicylic acid, ethylene, and other phytohormones (Chhajed et al., 2020).

In addition to the important biopesticidal role of these molecules, other bioactivities of interest to human health benefits as antimicrobial and anticancer compounds. They are considered to play a role in plant defense against external plant aggression, which is why

GLSs are also used as natural pesticides and biofumigants (Holst & Williamson, 2004; Ziedan & Moliszewska, 2022). In addition, recent studies suggest that glucosinolates have health benefits and may help reduce neurodegenerative and cardiovascular and cancer diseases when consumed in the diet (Kamal et al., 2022; Ma et al., 2018; M. H. Traka, 2016; M. Traka & Mithen, 2008).

- Polyphenols

Phenolic compounds are the most abundant group of secondary metabolites in nature; they are secondary metabolites synthesised only by plants and are characterised by the presence of one or more phenolic rings and one or more hydroxyl substituents (Truzzi et al., 2021; Xiao et al., 2013; Xie & Chen, 2013). Around 8000 molecules are currently known to be commonly found in food. They are mainly found in fruits, vegetables, tea and whole grains in a predominantly glycosylated form. Polyphenols are derived from a biosynthetic pathway involving precursors of the shikimic acid and/or acetate-malonate pathways; they are a very heterogeneous group and are divided into two main groups: flavonoids and non-flavonoids (Singla et al., 2019). Flavonoids are the most abundant group of phenolic compounds; they have the structure of phenylpropanoids (C3-C6-C3), consisting of two phenolic rings A and B linked to a heterocyclic ring C with a closed pyranosic structure (Singla et al., 2019), Figure 4.

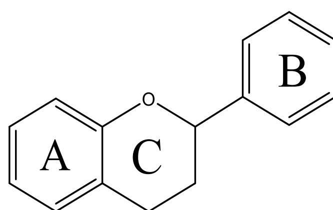

Figure 4: structural formula of a generic flavonoid

Flavonoids differ in their hydroxylation and oxidation state in the C ring and can be divided into several subclasses:

- Flavones and flavonols: their structure involve the presence of a carbonyl group in position 4 and the attachment of the B-ring to the heterocyclic ring in position 2. Finally, there is a double bond between the C2 and C3 positions (Singla et al., 2019; Tsao, 2010). These are molecules capable of absorbing light in the UV spectrum, which allows them

to be perceived by insects such as bees, and they play an important role in the pollination process, indicating to insects the location of pollen and nectar. These molecules are found not only in flowers but also in leaves, where they provide protection from excessive UV radiation. The two most important compounds in this class are luteolin and apigenin and their glycosylated forms.

- Isoflavones: These are structural isomers of flavonoids in which the B-ring is attached to the C3 of the C-ring instead of the C2 position. Found mainly in legumes, they protect plants from insects and predators (M. C. Dias et al., 2021; Panche et al., 2016).

- Flavanones: This subclass has many substituted derivatives. They are mainly found in the aglycone form in citrus fruits such as oranges and lemons. The glycosides in this group have a disaccharide at position C7 (De Oliveira et al., 2014).

- Flavanols: have a saturated heterocyclic ring, no double bond between C2 and C3 and a hydroxyl group in C3. This group of flavonoids is present in food in aglyconic form. The monomeric forms are the catechins and epicatechins, while the polymeric forms are the condensed tannins and are abundant in woody plants (Lattanzio, 2013; Singla et al., 2019).

- Anthocyanidins and anthocyanins: have two unsaturations in the heterocyclic ring. They are water-soluble pigments and are responsible for the blue, red, yellow, purple and violet colours of many fruits and flowers. They are also found in other parts of the plant such as leaves, bulbs and roots. Anthocyanins play an important role in attracting animals for pollination and seed dispersal. In mammals, they have some important functions: they reduce capillary permeability and fragility, reduce oedema, prevent collagen degradation and have an important antioxidant effect (Giada & Giada, 2013; Singla et al., 2019).

Flavonoids are formed by the condensation of intermediates of the shikimate and acetate pathways (Figure 5). In particular, cumaryl-CoA (a shikimate pathway intermediate) reacts with malonyl-CoA (an acetate pathway intermediate) to form flavanone and chalcone, from which flavones and isoflavones are derived. Hydrolysis of flavonone also gives rise to dihydroflavonol, which gives rise to anthocyanins, flavonols (catechins and

condensed tannins) (Marchiosi et al., 2020; Santos-Sánchez et al., 2019; Singh et al., 2017).

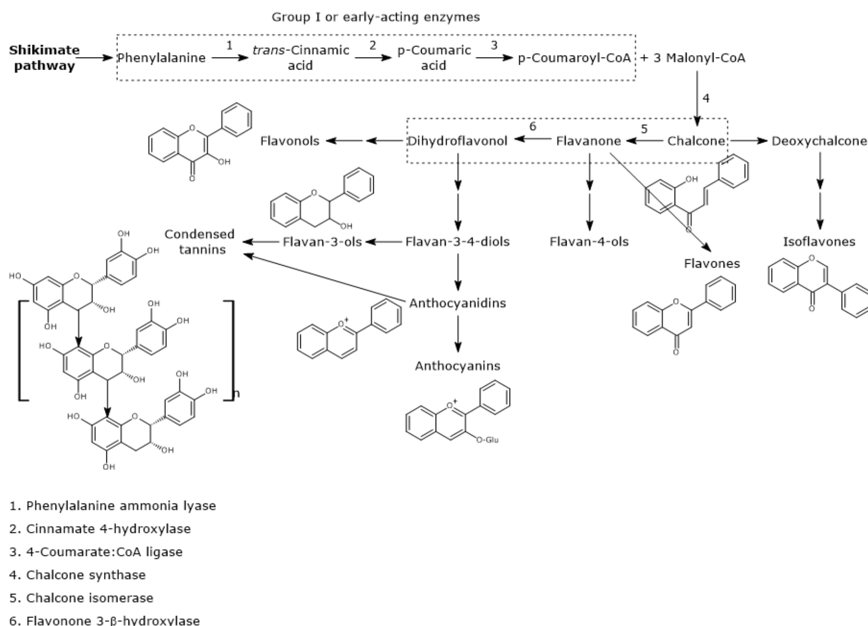

Figure 5: Favonoid biosynthesis diagram (From Singh et al., 2017).

The non-flavonoids are represented by three main subclasses: phenolic acids, stilbenes and lignans.

- Phenolic acids are the main group and are derivatives of the benzene or cinnamic ring, mainly in the form of hydroxycinnamic or hydroxybenzoic acids (Singla et al., 2019; Truzzi et al., 2021).

Polyphenols mainly play a protective role against pathogens, oxygen free radicals, UV radiation and peroxides. They are one of the most widely distributed secondary metabolites and there are numerous studies in the literature demonstrating significant anti-inflammatory, antioxidant, anticancer and cardiovascular activities (Truzzi et al., 2021).

Plants are therefore a valuable source of therapeutic molecules, but they also produce toxic substances that, in high concentrations, can have adverse effects on human health.

In the plant world, for example, alkaloids are a class of secondary metabolites responsible for a variety of effects on the central nervous system, some of which, when present in high levels, can cause serious illness, injury or even death, such as papaverine in poppy plants or atropine in nightshade berries (Beyer et al., 2009). In addition, plant organisms can develop interactions with fungi, microorganisms or other organisms that can produce toxins that remain in the plant matrix. A single fungal or plant species may produce many different toxins and a single sample may contain several contaminants (Alameri et al., 2023). Natural toxins are therefore natural substances produced by plants, animals or micro-organisms that are harmful to other living organisms (Hodgson, 2012; Salem et al., 2021). Other health hazards may be present as a result of environmental contamination during cultivation, e.g. soil contamination or pesticide use, harvesting or processing, and/or contact with certain materials (Debrauwer et al., 2005; Malik et al., 2010). Finally, the extraction processes themselves, which are necessary for the recovery of active molecules, can lead to the degradation of compounds and the production of toxic substances such as heterocyclic amines or acrylamides (Barceló-Barrachina et al., 2006). For this reason, it is important not only to consider plants as a source of bioactive molecules, but always to study the phytochemical composition of the matrix under investigation, to characterize the analytes present in the sample, assess the type of bioactive compounds present, but also the possible presence of hazardous substances. Techniques such as mass spectrometry are available to detect and quantify the presence of analytes in a single chromatographic analysis by its mass-to-charge ratio. Unlike spectroscopic techniques, mass spectrometry is usually combined with separation techniques (HPLC and GC) that allow the separation of analytes based on their affinity for stationary and mobile phases, allowing the identification of several analytes in a single analysis. MS is characterised by high sensitivity, selectivity and specificity, so that only a few micrograms of sample are required for analysis (Bartella et al., 2019; Malik et al., 2010; Picó et al., 2006). However, the possibility of observing a given analyte in MS is linked to its ability to ionise (acquire or release electrons); once in the gas phase, the ions produced are accelerated by an electric field and projected into an analyser, which separates them according to their m/z ratio and finally collects them in a detector. In tandem MS/MS analysis, it is also possible to use ionisation energy to fragment the analyte and obtain information about its structure. Characterization is based on spectral

characteristics (MS spectra $[M-H]^-$ and $[M+H]^+$), accurate mass, characteristic fragmentation, and as a function of information from various databases, literature or comparison with standards. Thus, thanks to MS, it is possible to define and quantify the molecules of interest present in a food product and also to detect the presence of any toxic compounds (Malik et al., 2010). For this reason, mass spectrometry was used in this project, both in the initial phase and throughout the extraction process, to verify the presence of the analytes of interest and the absence of high levels of phytotoxins or degradation products that could co-extract and prevent the safe use of the extract.

1.3 By-products: a new resource

The use of plants for the bioprospecting of metabolites for human health has been practised since ancient times, but attention is now turning to waste from the agro-food chain. There is currently a great deal of interest in agro-food by-products as a source of interesting molecules for well-being. The huge increase in population and urbanisation has led to a significant rise in food demand in the 21st century, resulting in an increase in cultivation and the generation of millions of tonnes of food waste (FW) per year, the disposal of which is becoming unsustainable (Garcia-Garcia et al., 2017; Grizzetti et al., 2013; C. Xu et al., 2015).

Food loss and waste is a serious threat to the sustainability of our food systems. According to the State of Food and Agriculture (2019) report of the Food and Agriculture Organisation of the United Nations (FAO), approximately 14% of the world's food (USD 400 billion per year) is lost after harvest and before reaching the shops; while the UNEP Food Waste Index report shows that a further 17% of our food ends up being wasted in retail and by consumers, particularly in households. The waste of food resources has a negative impact on the environment, as it is traditionally burned or disposed of in landfills, releasing huge amounts of CO_2 and increasing environmental pollution. In addition, the water used to grow food that is ultimately wasted and the use of land that is taken away from other possible uses must also be taken into account. Finally, agro-food waste contains many biodegradable compounds that can create a favourable

environment for the growth of pathogenic micro-organisms that can threaten public health (Socas-Rodríguez et al., 2021).

The terms "food loss" and "food waste" involve food leftovers from agricultural production, post-harvest handling and storage, further food processing, wholesale and retail commercial distribution, large consumer kitchens and private households (Edjabou et al., 2016; Sawaya, 2017). Specifically, 'food loss' occurs during the handling, storage, transport, processing and distribution of food due to limitations or problems associated with the agricultural process or processing technology. It therefore refers to a reduction in the amount of food that remains in the food chain after harvesting, before reaching the consumer (M. Schuster & Torero, 2016). Food waste, on the other hand, refers to food that is fit for consumption but is discarded, either at retail or by the final consumer, due to retail operations and consumer behaviour (Lipinski et al., 2013). A classification of types of food waste is proposed in (Figure 6), which takes into account the sources and patterns of food loss and waste during the food chain from field to fork.

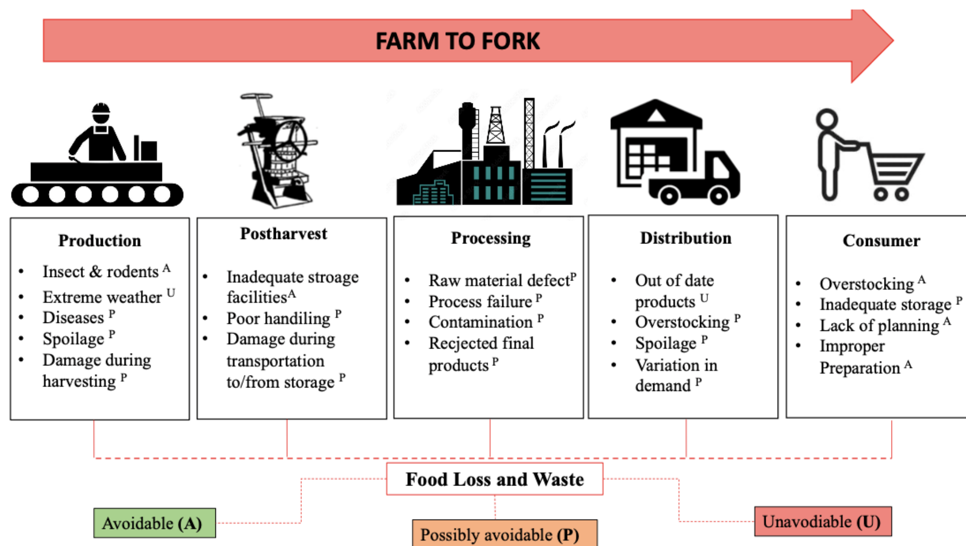

Figure 6 Food loss and waste throughout the food chain (From Ojha et al., 2020)

Food waste and losses can be divided into three broad categories: avoidable, partially avoidable (optional) and unavoidable food waste (Ojha et al., 2020; Santagata et al., 2021). By improving each stage of the food chain, in particular processing techniques,

product innovation, storage, distribution, marketing, labelling and cooking, avoidable or partially avoidable food waste, such as industrially damaged or contaminated products or consumer overpurchased, can be significantly reduced. However, it is not possible to eliminate waste production at the level of the agri-food chain, as waste is inevitably generated during food processing and consumption. For this reason, it is essential to identify strategies for the valorization and recycling of these by-products.

The need for sustainable development (SDG) was born, defined as development that meets the needs of the present without compromising the ability of future generations to meet their own needs (Korhonen et al., 2018). One of the SDG targets outlined by the UN in 2015 is to "halve global per capita food waste at the retail and consumer level by 2030, and reduce food losses along production and supply chains, including post-harvest losses". This would have a positive environmental impact, as well as an economic one, for both suppliers (increased agricultural production and income with reduced disposal costs) and consumers (less wasted money and better market prices).

In this context, the Circular Economy (CE) is attracting increasing interest and attention from the international scientific and policy communities, to help to maintain products, components, and materials at their highest level of usefulness and value. The CE is based on the implementation and promotion of circular flows to reduce environmental impacts while increasing resource efficiency (Moreau et al., 2017; Saavedra et al., 2018). The traditional linear model of energy flow, based on the production-use-discharge of the economic system, is now unsustainable. The global ecosystem is shrinking in size and volume due to past over-consumption of resources, but a circular rather than linear approach could help reduce this (Korhonen et al., 2018). According to the circular economy concept, waste from the agri-food chain is no longer considered waste but a by-product. The term by-product is therefore used to refer to process by-products that can still be used because they are rich in interesting bioactive compounds (Okino Delgado & Fleuri, 2015). In practice, CE aims to reduce waste and overuse of resources by converting end-of-life goods and waste generated during the production and use of goods into resources for the production of other products (Topi & Bilinska, 2017).

Strategies for the valorisation and reuse of food by-products are referred to as “second generation food waste management” and mainly involve their use as an ingredient in the

preparation of animal feed or as a biofuel for energy (Turrini et al., 2019). With these alternatives, producers minimise the cost of disposal, as farmers and energy companies bear the cost of transport and, in some cases, pay small amounts of money to receive tonnes of waste (Okino Delgado & Fleuri, 2015).

In recent years it has been increasingly exploited as a source of valuable molecules, also due to the growing demand for natural food additives with bioactive properties and reduced side effects. In fact, the non-edible parts of food can contain quantities of phytonutrients and valuable bioactive compounds such as pigments, sugars, organic acids, dietary fibres, flavourings and bioactive compounds, especially enzymes and antioxidants which may also be absent from edible fractions and if extracted, could be used as ingredients in nutraceuticals, cosmetics, food and pharmaceutical products (Chamorro et al., 2022; Socas-Rodríguez et al., 2021). These is of particular interest in fruit and vegetable waste, which is often generated after juice extraction or industrial processing. In fruit processing, 70% by weight of the starting material is discarded as pomace (a mixture of seeds, peel, pulp and stalk), which often contains more bioactivities than the commercialised juice. For example, the peel of citrus fruits, grapes and apples is known to contain around 15% more polyphenols than the juice. Another example is cocoa bean shells, obtained after roasting the beans, which can be used in food preparations as a low-cost cocoa substitute or as an ingredient for innovative and functional foods or beverages (Socas-Rodríguez et al., 2021). The use of agro-food by-products as a source of bioactive compounds increases the availability of sources while avoiding the need to increase agricultural cultivation to meet industrial demand. Bioprospecting for metabolites present in different by-products, even from the same plant, is part of metabolomics. Metabolomics is a science that has been actively practised for the last 20 years (Alseekh et al., 2021). Targeted and non-targeted metabolomics can be used to understand the metabolome of plants or by-products. Targeted metabolomics is a quantitative approach using a selective signal of known metabolites, whereas non-targeted metabolomics detects the presence of a large number of known and unknown metabolites, providing a complete description of the metabolome of the sample. Currently, both metabolomics approaches, when used in conjunction with various analytical platforms such as NMR spectroscopy, GC, UPLC and coupled mass

spectrometry, have greatly contributed to the detection and quantification of metabolites with high accuracy. This information is of particular importance in the study of active metabolites, as it can be cross-referenced with information on bioactivity through multivariate analysis, thus completing the information on an identified metabolome application.

1.4 The advantages of green extraction techniques and experimental design

The use of plant matrices and their by-products requires the development of appropriate extraction techniques for the safe and degradation-free recovery of bioactive metabolites. Given the wide range of natural molecules present in the phytocomplex, a detailed analysis of the chemical composition can reveal the quali-quantitative profile of the product, providing a preliminary assessment of the composition of the matrix that can potentially be used in the diet. Furthermore, the choice of solvent or extraction method will influence the yield of bioactive compounds. Conventional extraction methods such as maceration, Soxhlet, and hydrodistillation, produce low yields, require long extraction times and large amounts of sample and solvent (Garcia-Vaquero et al., 2020). Furthermore, they use organic solvents such as hexane, acetone, methanol, and their combinations (Viñas-Ospino et al., 2023). Due to the high structural complexity of the phytocomplex, characterised by mostly apolar metabolites, organic solvents are required. However, these solvents are often volatile, flammable and have harmful effects on the environment and human health (Chemat et al., 2019). The final extract may contain traces of these toxic organic solvents, as well as degradation products resulting from the hard extraction conditions used in such approaches. Conventional techniques are therefore unsuitable for the extraction of sensitive, thermolabile metabolites present at low concentrations (Mustafa & Turner, 2011). To overcome these problems, a green chemistry approach has been developed, based on the development of a process that reduces energy consumption, includes alternative solvents, uses renewable and natural products, and can guarantee a safe and high-quality extraction of bioactive compounds (Viñas-Ospino et al., 2023). The development of extraction methods aimed at reducing environmental impact for the production of healthy products is driving to search for alternative solvents and extraction processes. The shift in chemistry toward the use of sustainable tools presents new challenges for a rational combination of environmentally

friendly, less energy consuming and more economical analytical methods (Armenta et al., 2015). To meet this challenge, there is a wide range of alternative, unconventional solvent that are considered to be safe for health, such as natural deep eutectic solvents (NADES) and CO_2, as well as new extraction techniques. Currently, the most widely used techniques for the extraction of natural and botanical products are microwave-assisted extraction (MAE), ultrasound-assisted extraction (USAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) (Belwal et al., 2018). Their use has shown a positive upward trend in recent years in the scientific literature in the field of food and plant analysis (Figure 7). Generally speaking, the main advantages of their use are demonstrated by the short extraction time, the reduction in the volume of hazardous organic solvents, the achievement of a higher extraction yield and the automation of procedure, which guarantees the reproducibility of the extraction process with lower energy consumption (Diamanti et al., 2017; Picot-Allain et al., 2021).

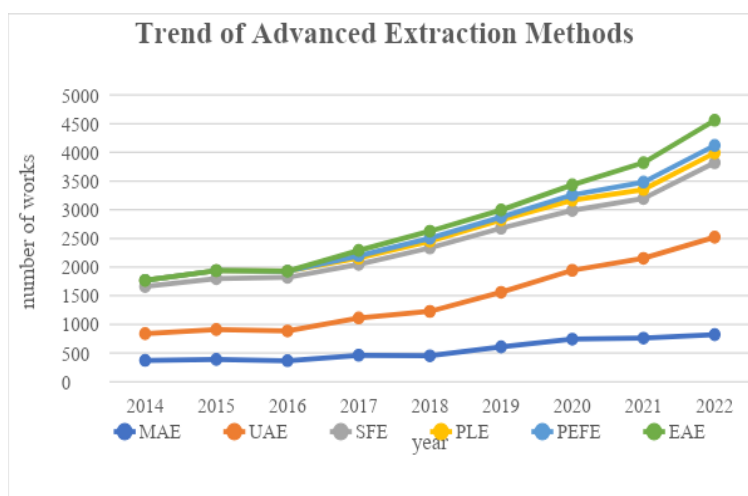

Figure 7. The trend of advanced extraction methods from 2014 to 2022.

During the development of this project, two techniques were selected as being the most suitable for the recovery of the compounds: PLE and USAE. PLE uses the solvent under subcritical conditions of high temperature and pressure, this makes the process more efficient than techniques that use atmospheric temperature and pressure (Alvarez-Rivera et al., 2019). Due to the high pressures, solvents remain close to the supercritical regions of the phase diagram remain in the liquid state at temperatures above boiling point

(Kitrytė et al., 2020). In addition, high pressure facilitates solvent penetration into the matrix by promoting the transfer of analytes in the solvent. Finally, high pressure helps to control the formation of bubbles in the matrix that would prevent the solvent from reaching the analytes (Richter et al., 1997). Many parameters can affect the efficiency of PLE, but the most important are matrix/solvent contact time, number of cycles and temperature. The matrix/solvent contact time influences the breaking of weak bonds that favour the interaction of the analytes with the matrix, thus favouring the diffusion process of the metabolites into the solvent. There are two extraction modes in PLE: static and dynamic. In static mode, the sample and solvent are held at constant pressure and temperature for a user-defined time, while in dynamic mode the solvent flows continuously through the sample (Carabias-Martínez et al., 2005). Another important parameter is the number of cycles (Moret et al., 2014). The number of cycles refers to the repetition of the extraction process within the same extraction vessel. The number of cycles is an important factor that can significantly impact on extraction efficiency, yield, selectivity, and the overall success of analytical procedure. Multiple cycles allow the repeated contact between the solvent and the sample matrix, avoiding the saturation of solvent, increasing the chances of extracting a higher proportion of the target compounds. More cycles can result in a higher yield of the target compounds and help to reduce the interference by progressively extracting the interfering compounds in earlier cycles and focusing on the target compounds in further cycles. The number of cycles is a critical parameter that should be adjusted to optimize the whole extraction process. Finally, temperature is the factor with the greatest influence on extraction efficiency with PLE. Firstly, temperature plays a crucial role in breaking the analyte-matrix bonds caused by intermolecular interactions(Mustafa & Turner, 2011). It reduces the activation energy of the desorption reaction, favouring the process of releasing the analyte from the matrix. High temperatures also favour a decrease in the viscosity and surface tension of the solvent and/or matrix by improving the wettability and penetration of the solvent into the matrix (Möckel et al., 1987). However, this can also lead to the co-extraction of unwanted molecules, reducing the selectivity of the process (Mustafa & Turner, 2011). It is therefore important to optimize the parameters while controlling the chromatographic profile of the extracts obtained. In addition, increasing the temperature at high pressure reduces the dielectric constant of the solvent, modifying the capacity

and extraction efficiency of the solvents and improving their solubility (Figueroa et al., 2018; Pavez et al., 2019). This phenomenon is particularly important because it allows the use of generally recognized as safe (GRAS) solvents such as ethanol or water, improving their solvent capacity. Water is the most environmentally friendly, non-toxic, non-corrosive, non-flammable, non-polluting, naturally abundant and inexpensive solvent available (Flórez et al., 2015). Unfortunately, it has low solubility for apolar compounds and is inefficient for the recovery of active metabolites. However, when water is in a subcritical state, its chemical and physical properties change. With increasing temperature and high pressure, as described above, its dielectric constant decreases due to the weakening of hydrogen bonds, bringing the solvency of water closer to that of organic solvents and improving the extraction efficiency of even apolar compounds (Chemat et al., 2019). On the other hand, a disadvantage of the PLE technique is the high initial instrumental cost and the need for qualified personnel to use it.

Ultrasound-assisted extraction is also widely used in food chemistry using a series of compression and rarefaction waves that propagate through the molecules of the medium. In particular, high-intensity rarefaction cycles create cavitation bubbles in the molecules of the medium, breaking up cell structures and facilitating solvent penetration (Bachtler & Bart, 2021; Vinatoru et al., 2017). Cavitation is related to the physical properties of the solvent, such as viscosity, surface tension, and vapor saturation pressure. Mass transfer is enhanced by the release of energy in the form of heat (Vernès et al., 2019; Vinatoru et al., 2017). The mechanical phenomena associated with USAE cause cell wall rupture and degradation of the matrix and increase mass transfer through particle size reduction (Wen et al., 2018). This technique can improve the efficiency of the process by reducing the extraction temperature and time, allowing optimal preservation of the bioactive properties of the compounds. However, a disadvantage of USAE is the presence of abrupt temperature changes due to the cavitation, which may cause undesirable degradation reactions (Kultys & Kurek, 2022). The USAE technique is also influenced by various factors such as ultrasonic power, temperature and solvent density (Saini & Keum, 2018).

Innovation in the technological development of advanced extraction methods requires the control and optimisation of several parameters to monitor the solubility of compounds and their degradation, while reducing extraction time, health risks and environmental impact. Solvent selection, time duration, operating temperature and pressure, and flow rates of the extraction processes are the main variable to be set. The large set of parameters control requires the necessity of new protocols and procedures with uniformed conditions to standardize the extraction of each natural matrix. Generally, two approaches for the optimization of the condition are performed. "One variable at a time" (OVAT) is a robust and empirical approach but a long time to set each parameter is required considering that only one factor is changed at a time, while all the others are held constant at their nominal values, which prevents the influence of factor interactions in the process from being evaluated (N. Rahman & Raheem, 2022). The main problem with OVAT is therefore the presence of a small experimental matrix with a small range of independent variable settings. It also requires a large number of experiments for process optimisation (Barrentine, 2001; Jacyna et al., 2019; Montgomery, 2017). Therefore, we can only define this approach as suitable for the optimisation of simple experimental processes with one or two independent variables. On the other hand, a rational approach may be employed as a Design of Experiments (DoE), considered a faster and more structured approach to design experiments for the investigation of input parameters (predictor variables) and the generated output (response variables), as well as various interactions that may exist between the input variables performed by statistical software (Yu et al., 2018). In order to evaluate the influence of each factor and to find the optimal settings, methods based on the multivariate design of experiments (DoE) focus on modelling and statistical analysis to define the factors that have a significant impact on the process. The correct planning of an experimental design is a fundamental step in obtaining reliable data capable of validating the experimental hypotheses. First of all, it is necessary to evaluate the independent variables, i.e. those factors that are considered capable of influencing the experimental process. The independent variables must be easy to control and vary according to the extraction technique, for example the factors that influence the PLE process are temperature, pressure, solvent composition, and extraction time (Sánchez-Camargo et al., 2017). Each of these factors is expressed in levels ranging from -1

(minimum) to 1 (maximum) with 0 being the central value (Condra, 2018). Finally, the third key element of an experimental design is the dependent variables, which represent the response variables that we want to define at the end of the experimental design, such as maximizing the recovery of specific bioactive molecules from plant matrices. However, it is important to limit the number of factors and levels in the experimental design, as this will determine the number of experiments to be carried out to optimise the extraction process, and for this reason different models for multivariate analysis are currently available. In particular, we can distinguish between methods for the preliminary evaluation of the factors, the most popular are factorial designs (full factorial design, fractional factorial design, Plackett-Burman design) and methods for the optimisation of the response based on quadratic models that allow the extremes to be defined as maximum and minimum, representing the critical conditions (Central Composite Design, 3-level factor design, Box-Behnken design) (Ferreira et al., 2019; Leyva-Jiménez et al., 2022). There are numerous applications of the DoE in different fields, both for target studies, where a group of defined metabolites is controlled, and non-target, where all metabolites of a sample are controlled (Ferreira et al., 2019).

1.5 Bioaccessibility and biodisponibility of bioactive compounds

Considering the bioactive properties of natural products ingested with food, it is important to consider their fate after exposure to the gastrointestinal digestive process. The need to elucidate the transformations of food and molecules after ingestion is crucial for studies related to metabolism and bioactivity, as well as for the development of products with functionalities that enhance the functional potential of food.

Human digestion is a complex, dynamic and continuous process that depends on the chemical and physical properties of the host (C. Li et al., 2020). In vivo studies would therefore be best suited to assess the true mechanism of digestion, including not only the enzymatic action but also the impact of the gut microbiota (Rasera et al., 2023). However, they are poorly reproducible due to large individual differences, as well as ethical and financial limitations and the need for invasive techniques to collect digesta (C. Li et al., 2020). Therefore, dynamic or static in vitro models are now used, which are

much easier to use, do not raise ethical issues and are more reproducible due to the simplification of the process. Dynamic in vitro models are active systems capable of maintaining dynamic digestion with continuous enzyme secretion and constant pH changes. Unfortunately, these methods require specialised instrumentation, large amounts of enzymes and samples, and are very expensive (Xavier & Mariutti, 2021). For this reason, the most common methods for simulating the digestion process use static in vitro models. In order to standardise studies in the literature and to be able to compare data from different laboratories, a standardised protocol called INFOGEST was created in 2014 (Minekus et al., 2014a). This static in vitro model requires the simulation of digestion in the oral cavity, stomach and intestinal tract. In particular, it details the amounts of enzymes, salts, pH and time for each step of the process, mimicking peristaltic movements through mechanical agitation. In addition, the experiment requires no special instrumentation, making it extremely accessible.

These studies provide information on the bioaccessibility and bioavailability of molecules and/or foods. Bioaccessibility is the amount of a compound that is solubilised in the small intestine and available for absorption (Peña-Vázquez et al., 2022). It depends on many factors, including the production process of the food, the composition and initial concentration of the food matrix, the ability of the biomolecules to cross the intestinal barrier and the characteristics of the host, i.e. the chemical and physical composition of the gastrointestinal fluids and the presence of enzymes, as well as, of course, the stability of the molecules during digestion (Helal et al., 2014; Tagliazucchi et al., 2012). Instead, bioavailability is the amount of compound that crosses the intestinal barrier as a result of absorption into enterocytes. This parameter is particularly relevant as it provides information on the actual passive and active absorption of the analyte (Toutain & Bousquet-Mélou, 2004). Today, several software tools are available, such as ADMET (https://admet.scbdd.com/), pkCSM (http://biosig.unimelb.edu.au/pkcsm/), which can predict the absorption of different metabolites in silico, and in vitro studies, which can provide information on whether an extract can be used as an ingredient in functional foods or nutraceuticals, or whether formulations that increase its bioaccessibility and/or bioavailability are required to preserve its biological activities.

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Chapter 2

Aim of PhD project

Strong population growth and urbanisation have increased the demand for food, resulting in the production of several tonnes of unavoidable waste each year from food collection and processing, with consequent negative environmental impacts. In many cases, the non-edible parts of food crops, which constitute waste in the supply chain, may contain high levels of secondary metabolites, even higher than the edible fraction, with interesting therapeutic properties. Thus, the use of by-products for the production of bioactive enriched extracts would reduce disposal costs and the huge environmental impact. It is therefore of interest to companies to investigate the potential applications of these by-products as a potential resource. However, traditional extraction techniques used to recover biomolecules are unsustainable and use large amounts of organic solvents. This could result in the presence of solvent residues in the sample, invalidating its use in food. For this reason, during the work was to develop and optimize, through experimental design, different unconventional extraction techniques, in particular ultrasound-assisted extraction, and pressurized liquid extraction, which allow the replacement of toxic organic solvents with safe food-grade solvents such as water and ethanol, while increasing extraction yields, reducing extraction time and environmental impact.

This Ph.D. project is part of the Chronos Excellence Project of the Department of Biotechnology and Biosciences of the University of Milan-Bicocca, which aims at studying the mechanisms underlying multifactorial diseases and ageing processes. There are many endogenous and exogenous factors involved in NCDs, but diet plays a key role in the development and prevention of these diseases. There is currently a strong interest in the search for new products and/or ingredients that can be used in food, pharmaceutical to improve the health of the population. Since ancient times, plants have always been a source for the identification and extraction of molecules with beneficial activity for human health. Therefore, objective of the present thesis was to study different metabolites in different food matrices in search of biomolecules with potential activities in the prevention of NCDs, with a focus on neurodegenerative diseases and cancer, which affect a significant part of society. In particular, this work has optimized extraction techniques for the recovery of three types of secondary metabolites. **Chapter 3** describes the phytochemical investigation of cocoa bean shells, one of the most cultivated by-

products with the greatest environmental impact in the world. Cocoa bean shells are a valuable source of methylxanthines, and the aim was to optimize their recovery by PLE. **Chapter 4** describes the use of *Camelina sativa* seeds by-product as a source of glucosinolates. Given the specificity of these molecules, different extraction techniques were compared to identify the most effective in terms of extraction efficiency. In addition, a purification method for the preparation of an extract enriched in GLSs was evaluated. **Chapter 5** describes the bioprospecting of different by-products (peel, pulp, albedo and seeds) of two important Sicilian lemon cultivars (Femminello Siracusiano e Femminello Zagra Bianca) at different stages of ripeness, to identify the fraction most enriched in polyphenols (important antioxidants), using a multivariate analysis. At all stages of the process, mass spectrometry played an important role by allowing the identification of the metabolites present in the extracts, while at the same time assessing the presence of any contamination of the matrix or the extraction process itself.

The potential activity of cocoa, camelina and lemon extract was evaluated in silico or usinig spectrophotometric and cellular assays due to verify the possible application for human health. Finally, since dietary intake exposes these molecules to the digestive process, the bioaccessibility and bioavailability of each analytes was evaluated by in vitro simulation of the gastrointestinal process.

Chapter 3

Extraction of methylxanthines by pressurized hot water extraction from cocoa bean shell by-product as a natural source of bioactive compounds.

Abstract

Cocoa beans are one of the largest cultivated crops all over the world, producing large amounts of by-products. For this reason, it is necessary to valorise cocoa by-products to obtain valuable source of bioactive compounds. In this paper, a pressurized hot water extraction process for recovery of theobromine and caffeine from cocoa by-product was developed and optimized. The extraction was carried out on ASE 200 using ethanol 15% (v/v), and all parameters affected by extraction efficiency were optimized by a chemometric approach. Theobromine and caffeine were quantified by UPLC-UV (283 nm), whereas antioxidant capacity was evaluated by in vitro assays (ABTS). By applying the PHWE under optimized conditions (90°C, 5 cycles and static time 6 minutes), the extraction efficiency increased by 156% for theobromine and 160% for caffeine in comparison with the results obtained using ultrasound assisted liquid extraction. The effect of the PHWE extract was evaluated using in silico analysis and the viability of colon and breast cancer cell lines was tested in detail. In addition, the biodisponibility after gastrointestinal digestion and the permeability of PHWE across the blood-brain barrier were calculated. In conclusion, the application of PHWE for the selective recovery of theobromine and caffeine from cocoa bean shell by-products is a green, automatic and rapid method, which represents a valid alternative to conventional extraction methods for obtaining ingredients for the food industry.

Keyword: *Theobroma cacao,* PHWE, Alkaloids, Flavonoids, Cacao bean shell by-products, UPLC-UV-HRMS, Cetuximab

Theobromine (PubChem CID5429) Caffeine (PubChem CID2519) Procyanidin B2 (PubChem CID122738) Catechin (PubChem CID9064)Epicatechin (PubChem CID72276)

3.1 Introduction

Nowadays food industry produces a large amount of waste during all production phases, and it often consists of inedible parts (Oreopoulou & Russ, 2007). Since their high contents of bioactive compounds, these food wastes should be considered as a food by-products. The huge by-product production causes an enormous economic loss and several environmental problems. In this context, there is an increasing interest in finding specific solutions to use food by-products as natural source of bioactive ingredients (Panak Balentić et al., 2018). Cocoa beans (*Theobroma cacao* L.) are widely used in food, pharmaceutical and cosmetic industries (Mazzutti et al., 2018). It is estimated that the world production of cocoa beans was about 4 million tons in the 2010 (FAO), and just a part of this production is exploited, whereas other parts such as cocoa pod husk, bean shell, and mucilage are usually considered wastes; Figure 1 shows the different fractions of cocoa beans. Cocoa bean shells are about 12-20% of the cocoa beans and represent one of the main by-products during the pre-roasting/roasting process (Panak Balentić et al., 2018). It can be estimated that the annual production of cocoa bean shells reaches approximatively 700 thousand tons, and most of this material is usually disposed as waste (International Cocoa Organization, 2017).

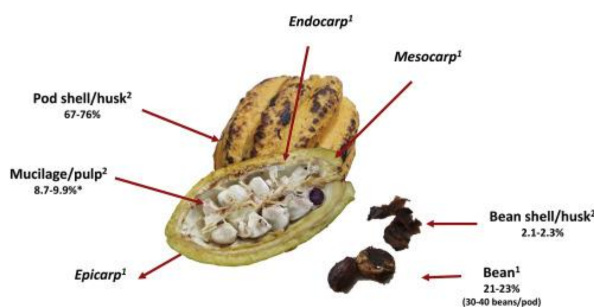


Figure 1 Anatomy of cocoa beans.

Several studies have demonstrated the antioxidant properties of cocoa bean shell extract due to high content of phenolic compounds, such as catechins, procyanidins (Lee et al., 2003; Othman et al., 2007), and its high content of alkaloids as caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) (Arlorio et al., 2005; Okiyama et al., 2018; Visioli et al., 2012). Due

to the interesting biological properties, cocoa bean shell extract has attracted the interest of scientific community and attention by food, pharmaceutical and cosmetic industries as natural and cheap source of bioactive compounds. Therefore, the valorisation of this residue may at same time reduce the environmental problems and create economic benefits. In this context, to obtain a good quality of extract and increase extraction yield, the extraction procedure used must be carefully selected. The conventional extraction methods (CSE) such as solid-liquid extraction (SLE), Soxhlet extraction (SE), and maceration extraction (ME), commonly used for the extraction of bioactive compounds from solid material, have a lot of disadvantages that render these applications quite uneconomical and highly polluting, due to excessive consumption of time, energy and toxic solvents (Ameer et al., 2017). In the last decade, in order to overcome the limitations of CSE several emerging technologies, such as ultrasound assisted extraction (USAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), and pressurize liquid extraction (PLE), have been developed and successfully applied for the extraction of food and food by-products (Galanakis, 2013, 2015). Among these extraction techniques, pressurized liquid extraction has been successfully used to produce functional ingredients from different raw materials, since it allows the extraction of several bioactive compounds in a short time frame (Mustafa & Turner, 2011). The use of liquid solvents at high temperature enhances the solubility and the solvation of analytes, whereas the high pressure facilitates the penetration of solvents into the sample matrix, reducing extraction time and solvent consumption. In particular, when water is used as unique solvent in PLE, this technique is called pressurized hot water extraction (PHWE). At first PLE has been widely used for the extraction of pollutants (Björklund et al., 2006) and contaminants (Campone et al., 2009, 2015) in foods and environmental samples and subsequently has been also widely applied to extract natural bioactive compounds, such as polyphenol (Alonso-Salces et al., 2001; Erdogan et al., 2011; Pagano, Piccinelli, et al., 2018; Pagano, Sánchez-Camargo, et al., 2018) alkaloids (Hossain et al., 2015; Mroczek & Mazurek, 2009; Okiyama et al., 2018) from food and food by-products. The objective of the present work was to develop a green, rapid, and inexpensive procedure for the extraction of dried cocoa bean shell, to obtain a valuable ingredient to be used in nutraceutical, cosmetics, and food industry. The extraction conditions of PHWE method have been carefully optimized by using a Box-Behnken

design to improve the recovery of caffeine and theobromine, and at the same time increase antioxidant capacity. Under optimized extraction conditions of PHWE process, the extraction efficiency increased drastically, while the antioxidant, considering the low content of phenolic compounds, remains roughly unchanged. Furthermore, a predictive in silico analysis of the biological activity of the main analytes identified in the PHWE extract was carried out to assist in knowing potential therapeutic application and to drive the future biological experiments.

Based on the obtained in silico information that underlines the potential neuroprotective activity of methylxanthine the transport across the blood-brain barrier was evaluated. In addition, due to the antioxidant capacity of catechin and procyanidin B2 the chemopreventive property of PHWE extract was tested on different health and cancer cell lines. The results showed no cytotoxic effects on human cell cultures and an interesting sensitising activity to an anticancer drug targeting in cancer cell lines expressing KRAS oncogenes. Finally, a simulation of gastrointestinal digestion was evaluated to explore the potential degradation of bioactive compounds and their bioavailability.

3.2 Materials and Methods

3.2.1 Chemicals and standards

Methylxanthines (Theobromine TB, theophylline and caffeine CF) and polyphenols (Catechin, Epicatechin and procyanidin B2) were purchased by Sigma-Aldrich (Milan, Italy). MS-grade solvents used for UPLC analysis, acetonitrile (MeCN), water (H_2O) and formic acid (HCOOH) were provided by Romil (Cambridge, UK); analytical-grade solvents, acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH) were supplied by Sigma-Aldrich (Milan, Italy). Polar brain lipid (PBL) was purchased by Avanti Polar Lipids, Inc. (Alabaster, AL). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). Gallic Acid (GA), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS, Folin & Ciocalteu’s phenol reagent, n-dodecane, phosphate buffered saline (PBS) 10mM pH=7.4, cholesterol, all salts and enzyme for digestion were purchased by Sigma-Aldrich (Milan, Italy).

3.2.2 Cocoa Samples

Cocoa bean shell mainly belong to Trinitario variety was kindly supplied by a cocoa processor after roasting process carried out at 225°C for about 20min. Cocoa bean shell was blended using a knife mill Grindomix GM 200 (Retsh, Haan, Germany) and ground samples were sieved through a test sieve to obtain homogeneous particle size powders in three different range 150-300 µm, 300-600 µm, 600-900 µm. Preliminary experiments showed no qualitative and quantitative differences between different particles sizes, therefore the range from 300 to 600 µm was selected for all the experiments.

3.2.3 Ultrasound assisted extraction (USAE)

The chemical composition of cocoa bean shell was preliminarily evaluated by the exhaustive extraction carried out by ultrasound assisted solid liquid extraction (USAE). One g of sample was consecutively extracted for 15 min at room temperature for three time, with fresh solvents, (H_2O; EtOH 15%; EtOH 50% and EtOH). At the end of each extraction cycle, the extract was collected, filtered, and dried under vacuum at 40°C in a rotary evaporator (Rotavapor R-200 Buchi Italia s.r.l, Cornaredo, Italy); the global yield was calculated as the mass ration between the total extract and sample weight.

3.2.4 Pressurized hot water extraction (PHWE)

Pressurized hot water extractions were performed using a Dionex ASE200 (Dionex Sunnyvale, CA). Extraction procedure was performed by using 1 g of dried sample (300 600 µm) packed into 5 mL stainless still extraction cell. The empty space of extraction cell was filled with solid-glass beads 4 mm (Sigma-Aldrich, Milan Italy), and a paper filter was placed at the bottom of the extraction cell (Whatman n°1). To select the extraction temperature range to employ into the experimental design, preliminary experiments were carried out at pressure 69 bar EtOH 7.5%, 3 cycles, static time 4 min. The extracts obtained were collected into a glass vial (60 mL) and solvent was evaporated by rotary evaporator (Rotavapor R-200 Buchi Italia s.r.l., Cornaredo, Italy) to calculate the extraction yield.

3.2.5 Extraction yield

The extraction yield was calculated gravimetrically and expressed as percentage $Y_{(\%)}$ of mass of extract (m_{Ex}) relatively to the total mass of raw material (m_{RM}).

$$Y_{(\%)} = {}^{m_{Ex}}/_{m_{RM}} * 100$$

3.2.6 Experimental design

Chemometric approach was used to find the best PLE extraction parameters using Statgraphic Centurion XVI 16.1 version (Rockville, USA.). A Box-Behnken design 2-factor interaction with 4 canter point, an error of 13 degree of freedom, for a total of 28 randomized run was used (Table 1). Four experimental factors: temperature (90-130 °C) number of cycles (1-5) modifier (EtOH 0-15%) and static time (2-6min.) were studied, whereas according to preliminary studies pressure flush volume and purge time were fixed at 69 bar 150% and 100s respectively. Theobromine (TB) and caffeine (CF) content (g/100g DM), and ABTS antioxidant activity (TEAC mmol/mg) were used as response variables. In Table 1, it was reported the experimental matrix design, with the experimental levels of the independent variables (factors) and the results obtained for the analysed response variables. Results from the CCD were subjected to regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of independent variable (A, B and C) contributions and their first order interaction. The effect of each factor on the response variables was analysed from the standardized Pareto chart, and response surfaces of the mathematical models were obtained. Finally, the optimized conditions extrapolated by Box-Behnken design were as follow: EtOH 15%, temperature 90 °C, 5 cycles and static time 6 minutes.

Table 1 Experimental conditions of the response surface design (Box-Behnken design 2-factor interactions) and experimental values of the response variables (theobromine, caffeine and AOC)

Run	Independent variables				Response Variables		
	Static (min)	T (°C)	C (n°)	Solvent[a] (%)	TB (g/100g DM)	CF (g/100g DM)	ABTS (µmol/mg)
DOE1	4	110	3	7.5	1.82	0.42	1.45
DOE2	4	110	3	7.5	1.84	0.44	1.46
DOE3	4	110	5	15	1.91	0.45	1.52
DOE4	4	90	1	7.5	1.41	0.32	1.22
DOE5	4	130	1	7.5	1.68	0.38	1.49
DOE6	4	90	5	7.5	1.78	0.41	1.31
DOE7	4	130	5	7.5	1.90	0.45	1.53
DOE8	2	90	3	7.5	1.76	0.41	1.28
DOE9	2	130	3	7.5	1.86	0.43	1.53
DOE10	2	110	5	7.5	2.03	0.47	1.48
DOE11	4	110	1	15	1.65	0.37	1.35
DOE12	2	110	3	0	1.86	0.43	1.29
DOE13	2	110	3	15	1.85	0.42	1.42
DOE14	6	130	3	7.5	1.85	0.43	1.44
DOE15	6	110	5	7.5	1.97	0.47	1.51
DOE16	4	130	3	15	1.77	0.41	1.44
DOE17	6	90	3	7.5	1.86	0.43	1.34
DOE18	4	90	3	15	1.95	0.45	1.48
DOE19	6	110	3	15	1.94	0.46	1.50
DOE20	6	110	3	0	1.96	0.46	1.35
DOE21	4	110	3	7.5	1.81	0.42	1.40
DOE22	4	110	5	0	1.90	0.44	1.28
DOE23	2	110	1	7.5	1.61	0.35	1.23
DOE24	4	110	3	7.5	1.75	0.41	1.35
DOE25	4	90	3	0	1.81	0.41	1.19
DOE26	6	110	1	7.5	1.58	0.35	1.32
DOE27	4	130	3	0	1.75	0.40	1.29
DOE28	4	110	1	0	1.59	0.33	1.05

[a] Refers to % ethanol in water.

3.2.7 Quantitative (UPLC-UV) and qualitative analysis (UPLC-HRMS)

Quantitative and qualitative analysis of theobromine and caffeine of PHWE cocoa bean shell extract was performed on Waters ACQUITY UPLC system coupled with a Waters Xevo G2-XS QTof Mass Spectrometer and UV detector (Waters Corp., Milford, MA, USA). The extract was chromatographed on a column Kinetex Biphenyl (100 x 2.1 mm I.D., 2,6 μm) from Phenomenex (Torrance, CA, USA). Water (A) and acetonitrile (B), both acidified with 0.1% HCOOH, were used as mobile phases at flow rate of 0.4 $mLmin^{-1}$. The gradient was programmed as follow: 0-2 min 5% B, 2-4 min 5-10% B, 4-6 min 10-15% B, held for 2 min 8-10 min 15-50% B, 10-13 min 50-98% B held for 3 min. Before injection, the initial conditions were held for 5 min as re-equilibration step. The injection volume was 5 μL, and the column was maintained at 30 °C. The UPLC system was coupled to a UV and three different wavelengths: 210, 283 and 325 nm were acquired, and external standard calibration method was used to quantify TB and CF in all samples. Stock solution of TB and CF at concentration of 1 $mgmL^{-1}$ was properly diluted with H_2O to obtain calibration curves (six level) in the range of 1-200 μgmL^{-1}. The linearity of each calibration curve was tested by the analysis of variance (ANOVA), and linear model was found appropriate over the concentration used.

The Xevo G2-XS QTof Mass Spectrometer equipped with an ESI source, was used in positive and negative ionization mode to acquire full-scan MS, and MS/MS, and the spectra were recorded in the range of m/z 100–1200. The source parameters were as follows: electrospray capillary voltage 3.0 kV, source temperature 150 °C and desolvation temperature 500 °C. The cone and desolvation gas flows were 10 and 1000 L/h, respectively. A scan time of 0.1s was employed. The cone voltage was set to 60V, and ramping collision energies for MS/MS analysis was set up at 20V to produce abundant product ions before detection at the TOF. The mass spectrometer was calibrated with 0.5 M sodium formate, and leucine-enkephalin (100 pg/μL) was used as LockMass at m/z 557.2771 in positive and m/z 554.2615 in negative ion mode and 2 kV ionisation voltage which was infused simultaneously with the flow of column at 5μL/min and acquired for 1s each 15s. The full mass base peak chromatograms (BPI) were acquired, and the molecular ion mass [M+H]+ and [M-H]- were obtained, from which the elementary composition was calculated (mass error <5ppm). From the peak identification of the molecular ion mass whereas, from MS/MS spectra acquired at 20V

collision energy the fragmentation pattern was obtained, and the information was used for the identification. TB and CF were identified comparing their retention time (t_R) accurate mass (positive) calculated molecular formula error ppm (between detected mass and calculated accurate mass), MS/MS fragmentation with pure standard. MassLynx software (version 4.2) was used for mass spectrometer control, data acquisition and data analysis.

3.2.8 Evaluation of antioxidant activity (ABTS)

ABTS assay was used to evaluate the antioxidant activity of all sample extracts. The experimental conditions were reported by Campone et al. (Campone et al., 2018). Briefly, 5 μL of PBS (control), Trolox (0.25-1 mg mL^{-1}) and extracts 1 $mgmL^{-1}$ were mixed with 500 μL of ABTS standard solution at a concentration of 1mM. 300 μL of each mixture were transferred into a 96 well plate and were incubated, protected from light, and after 60min the absorbance was read at 734 nm using a Multiskan Go spectrophotometer (Thermo Fischer Scientific). Results of ABTS assay were expressed as Trolox equivalent TEAC μmol/mg, and they were employed to quantify the antioxidant activity of the tested solution expressed as standard deviation (SD) of three measurements.

3.2.9 Prediction of biological activity using PASS software

The Prediction of Activity Spectra for Substances (PASS) (Way2Drug.com © Version 2.0) software was used to determine the biological activity of metabolites found at higher levels in Cocoa PHWE (Dadwal et al., 2021; Siddiqui et al., 2021). This in silico tool is capable of indicating the probable activity (Pa) and probable inactivity (Pi) of 6000 types of bioactivities. Both Pa and Pi values have a range between 0.000 and 1.000, and generally Pa, ≠ Pi = 1 calculates the probable values independently (Singh et al., 2014). However, values of Pa > 0.7 indicate a higher likelihood of bioactivity for future experimental models. Therefore, only predicted bioactivity values >0.7 were considered.

3.2.10 Gastrointestinal digestion simulation

Gastrointestinal digestion was simulated following the INFOGEST protocol described by Minekus (Minekus et al., 2014). Briefly, the oral, gastric, and intestinal phases were simulated by mimicking the salt, enzyme composition, pH, time and temperature of each phase of the digestive process. At the end of the intestinal phase, the digested extracts were analyzed by UPLC-HRMS in order to quantify the remaining compounds. Bioaccessibility and bioavailability was calculated as reported by Cañas et al. (Cañas et al., 2022):

$$\%\ bioaccessibility = \frac{digested\ fraction}{non - digested\ fraction} * 100$$

Where non-digested fraction and digested fractions are the concentration of compound before and after digestion simulation, respectively.

$$\%\ bioavailaility = \frac{digested\ fraction * absorption}{non - digested\ fraction} * 100$$

The absorption was estimated in silico using pkCSM-pharmacokinetics (http://biosig.unimelb.edu.au/pkcsm/, consulted on 5 September 2023) e ADMETlab (https://admet.scbdd.com consulted on 5 September 2023). Chemioinformatics free software.

3.2.11 In vitro artificial blood-brain barrier membrane permeability test (PAMPA-BBB)

The experiment followed the instructions reported by Sánchez-Martínez with some minor modifications (Sánchez-Martínez et al., 2022). The experiment uses a tracer and acceptor plate system separated by a 45um pore size membrane. In detail, 5uL of BBB solution (8mg PBL + 4mg cholesterol dissolved in 600 µL n-dodecane) is applied to the filter at the base of the donor plate. Add 350uL phosphate buffered saline (PBS) 10mM pH=7.4 to the well of the acceptor plate. The donor and acceptor plates are then sandwiched together and 200uL of extract (5mgmL-1) solubilised in PBS is added to the donor plate (above the filter). The system is then incubated for 5 hours at 37°C in the dark. At the end of the incubation, 150 µL is taken from the acceptor and donor wells for

LC-MS qualitative/quantitative analysis and barrier passage verification. The permeability across the artificial barrier was calculated according to the formula:

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

Where A is the filter's area, V_D and V_A are the volume in the donator and acceptor well respectively, t is the incubation time (sec), C_A and C_e the compound's concentration in acceptor and balance respectively after incubation. The C_e was calculated following the formula:

$$C_e = \frac{[C_{D(t)} * V_D + C_{A(t)} * V_A]}{V_D + V_A}$$

3.2.12 Cell culture and viability assay

Human breast adenocarcinoma cell line MDA-MB-231, and human colorectal adenocarcinoma cell lines Caco-2 and SW48 was routinely grown at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 (Sigma R0883) supplemented with 10% Fetal Bovin Serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 $mgml^{-1}$ streptomycin. For MDA-MB-231, RMPI medium was also supplemented with 4mM Sodium Pyruvate. SW48 (*KRAS WT/WT*) cell line and the isogenic SW48 expressing heterozygous KRasG13D (*KRAS WT/G13D*) or KRasG12V (*KRAS WT/G12V*) were obtained from Horizon Discovery Ltd. MDA-MB-231 and Caco-2 were obtained from the American Type Culture Collection. Cells were passaged using trypsin–EDTA.

For measuring cell viability, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) assay was performed. 4800 cells per well were plated in a 96-well plate and were allowed to attach for 24 h in DMEM w/o phenol red and supplemented with 10% FBS, 10 mM glucose and 2mM glutamine. Cells were then treated for 72h with different concentrations of PHWE extract and cetuximab (Erbitux®, Merck Serono). After the treatment, culture medium was removed and then MTT solution was added (1:11). After a 4h incubation, 100μL of a solution of Isopropanol

0.1N HCl and 10% Triton X100 was added to solubilize the formazan crystals formed. The plate was then read by spectrophotometer Victor X3 (PerkinElmer) at 570 nm wavelength.

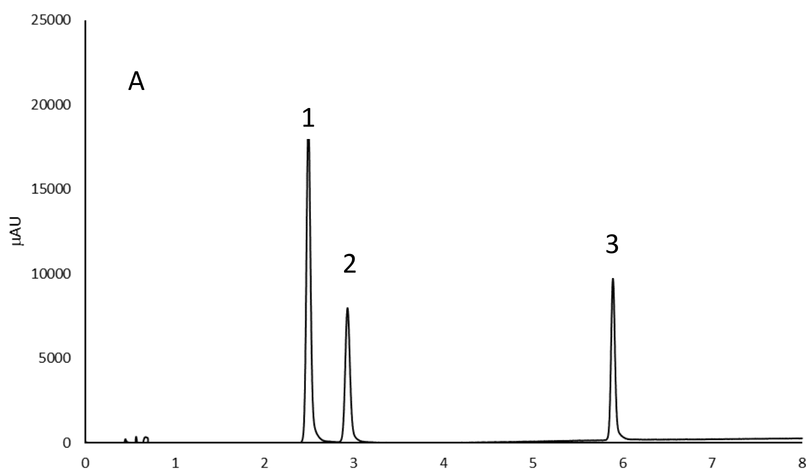
3.2.13 Statistical analysis

All data were performed in triplicate and results were presented as average ± standard deviation. Analysis of variance (ANOVA) was used to compare the means while Turkey's test was used to assess the statistically significant differences among treatments. For MTT assay, results were presented as average ± standard deviation and data are obtained from two or three independent experiments, each performed with three technical replicates. ANOVA test was used to compare the statistically significant differences among treatments, using JMP 14 software. Welch's correction was applied in the case of data violating the assumption of homogeneity of variances, by Levene's test. A p-value of ≤ 0.05 was considered significant.

3.3 Results and discussion

3.3.1 Analysis of USAE extract

The chemical composition of cocoa bean shell is quite variable, as is that of cocoa beans, and will depend, among other factors, on its origin and the processing to which it has been subjected (Okiyama et al., 2017). For this reason, to preliminary investigate the chemical composition of cocoa bean shell by-products, an ultrasound assisted solid liquid extraction was performed and analysed by UPLC-HRMS/MS. The UV chromatograms (280 and 300nm) showed the presence of only two main compounds eluted at 2.7 and 5.9min (Fig. 2 A).



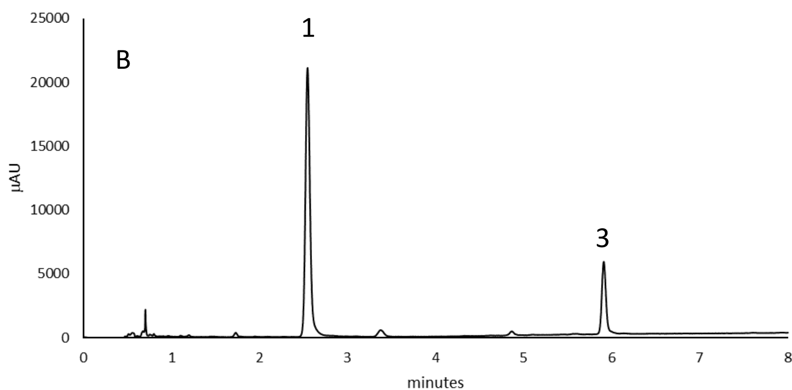


Figure 2 UV-Chromatograms of Cocoa by-product extract of standard compounds (a) and USAE (b) recorded at 283 nm.

However, to obtain a more detailed analysis useful to also identify the minor compounds in cocoa bean shell extract, the UHPLC-HRMS untarget analysis was performed in both positive and negative ionisation mode. The metabolites identification was carried out by using, HRMS data (accurate mass, isotopic distribution, and MS/MS characteristic fragmentation pathway) and using literature databases. Finally, the identified compounds were confirmed with standards. The UHPLC-MS/MS analysis in positive mode allowed the identification of 2 methylxanthines alkaloid, caffeine, and theobromine, corresponding at the two peaks shown in the UV chromatogram at 280nm.

The UHPLC-HRMS/MS in negative ion mode allowed the identification of 3 flavanols, catechin, epicatechin and procyanidin B2 which were not detectable in UV, perhaps due to poor concentration. The results of the qualitative analysis of cocoa bean shell were in accordance with the literature data on cocoa by-products (Okiyama et al., 2018). Once the main metabolites contained in the USA extract were identified, we carried out a quantitative analysis of the flavanols and alkaloids through the UPLC-UV-MS/MS analysis. The total extraction yield of USAE was 21.86 ± 1.3%, in particular theobromine and caffeine were the most abundant compounds in the extract at concentration of 35.9±0.21 and 7.03±0.15µg/mg Ext (1.32±0.01g/100g DM and 0.30±0.02g/100g DM) respectively while catechin, epicatechin and procyanidin B2 were found at concentration of 0.34±0.05; 1.77±0.21 and 0.35±0.05µg/mg Ext respectively. In general methylxanthine content were comparable to the data reported in literature (Barbosa-Pereira et al., 2018; Carrillo et al., 2014), while they are slightly higher than the results obtained by Okiyama et al. (Okiyama et al., 2018). These differences in the alkaloids content can be the consequence of a different migration of methylxanthines from bean to the shell during roasting process (Timbie et al., 1978) or due the fact that theobromine and caffeine levels depend on the origin of cocoa and cultivars (Arlorio et al., 2005). Regarding the concentration of flavonols the quantitative results were much lower comparing with the data reported in the literature (Nazaruddin et al., 2006; Okiyama et al., 2018), such variations in the levels of catechin, epicatechin and procyanidin B2 present in cocoa bean shell can be a consequence of several factors such as, climatic variations, genetic variability of the cultivars, harvest time, management, conditions which the cacao trees were grown (Oliveira et al, 2011). In addition, roasting processes could dramatically influence the polyphenol and flavanols content resulting in a reduction of portion of these compounds (Hurst et al., 2011).

3.3.2 Optimization of PHWE

3.3.2.1 Preliminary experiment for selection of PHWE temperature

After the qualitative and quantitative study of USA extract, which showed a low amount of phenolic substances, probably due to their degradation after roasting process our attention was focused mainly on methylxanthine being the majority compounds.

Therefore, the optimization of extraction conditions of PHWE process was carried out to primarily to improve extraction efficiency of caffeine and theobromine, but also trying to improve phenolic content. As commonly reported in literature, one of the most important parameters in PHWE process is the temperature (Pagano, Piccinelli, et al., 2018). For this reason, to select the temperature range to use in the experimental design, a preliminary experiment was performed increasing the temperature from 25 to 170 °C, and monitoring extraction yield (%), and TB and CF content. The other extraction parameters of PHWE system were set at central conditions of the experimental design (pressure 69bar, cycles 3, EtOH 7,5% static time 4 min.). As Figure 3A shows, the extraction yield percentage increases within the entire temperature range with a maximum of 49% ± 0.3 at 170°C, while the amount of TB and CF increases up to 110°C and subsequently decreases till 170°C, as shown in the Figure 3B and C respectively. This behaviour demonstrates that high values of extraction temperature correspond to an enhance of interfering compounds, without any benefit on extraction efficiency of target compounds. Based on these results, the temperature range between 90 and 130 °C was used in the next optimization performed by an experimental design.

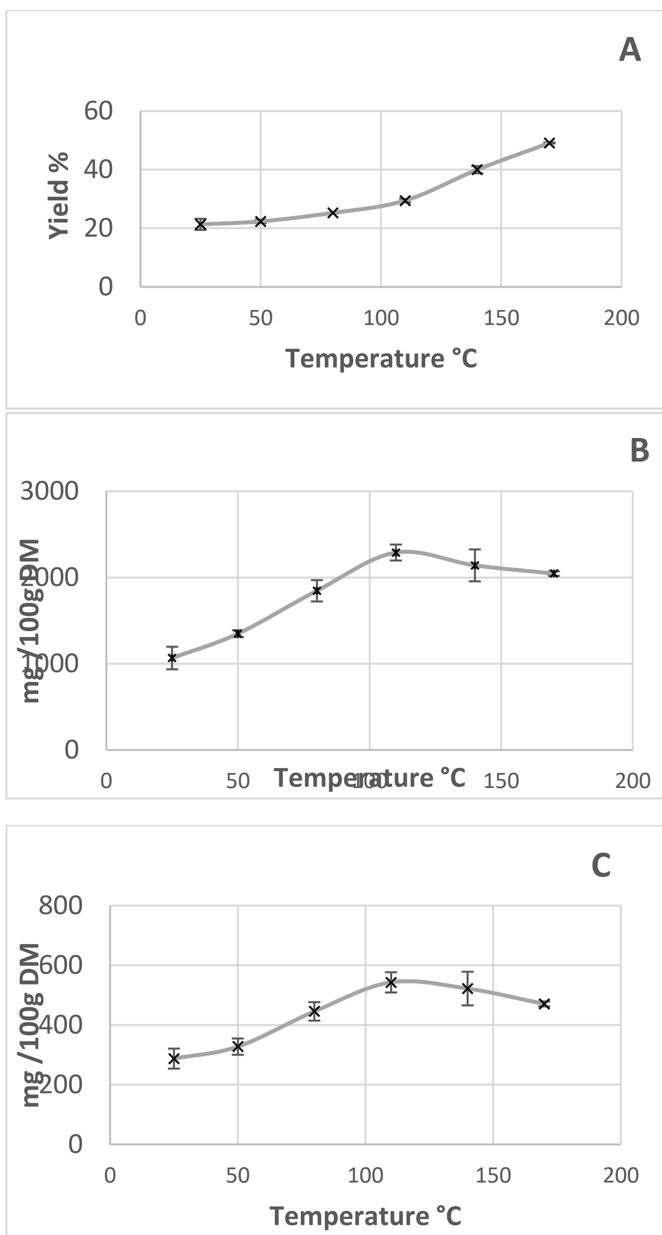


Figure 3 Preliminary experiments of extraction yield (A), theobromine (B) and caffeine (C) vs temperature at the central point of the response surface design experimental conditions: pressure 1000 psi; static time 4 minutes; cycles 3; and solvent EtOH 7,5%.

3.3.2.2 Response surface design of PHWE process

After the preliminary experiments performed to select the extraction temperature range, the optimization of the main extraction parameters that influence PHWE process was

carried out by experimental design. As well known, pressurized liquid extraction is a technique affected by several parameters (temperature, static time, solvent composition, pressure, and cycles etc.) simultaneously (Pagano et al., 2021), therefore to get the best extraction efficiency, it is extremely important to select the right parameters affecting the extraction process. The optimization of multivariate process such as PHWE can be carried out by one variable at time (OVAT) approach or using a chemometric analysis by an experimental design (DOE). The optimization performed by using OVAT approach requires many experiments, and it does not guarantee to find out the real optimum conditions, unless the variables are totally independent from each other. On the contrary, the use of an experimental design allows to evaluate the interaction among the variables, minimizing the experimental error and the number of experiments (Campone et al., 2018). In this study, the optimization was performed considering four of the most important PHWE parameters (temperature, cycles, static time, and solvent composition) with three response variables: the two methylxanthines content and the antioxidant activity (ABTS). Regarding the extraction solvent, its selection was carried out considering the analytes affinity and the environmental impact, thus water containing small amount of organic solvent (EtOH 15%) was selected to improve solubility of target analytes and at the same time keeping the extraction a green process (Pagano, Piccinelli, et al., 2018). Based on these considerations, a Box-Behnken design was selected to investigate the effect of extraction temperature, static time, numbers of cycles and EtOH % in water, on methylxanthines contents and antioxidant activity (ABTS) of PHWE extracts. Table 1 shows the experimental conditions for each run, and the experimental values of the responses at different experimental conditions. The statistical significance of the response variables studied can be observed from the standardized pareto chart for each experimental factor (figure 2A-C). The significance of the effects at 95% confidence level was highlighted by the vertical line in the chart whereas positive (grey) and negative (dashed) effect in the response variables were indicated by different bars colour. Cycles resulted to be the most important parameters in the model, as they make a positive contribution to the three response variables TB, CF, and AOC (Figure 4A, B and C). Besides, the quadratic effect of the cycles ($p < 0.05$) showed to be the second important term in two of the selected variables, making a negative contribution to TB and CF content (Figure 4A and B). At last, the other two experimental factors,

temperature and static time, also showed a positive influence ($p<0.05$) on the model of AOC. The influence of cycles and temperature were clearly highlighted for all the response variables in the response surface plots (Fig. 5A-C). They were also used for predicting and optimizing the responses. As it can be seen in Figure 3A and B, the two response variables, TB and CF show the same behaviour: the extraction efficiency improves as the number of cycles of the PHWE process increases, whereas an increase of the AOC (TEAC) is achieved by increasing both temperature and cycles. This behaviour was also observed in the desirability plot (Figure 6), where cycles are the parameter that has the greater influence on the desired effect, indeed increasing the numbers of cycles from 1 to 5 the desirability increases. The temperature has instead a slight influence on the desirability. Finally, considering the three response variables (caffeine, theobromine and AOC), the chemometric analysis suggested the flowing parameters in the optimized conditions: Temperature 90 °C, Cycles 5, EtOH 15% and Static Time 6 minutes with an optimized desirability = 0.984166 (98.42%). Previous studies have evaluated the capability of PLE as green extraction techniques, Okiyama et al. (Okiyama et al., 2018) studied the kinetics of PLE at different temperature on the extraction of flavanols and alkaloids from cocoa bean shell using as solvent pure ethanol. Their results provided a comparable amount of caffeine and theobromine but a higher content of flavonoids. In the study conducted by Jokić et al. (Jokić et al., 2018) regarding the optimization of subcritical water extraction of phenolic compounds and methylxanthine from cocoa bean shell. The authors demonstrated that the increase of the temperature in a range from 120°C to 220°C and reaction times from 15min to 75min resulted in a high content of target compounds in the same concentration range of those obtained in our study but they also highlighted, that high temperatures lead to the extraction of unwanted compounds.

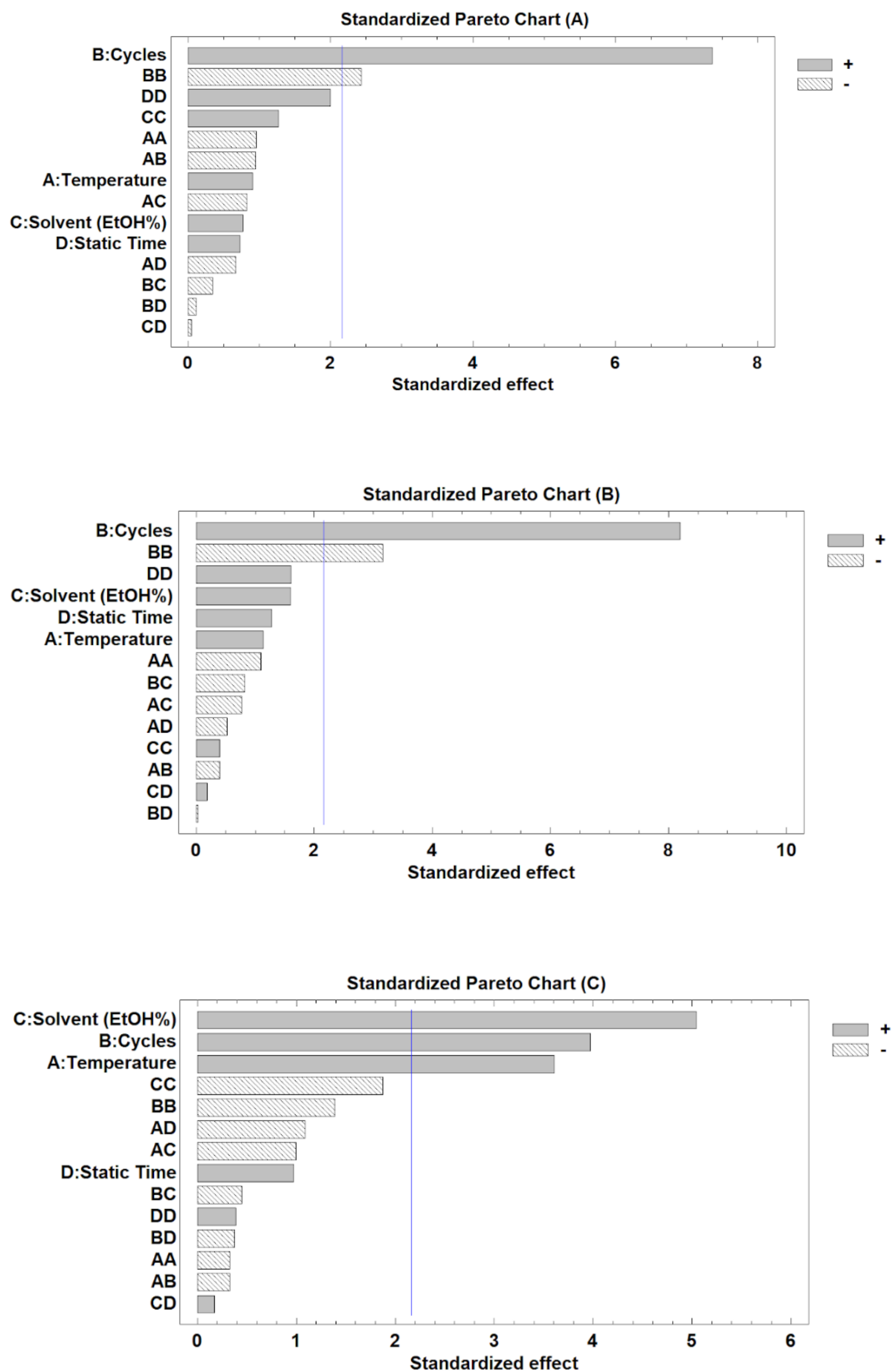


Figure 4 Standardized Pareto Chart of Theobromine (A) Caffeine (B) and AOC-ABTS (C) shows the estimated effects and interactions of each term in the Box-Behnken design

model in decreasing order of significance. Bars beyond the vertical line are statistically significant with confidence level of 95%.

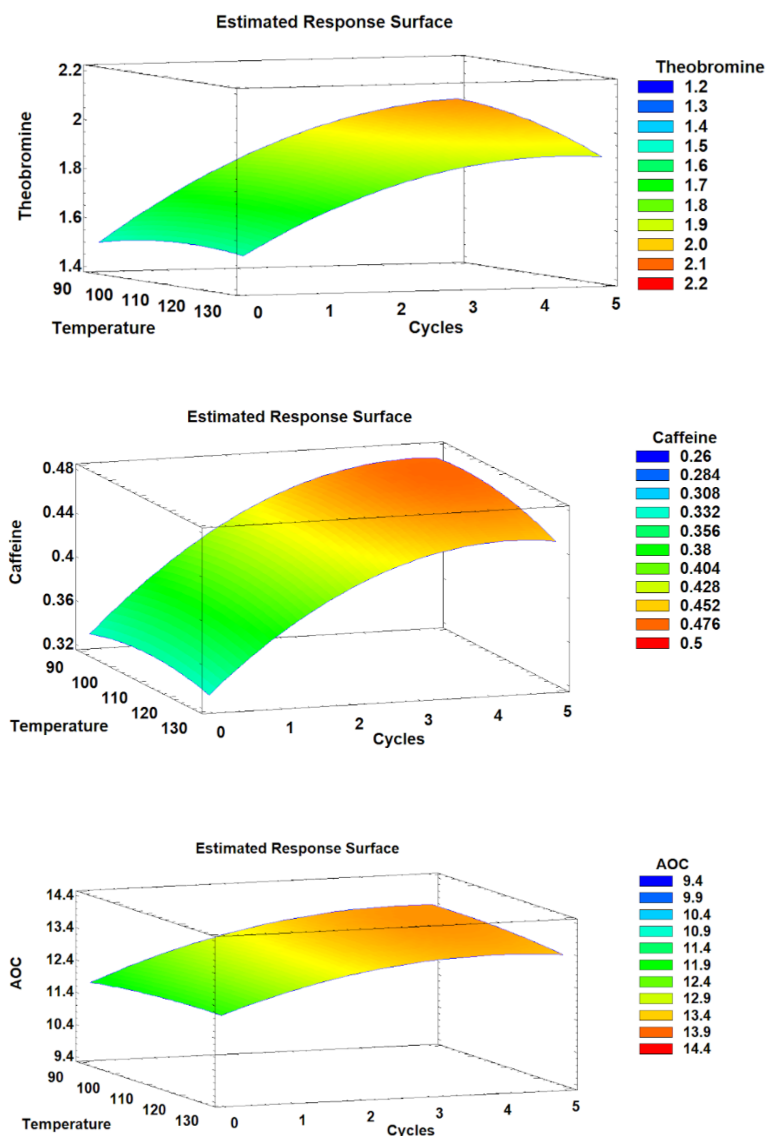


Figure 5 Response surface plot representing the predicted value of each response variables theobromine (g TB/100g DM), caffeine (g CF/100g DM) and AOC-ABTS (TEAC) over the space of temperature (90-130 °C) and cycles (1-5). The other two factors were held constant at their higher values (optimized condition).

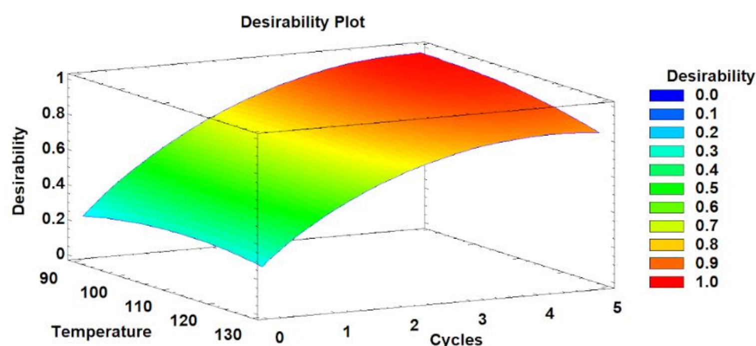


Figure 6 Response surface plot displayed the overall desirability versus the space of two factors temperature (90-130 °C) and cycles (1-5) at same time, the other two factors, the other two factors were held constant at their higher values (optimized condition).

3.3.3 Quantitative analysis and antioxidant activity of PHWE extract

From a quantitative point of view optimized experimental conditions of response surface design provided an increase in total extraction yield of 10%. To evaluate the improvement of extraction efficiency of target compounds (TB and CF) obtained under optimized conditions, a recovery study was carried out by an UPLC-UV analysis. The recoveries were calculated by comparing the amount of methylxanthine compounds in PHWE extract with those obtained by the ultrasound extraction technique. The results were TB 156.4% ± 2.8 and CF 160.8% ± 3.5. This demonstrates the possibility of developing a method capable of providing an exhaustive extraction efficiency, even greater than usual (USAE) extraction techniques.

Finally, the quantitative analyses of methylxanthines in the PHWE extract under optimized condition were carried out by UPLC-UV using the external standard method. Theobromine and caffeine calibration curves in the concentration range of 0.1-10 μgmL^{-1} were used to quantify their content into PHWE extract. The external standard calibration curves for theobromine and caffeine provided food linearity within the investigated concertation range with correlation coefficients (R^2) of 0.9993 and 0.9992 respectively. The quantitative analysis of PHWE extracts revealed TB and CF contents were 2.06 ± 0.06 and 0.48± 0.02 g/100g DM respectively (56.2 ± 0.16 and 11.3 ± 0.32

µg/mgEXT). In general, quantitative results concerning caffeine and theobromine were in accordance with the range of data reported in the literature for cocoa bean shell. In particular theobromine content was similar than that observed by Okiyama 2018 et all for the cocoa bean shell using pressurized liquid extraction (Okiyama et al., 2018), but it was much higher than that reported by Ortega et al. (Ortega et al., 2010) and Soares et al., (Soares et al., 2020). Regarding the caffeine content, it was so much higher than that observed by Soares et al. and Ortega et al. (Okiyama et al., 2018; Soares et al., 2020). In addition, the antioxidant capacity (AOC) of PHWE extract under optimized conditions was evaluated. The AOC in the PHWE was 0.266 ± 0.12 (µmol TE/mg EXT), this result confirms the low radical scavenger activity of PHWE extract even after the optimization process. This data depends to the low content of catechin, epicatechin and procyanidin B2 that remain comparable to the result obtained in preliminary experiment by using ultrasound assisted extraction. This result was close to those report by Soares et al. (Soares et al., 2020) but lower to the result reported by Hernanz et al. (Rebollo-Hernanz et al., 2021). This variability can be explained throughout different production process steps, such as fermentation, roasting, and alkalizing but also to the origin and variety of cacao.

3.3.4 in silico prediction activity and molecular mechanism of cocoa extract

Methylxanthines have been used in the diet since ancient times for their stimulant activities on the central nervous system (SNC) and beneficial properties acting against respiratory diseases, cardiovascular diseases, obesity, diabetes, and especially against neurodegenerative diseases and cancer. The PASS online software was used for predictions of bioactivities and/or molecular targets *in silico*. The analysis was obtained by using the simplified molecular input line entry system (SMILES) code of each molecule into the software and obtaining in response a table containing the probable activity (PA) and probable inactivity (PI) values with a biological activity variability between 0 and 1. Biological predictions with PA > 0.7 show the maximum potential biological activity. In detail for caffeine, vasodilator and diuretic capacity was shown with Pa > 0.7, which is important in counteracting cardiovascular disorders, which are also responsible for serious pathological conditions. Theomobrine, showed many properties similar to that of caffeine, but also exhibited oxygen scavenger power with Pa

> 0.6. Finally, both methylxanthines revealed a high probability (Pa > 0.8) of inhibiting Cyclic AMP phosphodiesterase. This information is very important because phosphodiesterases are a group of 11 enzymes that perform numerous functions in the body, but they appear to play a key role in neurodegenerative diseases such as Alzheimer's. In particular, studies have shown that the synthesis and degradation of cyclic nucleotides (including cyclic AMP) are associated with cognitive impairment, as cAMP levels are important for the maintenance of cognitive function. In addition to inhibiting phosphodiesterases, other studies have shown that theobromine and caffeine intervene in the regulation of calcium mobility, as antagonists of adenosine receptors, and in the modulation of GABA receptor function, thus playing an active role in prevention, but the mechanism is not completely understood.

Finally, the possible activities of catechin, epicatechin, and procyanidin B2 were also considered, showing particular efficacy as antioxidant, chemopreventive, and anticarcinogenic (Pa > 0.7). All possible targets and properties of each molecule identified in the extract were resumed in Table 2

Table 2 Main potential biologic activities predicted using Pass online based software.

PHWE compounds	predicted bioactivities	Pa	Pi
caffeine	Vasodilator	0.714	0.007
	Diuretic	0.720	0.003
	Cyclic AMP phosphodiesterase inhibiter	0.943	0.003
	Neurotrasmitter antagonist	0.402	0.091
theobromine	Vasodilator	0.695	0.010
	Oxygen scavenger	0.616	0.019
	Neurotrasmitter antagonist	0.444	0.068
	Cyclic AMP phosphodiesterase inhibiter	0.858	0.003
Catechine/	Lipid peroxidase inhibitor	0.888	0.003
epicatechine	Antioxidant	0.810	0.003
	Anticarcinogenic	0.795	0.005
	Chemiopreventive	0.788	0.004
Procianidine B2	Lipid peroxidase inhibitor	0.741	0.004

Antioxidant	0.803	0.003
Anticarcinogenic	0.757	0.007
Chemiopreventive	0.830	0.003
Antihemorrhagic	0.884	0.002
Antimutagenic	0.878	0.002

3.3.5. *Blood-brain barrier permeability of PHWE cocoa bean shell*

Given the predictive neuroprotective activity and the known activity on the central nervous system, the permeability of PHWE extract across the blood-brain barrier (BBB) was measured. The BBB is a selective barrier with a protective function in the brain, consisting of endothelial cells that form micro vessels in the brain. One of the most widely used methods is the application of Parallel Artificial Membrane Permeability (PAMPA) technology to assess the ability of molecules to cross it and exert a function on the nervous system. This approach uses non-biological membranes to simulate passive passage across them and can therefore be used to mimic passive transport across the blood-brain barrier of drugs or natural compounds (Mensch 2010, Simon 2020). Firstly, it is important to consider that the permeability of a compound depends on numerous factors such as lipophilicity, topological polar surface area (TPSA), molecular weight and ionisation at physiological pH, as well as the presence of specific transporters that may interfere with and/or favour the entry of substances. The results obtained by applying PAMPA-BBB to PHWE cocoa extract, after UHPLC-HMRS qualitative-quantitative analysis, showed the presence in the acceptor plate of only two compounds present in the PHWE extract: theobromine and caffeine. This may be related to certain physicochemical properties of these two methylxanthines. Although they are polar compounds, they have a low molecular weight (< 500 Da) of 180.16 g/mol (TB) and 194.19 g/mol (CF), which could explain their ability to cross the BBB equally well. In addition, these molecules have a TPSA of 72.454 $Å^2$ (TB) and 79.029 $Å^2$ (CF), and it is known that substances with a TPSA of less than 90 $Å^2$ have sufficient blood-brain barrier permeability. Finally, simulation of passage through the PAMPA-BBB system showed higher permeability of caffeine than theobromine with LogPe of -5.68 and -6.83 cm s^{-1}

respectively. These values are similar to the permeability coefficients of some drugs used in the therapy of neurodegenerative pathologies, such as galantamine (Sánchez-Martínez et al., 2022), thus highlighting their good BBB crossing ability. Considering the great permeability of caffeine and theobromine in the cocoa PHWE extract, its could be considered for further studies to assess its effective role in the prevention of Alzheimer's and neurodegenerative diseases, which are rapidly increasing in society.

3.3.6 Cytotoxicity evaluation of PHWE extract, alone or in combination with cetuximab, on human cancer cell lines

According to the in silico online analysis, the phenolic fraction of PHWE extract also showed probable anti-cancer activity. Therefore, to evaluate the capability of PHWE extract of altering the viability of human cells, colorectal cancer cell lines isogenic for KRAS gene, one of the proto-oncogenes more frequently mutated in human cancers, including the deadliest ones, and a breast cancer cell line were treated with increasing doses of extract from 0.016 to 250ng/µl. In detail, SW48 cells expressing wild type KRas (SW48 KRAS wt/wt) or the oncogenic mutant KrasG13D (SW48 KRAS wt/G13D), and the triple negative breast cancer cells expressing KrasG13D, MDA-MB-231 were used. Once verified that the extract alone had no cytotoxic effect, even at high doses (Figure 7A), the extract was tested in co-treatment with cetuximab, a known anti-cancer agent that is used against some cancers, including the colorectal one (Galizia et al., 2007). This drug is a monoclonal antibody directed against the EGF receptor, which acts by attenuating the mitogenic and pro-survival signalling pathway mediated by the Ras/Raf/MAPK axis (Molina & Adjei, 2006). Tumours harbouring specific oncogenic mutations in signalling elements downstream of the EGF receptor, such as KRas and BRAF, show intrinsic resistance to cetuximab and do not benefit from treatment (Bray et al., 2019). Unfortunately, these mutations accounts for ~40% of colorectal cancer cases. The viability of cells treated with the higher dose of PHWE extract (250 ng / µl) in combination with increasing doses of cetuximab (0.5-50nM) was evaluated by using MTT assay. In detail, a cell lines group partially responsive to cetuximab, such as Caco-2 cells and SW48 cells expressing wild type KRas or $KRas^{G13D}$ (Tisi et al., 2021, McFall & Stites, 2020), and a cetuximab-insensitive cell line group, such as SW48 cells expressing $KRas^{G12V}$ (Tisi et al 2021, McFall & Stites, 2020) and triple negative MDA-

MB-231 cells, which express the oncoprotein Raf^{G464V} in addition to $KRas^{G13D}$ were treated. From the analysis, it emerged that the PHWE extract significantly sensitizes to cetuximab the cells expressing $KRas^{G13D}$, including partially responsive (SW48 $KRas^{G13D}$) and resistant (MDA-MB-231) cells (Figure 7B). In the cetuximab-insensitive SW48 cells, expressing the $KRas^{G12V}$, the combined treatment resulted in a reduction in cell viability if compared to cetuximab alone, albeit below the threshold of statistical significance, while no effect was detected in the Ras wt expressing cells, i.e. Caco2 and SW48 w.t. cells (Figure 7B). The observed effect could be due to the peculiar allele-specific signalling network generated by $KRas^{G13D}$ mutant in a context with over-expressed EGFR, as previously described (Tisi et al., 2021).

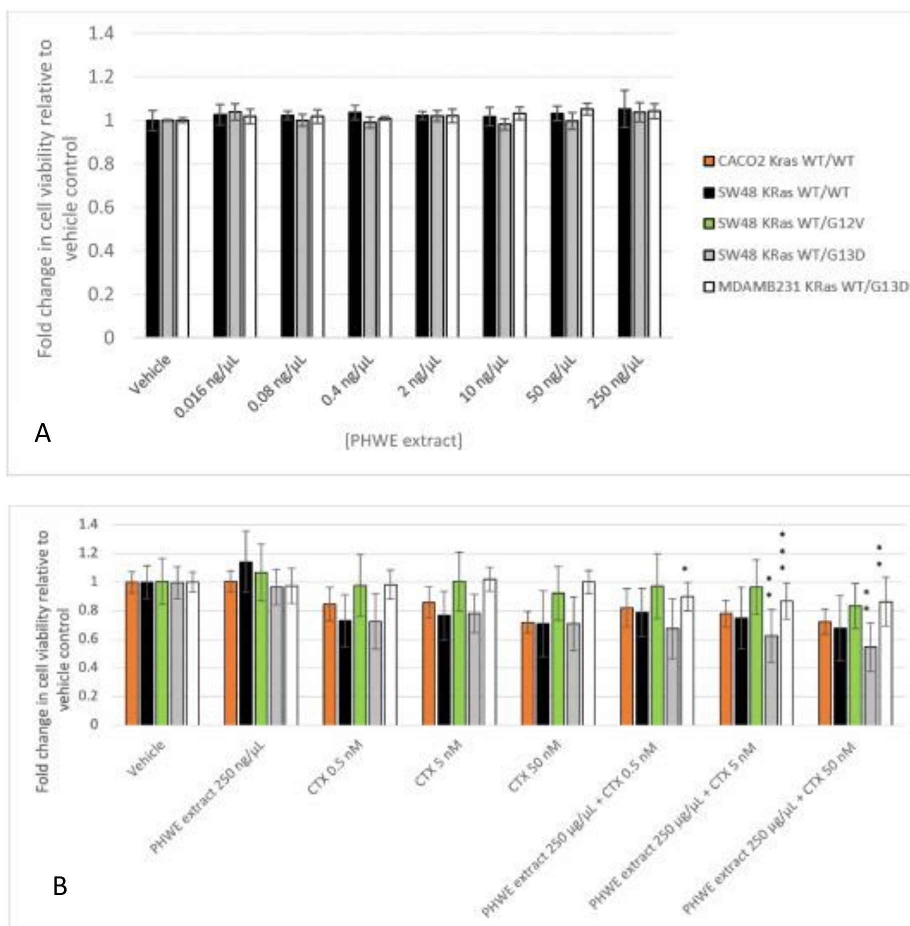


Figure 7 Effect of 72h treatment with PHWE extract, alone (A) or in combination con cetuximab (CTX) (B), on cell viability of human colorectal cancer SW48 isogenic cell

lines, colorectal cancer Caco2 cell line and breast cancer MDAMB231 cell line, as measured by MTT assay. Triple, double, and single asterisk above histograms refer to the comparison to the same cell lines treated with cetuximab alone and indicate a statistical significance of 99,999 and 95% respectively. Welch's correlation was applied to the comparison between treatment with 50nM CTX and the cotreatment with 50nM CTX+PHWE inMDAMB231 cells since Levene's test did not confirm the homogeneity of variances.

3.3.7 *In vitro gastrointestinal digestion simulation by INFOGEST protocol*

Considering the possible use of PHWE extract as dietary supplements, it is important to evaluate the effects of gastrointestinal digestion on the identified compounds, with particular attention to caffeine and theobromine. For this purpose, the INFOGEST protocol was selected from among various static digestion models, which have a set of digestion parameters defined according to human physiological knowledge and internationally agreed to adequately simulate the digestion of food or food ingredients in the adult organism. INFOGEST is widely used for bioaccessibility studies of phytochemical compounds such as polyphenols, methylxanthines and numerous secondary metabolites, as well as for observing the digestive fate of proteins, lipids and carbohydrates (Brodkorp et al.,2019; Minekus et al., 2014).

The results obtained showed a significant resistance of methylxanthines (caffeine and theobromine) to the enzymatic digestion process. Specifically, after the intestinal digestion step, theobromine and caffeine concentrations remained at 46.65 ± 0.15 and 9.61 ± 0.09 µg/mg EXT, respectively, with a bioaccessibility of approximately 80% (83% for theobromine and 85% for caffeine). Bioaccessibility refers to the amount of compost that resists digestion and is available for absorption. However, in addition to this information, it is important to calculate bioavailability, which refers to the percentage of the compost that is absorbed through the gastrointestinal wall. Specifically, a bioavailability of 84.8% was calculated for caffeine and 81.7% for theobromine, indicating a high ability of the molecules to cross the intestinal barrier. This can be attributed to a high hydrophilicity, confirmed by negative values of the partition coefficient (LogP= -1.0293 for caffeine and LogP= -1.0397 for theobromine), as well as

a reduced molecular weight and a marked molecular stability. These results suggest that caffeine and theobromine have high bioaccessibility and bioavailability, which certainly allows their evaluation in functional foods by influencing their activities after oral ingestion. Instead, after gastrointestinal digestion, the three flavanols present in the initial extract (catechin, epicatechin and procyanidin B2) are not found. It is known that catechins and their polymers are easily degraded by the digestive process, they can easily undergo autoxidation processes due to the alkaline pH of the intestine and the presence of bile salts (Pagliari et al., 2023).

3.4 Conclusions

Experimental results have shown that cocoa bean shells obtained after bean roasting are a good source for the recovery of methylxanthines. In particular, pressurized hot water extraction proved to be an efficient and sustainable method to increase the recovery of theobromine and caffeine from this plant matrix. Extraction temperature and number of cycles play a critical role in extraction efficiency. In particular, under optimized conditions (90°C, 5 n° of cycles, EtOH 15%, static time 6min) a good recovery of TB e CF of 2.06 ± 0.06 and 0.48 ± 0.02 g/100g DM, respectively, were obtained.

In silico analysis of the potential bioactivities of the extract showed interesting activity as phosphodiesterase enzyme inhibitors for the treatment of neurodegenerative diseases such as Alzheimer's disease. Following this result, the ability of the extract to cross the blood-brain barrier was evaluated and good permeability of caffeine and theobromine alone was observed, similar to that of galantamine, used in the treatment of neurodegenerative diseases. Moreover, the chemopreventive activity associated with most of the minor compounds was evaluated using cellular assays, which revealed an interesting mechanism of PHWE extract to sensitise human cancer cells to the anticancer drug cetuximab, similar to that of other natural products such as lauric acid (Weng et al., 2016) and honokiol (Pearson et al., 2018). Furthermore, the excellent resistance to gastrointestinal digestion and good availability of methylxanthines allow PHWE extract to be considered as an ingredient for nutraceuticals or functional foods with good prospects for industrial scalability.

Acknowledgement

I want to thanks Professor Elena Sacco of the University of Milan-Biccoca (Italy) for the data provided on the in vitro cellular tests. Also, a big thanks to Professor Alejandro Cifuentes and all laboratory team for welcoming me at Instituto de Investigación en Ciencias de la Alimentación (CIAL), in Madrid (Spain) and to supporting me in the bioactivity and permeability studies of the extracts.

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Chapter 4

Optimization of two different green extraction techniques for recovery of naturally occurring glucosinolates from by-products of *Camelina sativa* Crantz (L.) and their effect on human colorectal cancer cell line

Abstract

The food waste generated by small and medium agro-industrial enterprise requires appropriate management and valorization in order to decrease environmental problems and recover high-value products, respectively. In this study, the *Camelina sativa* seed by-product was used as a source of glucosinolates. To begin, the chemical profile of the extract obtained using an international organization for standardization (ISO) procedure was performed by UPLC-HRMS/MS analysis. In addition, two extraction methods based on ultrasound-assisted extraction and pressurized-liquid extraction, were developed as an alternative and green methods to recover glucosinolates. The main parameters that affect extraction efficiency, for both extraction techniques, were optimized using a response surface design. Under optimized conditions, the USAE and PLE extracts showed an improvement in extraction yield with a reduction of organic solvent amount and extraction time compared to those obtained using the ISO procedure. Finally, the optimised extract was purified to obtain an extract enriched in GLSs to evaluate its therapeutic properties. In silico analysis using software and in vitro analysis on human colorectal cancer cell lines showed promising results in chemopreventive activity. Finally, to assess the degradation of GLSs potentially ingested with the diet, a simulation of *in vitro* gastrointestinal digestion was performed.

4.1 Introduction

Camelina sativa is a herbaceous plant in the Brassicaceae family that is easy to grow due to its high adaptability and low water, fertilizer, and pesticide requirements compared to other oilseed crops; so C. sativa was identified in the 1960s as a crop suitable for sustainable farming systems (Ergönül & Özbek, 2020). Today, its cultivation for oil production is rapidly increasing in Europe and North America. *Camelina sativa* seeds contain 30-40% oil rich in unsaturated fatty acids. Cold-pressed camelina seed oil appears to be an excellent vegetable alternative to fish and flax oil due to its unique profile of essential fatty acids such as linoleic (omega-6) and linolenic (omega-3) and high levels of active molecules such as tocopherol, phytosterol, and phenolic compounds, glucosinolates, which place it among alternative oil plants that can help reduce health problems caused by poor diet (Ergönül & Özbek, 2020; Gugel & Falk, 2011; Piravi-vanak et al., 2022). However, following to the oil production generate large amount of by-product called camelina cake, which has to be disposed. Nowadays, huge population growth and urbanization have led to a sharp increase in food production worldwide (K. Xu et al., 2015), resulting in the generation of food waste (FW) and the search for alternatives in the waste management system (Garcia-Garcia et al., 2017). For this purpose, agricultural food by-products are often used as sustainable source of bioactive molecules, in particular, the camelina cake obtained after pressing still contain several interesting compounds that could be used in different industrial applications. For examples due to its good content of amino acid and protein, the camelina cake could be suitable for animal feed (Erickson et al., 2012; Zubr, 1997), however, this use is currently limited by the presence of antinutritional compounds such as glucosinolates (GLSs), sinapin, inositol phosphates, condensed tannins and erucic acid. GLSs are particularly abundant in the Brassicaceae family; they are secondary metabolites with a similar basic structure, consisting of a D-thioglucose group linked to a sulfonated aldoxy group and a variable side chain derived from an amino acid. Today, more than 120 different glucosinolates have been identified in many plants such as mustard, cabbage, cauliflower, broccoli, radish, etc. It has been shown that the composition and concentration of GLSs vary between species and within the same variety depending on the environment, cultural conditions, age and health of the plant (Fahey et al., n.d.;

Mithen et al., n.d.; Ram Bhandari et al., 2015). GLSs play an important role in plant protection (Bohinc et al., 2012; RADOJČIĆ REDOVNIKOVIĆ et al., 2008). In fact, these compounds remain inactive unless they interact with an enzyme called myrosinase, which converts them first to glucose and aglycones, and then to other molecules such as nitriles or isothiocyanates (N. Li et al., 2021). The glucosinolate-myrosinase system is used as a defence against external plant aggression, which is why GLSs are also used as natural pesticides and biofumigants (Holst & Williamson, 2004; Ziedan & Moliszewska, 2022). In addition, recent studies suggest that glucosinolates have health benefits and may help reduce neurodegenerative and cardiovascular diseases when consumed in the diet (Kamal et al., 2022; Ma et al., 2018; M. H. Traka, 2016; M. Traka & Mithen, 2008). Because of their diverse chemical and biological properties, these phytochemicals have attracted the attention of many researchers. Plant species containing high concentrations of individual GLS or a limited number of GLS are one of the most suitable sources for extracting and purifying discrete amounts of these compounds. However, to exploit *Camelina sativa* pressed cake (PC) as a source of bioactive compounds, efficient and suitable extraction methods need to be developed (Pagano et al., 2021). The recovery of GLSs procedures involve several time-consuming and potentially hazardous steps. These steps are freeze-drying, tissue disruption and laborious and polluting extraction protocol involving the use of toxic extraction solvent. Therefore, it is important, to use sustainable processes with low environmental impact to improve the recovery of metabolites from by-products such as camelina cake. Currently, the use of new green techniques such as microwave extraction, ultrasound (USAE), pressurised liquid extraction (PLE) or supercritical CO_2 is becoming more widespread for the extraction of broad class of analytes. These green extraction techniques have many advantages over conventional one (maceration, distillation, Soxhlet, etc.) such as reduce energy consumption, waste management, time consuming and cost of production, improving at the same time the safety and health of consumer, the regulatory compliance, and preserving the bioactive compounds in the matrix.

In the present work, initially, the international standard method ISO9167-1 (Norm, 1992) for GLSs extraction was used to obtain an extract to be used as reference and also to develop a an analytical procedure based on ultra-pressure liquid chromatography

(UPLC) coupled to a high-resolution mass spectrometry (HRMS) to investigate the chemical composition of PC. After chemical characterization, two different methods based on ultrasound assisted extraction (USAE) and pressurized liquid extraction (PLE) was developed in order to increasing the extraction efficiency and replace toxic traditional solvents, employing in the ISO procedure, with sustainable solvent. The main parameters of USAE and PLE were carefully optimized using an experimental design to improve extraction efficiency and reduce organic solvent consumption. Under optimized extraction conditions, the developed PLE method showed better efficiency than that obtained by the ISO and USAE procedures. In addition, a purification method based on a rapid solid-phase extraction (SPE) was developed to isolate and concentrate the glucosinolate compounds. Furthermore, the biological activities of GLSs in the purified extract was investigated by in silico and in vitro assays, the putative properties and their anticancer activity respectively. Finally, *in vitro*, gastrointestinal digestion was simulated to evaluate the bioaccessibility and bioavailability of the compounds.

4.2 Materials and methods

4.2.1 Standards and materials

MS-grade solvents used for UPLC analysis acetonitrile (MeCN) water (H_2O) and formic acid (HCOOH) were provided by Romil (Cambridge, UK); analytical-grade solvents methanol (MeOH) and ethanol (EtOH) were supplied by Sigma-Aldrich (Milan, Italy). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). Acetic acid (Sigma-Aldrich) and ammonium hydroxide solution naphthylethylene diamine dihydrochloride, phosphoric acid, ascorbic acid, fluorescein sodium salt, Trizma hydrocloride (Tris-HCl) monopotassium phosphate, Dipotassium phosphate, sodium nitroprusside dehydrate (SNP), sulphanilamide were provided by Sigma-Aldrich (Milan, Italy). 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from TCI Chemicals (Tokyo, Japan). Glucoarabinin potassium salt, glucocamelin potassium salt and homoglucocamelinin potassium salt were purchased from Extrasynthese (Lyion, France). Stock standard solutions (1 mg mL^{-1}) of each compound were prepared using methanol and stored at 4°C. Diluted solutions and mixtures were made in MeOH:H_2O 1:1, (v:v).

4.2.2 Samples

Camelina sativa pressed cake (PC) was supplied by FlaNat Research srl (Milan Italy). After cold oil extraction, the by-product was immediately finely blended using a knife mill, Grindomix GM-200 (Restek GmbH Germany) operated at 6000 rpm for about 30 short cycles of approximately 15 seconds each, to prevent samples from heating. The ground samples were sieved through a test sieve in the range of 300-600 μm to obtain a powder with a homogeneous particle size distribution and stored in the dark at -80°C in polyethylene bags until the analysis.

4.2.3 Optimization of ultrasound-assisted extraction

Extraction of GLS compounds from PC was performed by ultrasound-assisted solid liquid extraction (USAE). For each extraction, 1 g of finely ground sample was placed in a 50 mL polypropylene tube, and an extraction solvent was added to the sample. Then, the tube was gently shaken by hands for a few seconds and immerse in the ultrasonic bath (frequency, 35 kHz; power, 60-120 W; Sonorex TK 52; Bandelin electronic, Berlin, Germany). During the extraction, the water bath temperature was continuously monitored and adjusted to maintain a constant temperature of 30°C. At the end of each extraction cycle, lasting 5 minutes for each, the samples were centrifuged (ALC centrifuge PK 120, Thermo Electro Corporation, San Jose, CA, USA) at 19.8 g. The supernatant was collected with a Pasteur pipette, filtered (Whatman No. 1 filter) and analysed by UPLC-HRMS. A central composite experimental design (CCD) was performed to select the best extraction conditions using Statgraphic Centurion XVI Version 16.1 (Rockville, MD, United States). The effect of four independent factors on extraction efficiency and the total amount of EtOH were studied through an experimental design. The range within each factor, were selected by preliminary experiments. In particular, a response surface Box-Behnken design 2-factor interaction was carried out considering three variables at three different levels (low, medium, and high): solvent volume (vol) at 5, 10 and 15 mL, number of cycles (n°) 2, 3 and 4 and composition of solvent (EtOH %) 40, 60 and 80%. Four response variables were individually considered in the optimization of the extraction conditions: the extraction yield of each GLS was expressed as μg g^{-1} of dry matter (μg/g DM), and total ethanol used (mL). A total of 16 experiments (16 points of the factorial design, 4 center points, 6 freedom degree) were

carried out in randomized run. Optimal experimental conditions that independently maximized extraction efficiency and minimized the total amount of EtOH used, were obtained from the fitted model. Analysis of variance (ANOVA) was conducted to evaluate the statistical significance of independent variable contributions and their first order interaction. The experimental matrix design, with the experimental conditions of each independent variables and the results of experimental GLS extraction yield (mg/g DM) and the total EtOH used (mL) from 16 selected combinations of the independent variables, were reported in Table 1.

Table 1 Experimental conditions of the response surface design and experimental values of the response variables

	Independent Variables			Response Variables			
n°	EtOH (%)	Volume (mL)	Cycles (n°)	GLS 9 (µg/gDM)	GLS 10 (µg/gDM)	GLS11 (µg/gDM)	Tot EtOH (mL)
1	40	5	3	493	1047	215	6
2	60	5	2	911	1984	387	6
3	80	10	2	832	1900	380	16
4	40	10	4	264	443	178	16
5	80	5	3	981	2234	428	12
6	80	10	4	1268	2981	568	32
7	80	15	3	1417	3240	631	36
8	40	10	2	526	1181	222	8
9	60	15	2	1304	3046	562	18
10	60	10	3	1166	2722	501	18
11	60	10	3	1221	2773	510	18
12	60	10	3	1269	2855	532	18
13	40	15	3	274	408	183	18
14	60	10	3	1142	2649	490	18
15	60	5	4	1180	2553	463	12
16	60	15	4	1354	2910	601	36

4.2.4 Pressurized liquid extraction (PLE)

The Dionex ASE300 was used for pressurized liquid extractions (Dionex Sunnyvale, CA). For the extraction operation, 1 g of dried material was packed into a 10 mL stainless steel extraction cell. The extraction cell's empty area was filled with 4 mm solid-glass beads (Sigma-Aldrich, Milan, Italy), and a paper filter (Whatman n°1) was inserted at the bottom of the extraction cell.

Preliminary tests were performed with EtOH 80%, 3 cycles, static duration 4 min to identify the extraction temperature range to be used in the experimental design. The extracts were collected in a glass vial (60 mL) and the solvent was evaporated using a rotary evaporator (Rotavapor R-200 Buchi Italia s.r.l., Cornaredo, Italy) to calculate the extraction yield.

4.2.5 Experiments of Design (DoE)for PLE extraction optimization

The Statgraphic Centurion XVI Version 16.1 (Rockville, USA) for central composite experimental design (CCD) analysis and data processing was used to improve the PLE settings for the recovery of glucosinolates found in extract using a small number of tests. A response surface Box-Behnken design with two factors was used. Four variables were examined at three levels (low, medium, and high) using this design: extraction temperature (Temp) at 70, 110, and 130 °C, number of cycles 2, 4, and 6, composition of solvent modifier (EtOH%) at 60, 80, and 100%, and static time 2, 4, and 6 minutes. The ranges within each factor were chosen according to preliminary tests. The response variable was the UHPLC-HRMS peak area of each analyte. In a randomized run, 27 trials (27 points of the factorial design, 3 center points, and 12 freedom degrees) were performed. The experimental matrix design was given in Table 2, along with the experimental levels of the independent variables (factors) and the findings obtained for the investigated response variables (peak area of Glucoarabinin, glucocamelinin and homoglucocamelinin). To get the parameters of the statistical models, data from the CCD were submitted to regression analysis using least square regression methods. ANOVA was used to determine the statistical significance of independent variable (A, B, C, and D) contributions and their first order interaction. The standardized Pareto chart was used to analyze the influence of each component on the response variable, and the response surfaces of the mathematical models were created. We've defined three response variables and four experimental factors. The chosen design comprises 27 runs, with one sample gathered throughout each run and the quadratic model was the default. The optimum Box-Behnken design settings were as follows: EtOH 65%, temperature 70°C, 6 cycles, and static duration 2 minutes.

Tab.2 Experimental condition of the response surface design and experimental quantitative value of the response variable (GLS9, GLS10 and GLS11)

Run	**EtOH (%)**	**Cycles (n°)**	**Temp (°C)**	**S.time (min)**	**GLS 9 (µg/gDM)**	**GLS 10 (µg/gDM)**	**GLS 11 (µg/gDM)**
1	100	6	100	4	3947	7804	2710
2	80	4	130	2	7455	15270	3957
3	80	4	100	4	8943	17514	4113
4	100	2	100	4	5142	10769	3065
5	80	6	130	4	6585	12813	3887
6	100	4	100	2	5041	10657	2983
7	80	4	70	6	8740	18294	3994
8	60	4	130	4	8641	16216	4149
9	80	6	100	2	10288	21792	4707
10	60	4	70	4	6403	13497	3726
11	100	4	130	4	6734	11910	3593
12	60	4	100	6	7462	14224	3372
13	80	2	70	4	7948	15659	3992
14	80	2	130	4	7566	16288	3429
15	60	4	100	2	7711	15197	3700
16	80	2	100	2	8152	15199	3895
17	100	4	100	6	4368	8830	2762
18	60	6	100	4	11616	22122	4692
19	80	4	70	2	6706	14352	3359
20	80	6	70	4	7015	14208	3460
21	80	4	100	4	7439	14848	3634
22	60	2	100	4	5540	10381	2715
23	80	2	100	6	9381	19054	4396
24	80	4	130	6	6477	12670	3493
25	80	6	100	6	10138	21895	4407
26	80	4	100	4	8659	15967	3992
27	100	4	70	4	3135	6434	2246

4.2.6 Purification of glucosinolates by solid phase extraction

A solid-phase extraction procedure was developed to obtain a GLSs rich extract to be used into in vitro assays. Briefly, a strong anion exchange (SAX) Mega Bond Elute NH_2 cartridges (1 g) were activated with 10 mL of MeOH and equilibrated with 10 mL of H_2O 1% acetic acid. The ultrasound-assisted solid liquid extract was loaded onto the NH_3^+ cartridge, washed with 5 mL of MeOH 1% acetic acid; finally, the glucosinolate

fraction was eluted with 10 mL of freshly prepared H_2O 2% NH_4OH solution. The purified extract was evaporated to dryness in a vacuum evaporator at 40° C, dissolved in water at a concentration of 1 mg mL^{-1} and filtered with 0.22 μm PES filter before in vitro assays.

4.2.7 Qualitative and quantitative analysis by HRMS/MS analysis

Qualitative and quantitative analyzes of GLSs were carried out using an acquity UPLC system coupled with a Xevo G2-XS QTof mass spectrometer (Waters Corp., Milford, MA, United States). The mass spectrometer equipped with an electrospray ion source (ESI), was used in negative and positive ionization modes to acquire full-scan MS, and spectra were recorded in the range of 50-1000 m/z. The source parameters were as follows: electrospray capillary voltage 2.0 kV, source temperature 150 °C and desolvation temperature 600 °C. The cone and desolvation gas flow were 20 and 900 L h^{-1}, respectively, and a scan time of 0.3 s was employed. Cone voltage was set at 70 V, and source offset at 20. The mass spectrometer was calibrated with 0.5 M sodium formate, and 100 pg μL^{-1} of standard leucine-enkkephaline at m/z 554.2615 was infused with the flow of column at 5 μL min^{-1} as the lock mass and acquired for 1 s each 30 s. The total ion current (TIC) used for qualitative analysis was acquired, and an MS/MS spectrum of each compound at different collision energy was acquired and compared to reference standards from which the GLS identification was performed. A quantitative analysis was performed using multiple reaction monitoring (MRM) data acquisition mode by monitoring three characteristic fragments for each target compounds of [M+H]- ion of glucoarabinin (506.1523 > 442.14, 248.11, 96.96) glucocamelinin (520.1684 > 456.16, 262.12. 96.96) and homoglucocamelinin (534.1819 > 470.18, 276.14, 96.96) and ramping collision energy from 25 to 30 V to produce abundant product ions before the detection. In order to quantify the GLS compounds in the extracts, an external standard calibration was conducted six points between 0.01 and 10 μg mL^{-1}. Each level was acquired in triplicate. The analysis of variance (ANOVA) was carried out to test the regression curves, and the linear model was found appropriate over the concentration range (R^2 values > 0.9992). Precision and intraday repeatability were also estimated in all the concentration levels with a coefficient of variation lower than 5%. The results of the quantitative analysis for each analyte were expressed as μg g^{-1} of dry matter (DM).

The Mass Lynx software (version 4.2) was used for instrument control, data acquisition, and processing.

4.2.8 Antioxidant activities

4.2.8.1 ABTS assay

ABTS assay was used to evaluate the antioxidant activity of all sample extracts. The experimental conditions were reported by Pagano et al. (Pagano, Sánchez-Camargo, et al., 2018). Briefly, 5 mL of PBS (control), Trolox (0.25-1 mg mL-1) and extracts 1 mgmL-1 were mixed with 500 mL of ABTS standard solution at a concentration of 1mM. 300 mL of each mixture were transferred into a 96 well plate and were incubated, protected from light, and after 60 min the absorbance was read at 734 nm using a Multiskan Go spectrophotometer (Thermo Fischer Scientific). Results of ABTS assay were expressed as Trolox equivalent TEAC mmol/mg, and they were employed to quantify the antioxidant activity of the tested solution expressed as standard deviation (SD) of three measurements.

4.2.8.2 Oxygen radical absorbance capacity (ORAC)

The scavenging capacity of extracts against oxygen radicals was evaluated using the ORAC assay. The experiment is performed in black 96-well plates according to the protocol reported by Sánchez-Martínez et al. (Sánchez-Martínez et al., 2022). Each well is filled with 100 μL of extract at different concentrations in water, 100 μL of AAPH (590mM) in 30mM phosphate buffer saline (PBS) at pH=7.5, 25 μL of fluorescein in PBS and 100 μL of PBS to make up the volume. Fluorescence was measured with λ excitation= 485 nm and λ emission= 530 nm every 5 minutes for 1 hour at 37°C. Ascorbic acid was used as a positive control. The peroxyl radical scavenging ability of the extract was expressed as IC50 (ug of extract able to inhibit 50% of the AAPH radical).

4.2.8.3 *RNS scavenging capacity*

RNS radical scavenging capacity was measured by a point spectrophotometric assay capable of measuring the ability of the sample to neutralize nitric oxide (NO) radicals. The assay was prepared according to Sánchez-Martínez et al. (Sánchez-Martínez et al.,

2022). The assay was performed in transparent 96-well plates. Each well was filled with 100 µL of the extract at different concentrations in water and 50 µL of SNP (5mM) solubilized in 30mM PBS at pH = 7.5. After incubation for 2 h under the white light of a lamp at room temperature, 100 µL of Griess reagent (prepared by mixing 500 mg sulfanilamide with 50 mg naphthyl-ethylenediamine dihydrochloride and 1.25 mL phosphoric acid in 48.5 mL water) is added. After incubation for 5 minutes, the absorbance at 734 nm is recorded to measure the concentration of nitrite radicals. Ascorbic acid is used as a reference standard. The results are expressed as ug ascorbic acid equivalent/mg extract.

4.2.9 Cell cultures

CCD841 (ATCC® CRL-1790™) healthy human mucosa cell lines and CaCo-2 (ATCC® HTB 37™) human colorectal cancer cells were grown in an EMEM medium supplemented with heat inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. E705 (kindly provided by Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) and SW480 (ATCC® CCL-228™) human colorectal cancer cells were grown in an RPMI 1640 medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. All the cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. ATCC cell lines were validated by short-tandem repeat profiles that are generated by simultaneous amplification of multiple short-tandem repeat loci and amelogenin (for gender identification). All the reagents for cell cultures were supplied by Lonza (Lonza Group, Basel, Switzerland).

4.2.10 Viability assay

Cell viability was investigated using MTT-based in vitro toxicology assay kit (Sigma, St. Louis, MO, United States), according to the manufacturer's protocols. The different cell lines were seeded in 96-well microliter plates at a density of 1×10^4 cells/well, cultured in a complete medium, and treated after 24h with 400 and 800 µg mL^{-1} of glucosinolates purified extract. After 48h at 37°C, the medium was replaced with a complete medium without phenol red containing 10 µL of 5 mg mL^{-1} MTT [3-(4,5-Dimethylthiazol-2-yl)-2.5-Diphenyltetrazolium Bromide]. After 4 h of additional

incubation for CCD841 and 2 h for CRC cells lines, formazan crystals were solubilized with 10% Triton X-100. 0.1N HCl in isopropanol and the absorbance was measured at 570 nm using a microplate reader. Cell viabilities were expressed as a percentage against the untreated cell lines used as controls. Two types of statistical analyzes were performed using R (version 4.0.0) and GraphPad Prism 8. A general linear model (GLM) was used to evaluate the dose-dependency of the cell lines, while a 2-way ANOVA with Tukey multiple comparison test was conducted to understand differences between the lines at the same concentration of the extract. The significance threshold was set at $p=0.05$.

4.2.11 Enzyme Assays

To evaluate the effect of glucosinolates on enzymatic activities, CRC cell lines, and the healthy cell lines were seeded at 1×10^6 cells/100 mm dish and treated for 48 h with the extract at 400 and 800 µg mL^{-1}. The cells were rinsed with ice-cold PBS and lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% NP-40, containing protease inhibitors (1 µM leupeptin, 2 µg mL^{-1} aprotinin, 1 µg mL^{-1} pepstatin and 1 mM PMSF). Homogenates were obtained by passing the cells 5 times through a blunt 20-gauge needle fitted to a syringe and then centrifuging them at 15,000g for 30 min at 4°C. Enzyme activities were assayed on supernatants. Glutathione S-transferase (GST) was measured as reported in Habig, (Habig et al., 1974) using 1 mM reduced glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrates in the presence of 90 mM potassium phosphate buffer (pH 6.5), and the reaction was monitored at 340 nm. Superoxide dismutase (SOD) was measured using an indirect method according to Vance (Vance et al., 1972). This technique is based on the ability of SOD to compete with ferricytochrome c for superoxide anions generated by the xanthine oxidase system and, thus, to inhibit the reduction of ferricytochrome c. Briefly, the protein samples were incubated with 0.01 mM ferricytochrome c in 10 mM HEPES-Tris (pH 7.5), 0.1 mM EDTA, 0.01 mM xanthine in 1 mM NaOH and xanthine oxidase at a final concentration of 0.006 U/mL. Under these conditions, one unit of SOD is the amount of enzyme able to yield a 50% decrease in the rate of ferricytochrome c reduction followed at 550 nm. All the assays were performed in triplicate at 25°C with a Jasco V-550 spectrophotometer and analysed with the Spectra Manager (version 1.33.02) software of Windows. A linear model was chosen for statistical analyzes of enzymatic

assays to evaluate differences against a control set at fold=1. The significance threshold was set at p=0.05.

4.2.12 In vitro gastrointestinal digestion by INFOGEST protocol

Gastrointestinal digestion was simulated following the INFOGEST protocol described by Minekus (Minekus et al., 2014a). Briefly, the oral, gastric and intestinal phases were simulated by mimicking the salt, enzyme composition, pH, time and temperature of each phase of the digestive process. At the end of the intestinal phase, the digested extracts were analyzed by UPLC-HRMS in order to quantify the remaining compounds. Bioaccessibility and bioavailability was calculated as reported by Canas (Canas et al., 2022):

$$\% \, bioaccessibility = \frac{digested \, fraction}{non - digested \, fraction} * 100$$

Where non-digested fraction and digested fractions are the concentration of compound before and after digestion simulation, respectively.

$$\% \, bioavailaility = \frac{digested \, fraction * absorption}{non - digested \, fraction} * 100$$

The absorption was estimated in silico using pkCSM-pharmacokinetics (http://biosig.unimelb.edu.au/pkcsm/, consultato il 5 september 2023) e ADMETlab (https://admet.scbdd.com/, consultato il 5 september 2023). Chemioinformatics free software.

4.2.13 Statistical Analysis

All quotative data were performed in triplicate and results were presented as average ± standard deviation. Analysis of variance (ANOVA) was used to compare the means while Turkey's test was used to assess the statistically significant difference among extraction conditions using JMP 14 software. A p-value of ≤ 0.05 was considered significant.

4.3 Results and discussion

In order to explore the chemical composition of *Camelina sativa* by-products, a detailed characterization of phytochemical compounds was performed by UPLC-HRMS. The full MS chromatograms was shown in Figure 1, and the list of the tentatively identified compounds numbered according to elution order was shown in Table 3. The untarget analysis in negative ion mode allowed the identification of 11 metabolites belonging mainly to two classes, polyphenols and glucosinolates. The results of the qualitative analysis was in accordance with the literature data (Berhow et al., 2013; Quéro et al., 2016; M. J. Rahman et al., 2018; Terpinc et al., 2012). However, among all the compounds found in the extract, our attention was focused on glucosinolates. The analysis of chromatogram in full MS and MS/MS mode allowed to identify the presence of three main glucosinolates in the extract, which were assigned as glucoarabinin (GLS9), glucocamelinin (GLS10), and homoglucocamelinin (GLS11) (Figure 2). The identification was based on the retention time and the UV and MS/MS spectra and finally confirmed with commercial standards. The results of the qualitative analysis were in accordance with the literature data (Berhow et al., 2013; Russo & Reggiani, 2012; A. Schuster & Friedt, 1998). The individual and total GLS contents in *Camelina* sativa seeds were investigated by using an international standard method (ISO 9167-1) with some slight modifications and avoiding the desulfation step. The quantitative analysis of the ISO method was carried out by using a selective MRM method, and the results showed that the amount of glucoarabinin, glucocamelinin, and homoglucocamelinin were 304.3 ± 62, 403.3 ± 23 and 262.6± 87 µg g^{-1} DM respective.

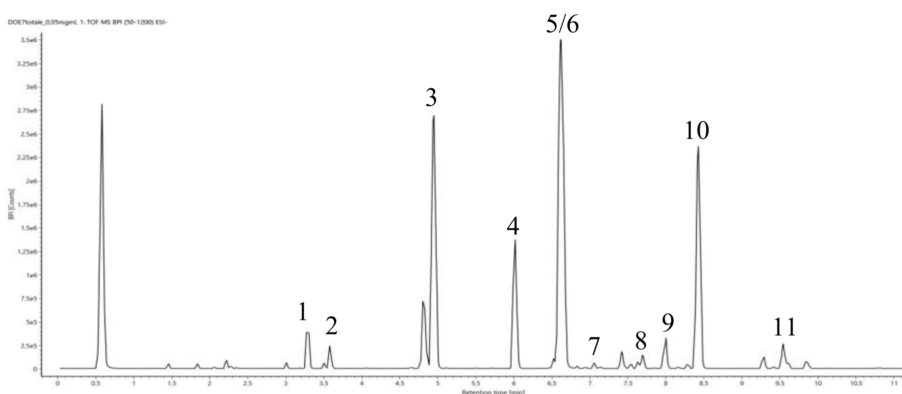


Figure 1 UPLC Full MS chromatogram of *Camelina sativa* PC extract acquired in negative ionisation mode

Table 3 HRMS and MS/MS data of detected compounds in the *Camelina sativa* PC extract.

N°	Rt (min)	$[M-H]^-$	formula	compounds	fragments	Δ (ppm)
1	3.27	451.1246	$C_{21}H_{24}O_{11}$	epicatechin-O-glucoside isomer	289.071; 245.0818	-0.1
2	3.58	451.1246	$C_{21}H_{24}O_{11}$	epicatechin-O-glucoside isomer	289.071; 245.0818	0.1
3	4.95	506.1194	$C_{17}H_{33}NO_{10}S_3$	glucoarabinin	491.0946, 442.121; 248.096; 96.9598	0.1
4	6.02	741.2214	$C_{32}H_{38}O_{20}$	rutin-2-O-apioside	609.147; 301.0337	1.4
5	6.62	520.1356	$C_{18}H_{35}NO_{10}S_3$	glucocamelinin	505.1126; 456.1375, 262.1116; 96.9596	1.2
6	6.62	609.1468	$C_{27}H_{30}O_{16}$	rutin	301.034; 283.0245	1.1
7	7.04	755.2051	$C_{33}H_{40}O_{20}$	Kaempferol-3-O-gentiobioside-7-O-rhamnoside	623.162; 489.1033, 315.0497	1.4
8	7.63	593.1518	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-neohesperidoside	285.040; 151.0033	1.1
9	8.00	623.1619	$C_{28}H_{32}O_{16}$	Isorhamnetin-3-O-β-rutinoside	315.062; 151.0034	0.3
10	8.43	534.1519	$C_{19}H_{37}NO_{10}S_3$	homoglucocamelinin	519.128; 470.153; 276.127; 96.9598	3.6
11	9.54	623.1627	$C_{28}H_{32}O_{16}$	Tamarixetin-7-O-rutinoside	209.045; 108.0207	-0.1

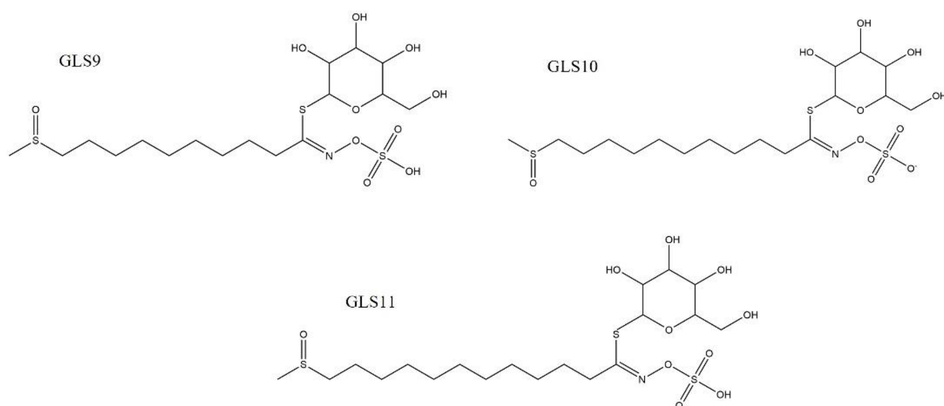


Figure 2 Structures of the main glucosinolates identified in *Camelina sativa* PC extract

4.3.1 Develop and Optimization of USAE and PLE method for the recovery of GLS

Given that the conventional ISO method requires large amounts of organic solvent (methanol) and long extraction times, two green extraction methods, ultrasound-assisted extraction, and pressurised liquid extraction, were developed, optimized, and compared to find more sustainable and efficient extraction for the recovery of GLSs

4.3.1.1 Optimization of glucosinolates extraction by USAE

- *Selection of solvent composition*

Given the interesting content of GLSs in the extract of *C. sativa* PC, especially considering that the matrix used is an industry by-product, we decided to develop and optimize a green extraction method based on ultrasound-assisted extraction (USAE) to improve extraction yield and reduce the use of chemicals and environmental impact. As commonly reported in the literature, one of the most important parameters that affect the extraction efficiency in USAE process is the composition of the extraction solvent. For this reason, to replace methanol with a green solvent such as EtOH (generally recognized as safe) and to select a solvent composition to be used in the further experimental design, preliminary experiments were carried out by increasing from 0 to 100 the organic solvent percentage (ethanol and methanol) in water, and GLS content was monitored. The other parameters of USAE, namely, solvent volume, extraction cycle, and extraction time were kept

constant at 10 mL, 2 cycles, and 5 minutes respectively. The results indicate that an extraction solvent with a water content higher than 80% formed a mucilaginous agglomerate that makes the injection in the chromatographic system impossible. However, as shown in Figure 3, the quantitative trend of monitored GLSs is comparable using both methanol and ethanol. GLSs content increases proportionally to the increase in organic solvent from 20 to 60%, but beyond this value it begins to decrease. Based on these results and considering that the behaviorof methanol and ethanol was comparable, EtOH in the range of 40 to 80% was selected as extraction solvent in the next optimization step because of its lower environmental impact and toxicity.

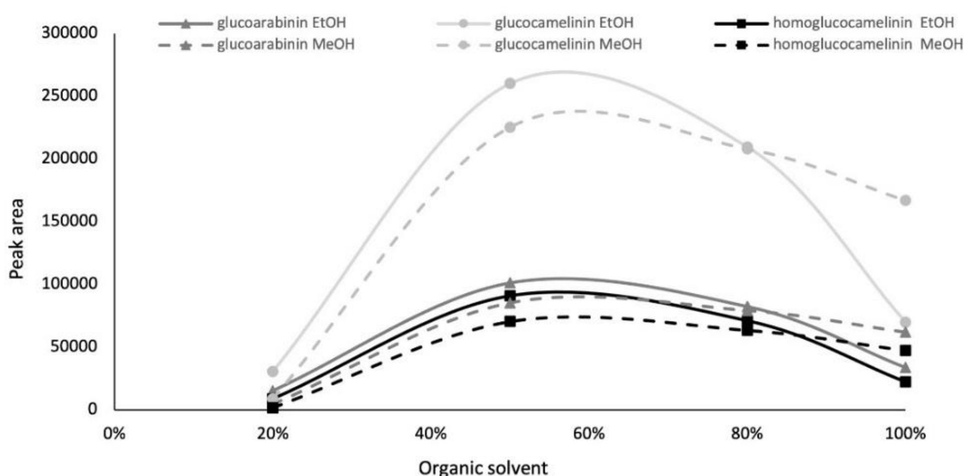


Figure 3 Glucosinolate peak area vs. organic solvent percentage, ethanol (solid line) and methanol (dashed line).

- *Response surface design*

After the preliminary experiments were carried out to select the organic solvent and its content, a response surface methodology (RSM) was used to maximize the extraction of GLSs and at the same reduce the consumption of the organic solvent. In this study, the influence of the three independent variables (solvent volume, solvent composition, and extraction cycles) on the extraction efficiency of each glucosinolate and on the total EtOH consumption was simultaneously evaluated by a Box-Behnken 2 factor interaction

design. GLS contents (µg g^{-1} DM) were considered as response variable to be maximized and total consumption of EtOH (mL) as variable to be minimized considering that the reduction of the organic solvent has a positive influence on the cost of the analysis and on the environmental impact. Table 1 reports the total volume of EtOH used and the amount of glucosinolates for each run provided by the CCD used as a response variable. The statistical parameters of the experimental design are summarized in Table 4.

Table 4 Analysis of variance of the regression model

	sum of squares				mean square			
	GLS 9	**GLS 10**	**GLS 11**	**Tot EtOH**	**GLS 9**	**GLS 10**	**GLS 11**	**Tot EtOH**
A: EtOH %	10801.6	66195.2	1826.8	286.8	10801.6	66195.2	1826.8	286.8
B: Volume	769.3	3988.1	293.5	836.4	769.3	3988.1	293.5	836.4
C: Cycles	303.7	749.0	83.3	187.2	303.7	749.0	83.3	187.2
A^2	7626.5	40568.0	961.2	6.8	7626.5	40568.0	961.2	6.8
B^2	31.6	4.2	4.9	5.5	31.6	4.2	4.9	5.5
C^2	66.0	539.9	10.4	5.8	66.0	539.9	10.4	5.8
AB	1073.6	6761.8	137.5	34.2	1073.6	6761.8	137.5	34.2
AC	1218.4	8271.0	135.3	20.3	1218.4	8271.0	135.3	20.3
BC	119.0	1241.9	3.5	109.2	119.0	1241.9	3.5	109.2
Lack of fit	309.1	2120.5	42.0	27.0	103.0	706.8	14.0	9.0
Pure error	97.1	224.9	9.9	0.1	32.4	75.0	3.3	0.0
Total	22415.9	130665.0	3508.2	1519.2				
R^2	98.2	98.2	98.5	98≅.2				
Adj. R^2	95.5	95.5	96.3	95.5				

	F-value				Pvalue			
	GLS 9	**GLS 10**	**GLS 11**	**Tot EtOH**	**GLS 9**	**GLS 10**	**GLS 11**	**Tot EtOH**
A:EtOH %	333.6	882.9	550.8	7821.9	**0.0004**[a]	**0.0001**[a]	**0.0002**[a]	**0.0000**[a]
B: Volume	23.8	53.2	88.5	22811.1	**0.0165**[a]	**0.0053**[a]	**0.0025**[a]	**0.0000**[a]
C: Cycles	9.4	10.0	25.1	5105.8	0.0549	0.0508	**0.0153**[a]	**0.0000**[a]
A^2	235.5	541.1	289.8	184.4	**0.0006**[a]	**0.0002**[a]	**0.0004**[a]	**0.0009**[a]
B^2	1.0	0.1	1.5	150.6	0.3958	0.8273	0.3131	**0.0012**[a]
C^2	2.0	7.2	3.1	157.1	0.2486	0.0748	0.1749	**0.0011**[a]
AB	33.2	90.2	41.5	552.3	**0.0104**[a]	**0.0025**[a]	**0.0076**[a]	**0.0001**[a]
AC	37.6	110.3	40.8	2978.3	**0.0087**[a]	**0.0018**[a]	**0.0078**[a]	**0.0002**[a]
BC	3.7	16.6	1.1	245.4	0.151	**0.0268**[a]	0.3812	**0.0000**[a]
Lack of fit	3.2	9.4	4.2		0.1836	**0.0489**[a]	0.1338	**0.0004**[a]
Pure error								
Total								
R^2								
Adj. R^2								

R^2 = Quadratic correlation coefficient. [a] Significant ($p < 0.05$).

Based on the results, the model showed a high correlation ($R^2 \cong 98\%$), indicating a slight variance of the data and a good prediction of the model with respect to all the all

considered response variables. Two independent variables, percentage of EtOH and its volume, had a significant influence on both GLS extraction and the volume of total EtOH ($p<0.05$) while the extraction cycles had a significant influence only on the extraction efficiency of homoglucocamelinin and on the volume of EtOH. Moreover, the quadratic effect of multiple parameters as well as the interaction among many parameters was statistically significant ($p<0.05$) for the response variables considered (Table 2). As shown in the desirability plot (Figure 4), the volume of the extraction solvent linearly influences the desired effect; in fact, by increasing the extraction volume from 5 to 15mL there is an increase in the desirability. Regarding the percentage of EtOH, the desirable effect increases proportionally to the increase in organic solvent from 40 to ≅70%, but beyond this value it begins to decrease. This result was also in agreement with those obtained in our preliminary results. Finally, the optimized conditions to maximize the extraction of glucosinolate compounds and at same time reduce the consumption of organic solvent were calculated as EtOH 65%, cycles 2, and solvent volume 5 mL. After selecting the optimized extraction conditions to evaluate the improvement in extraction efficiency, these results have been comparted with those obtained using the ISO method. A quantitative analysis of the three glucosinolates was carried out by UPLC-HRMS using the external standard method. The calibration curve of glucosinolates in the concentration range of 0.1-10 $\mu g\ mL^{-1}$ were used to quantify their content in both extracts. The external standard calibration curves for all the analytes provided good linearity within the investigated concentration range with correlation coefficients (R^2) ranging from 0.9993 and 0.9998. The quantitative analysis of the extract obtained by using USAE shows that the content of glucoarabin, glucocamelin, and homoglucocamelin were 1525.6± 53, 3544.6± 209 and 615.5± 68 $\mu g\ g^{-1}$ DM respectively. This result highlights a huge increase in extraction efficiency for all target compounds, in particular, the recovery of glucoarabin, glucocamelin, and homoglucocamelin were 501, 878 and 234% respectively. These results can be explained by the increased chemical stability of glucosinolates under the milder extraction conditions of the developed USAE method, compared to the ISO procedure which uses methanol at 75°C as extraction solvent. These conditions can cause the thermal degradation of glucosinolates as highlighted by some authors (Hanschen, Rohn, et al., 2012; MacLeod et al., 1981; Mao et al., 2019; Oerlemans et al., 2006).

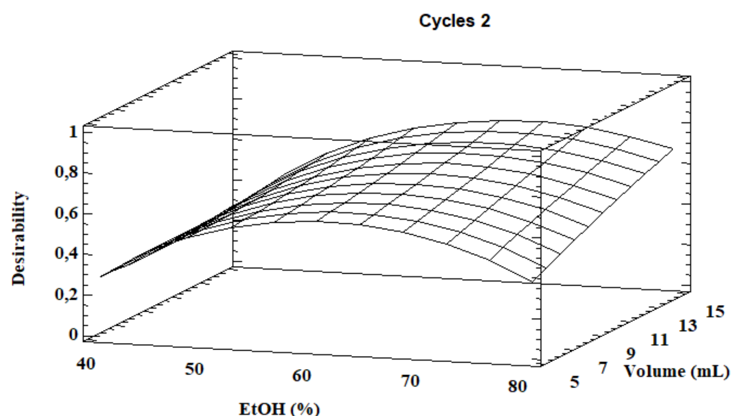


Figure 4 Desirability plot for total glucosinolate extraction as functions of ethanol percentage and total solvent volume.

4.3.1.2 Preliminary experiment for PLE extraction

After the optimization of USAE, in order to increase the recovery of GLSs the Pressurised Liquid Extraction (PLE), a green technique commonly used to improve the recovery of analytes was performed. Before the optimization of PLE procedure, a preliminary extraction at mild condition (70°C, two cycles of extraction, and 65% Ethanol) was carried out to evaluate the metabolic profile and recovery of glucosinolates. In fact, given the high temperatures and pressures, which can modify the chemical-physical properties of the solvent and facilitate its penetration into the matrix, the extraction of different molecules could be observed. In this condition the same USAE chromatographic profile was obtained (Pagliari, Giustra, et al., 2022).

4.3.1.3 Optimization of glucosinolates extraction by PLE

- *Selection of solvent composition and temperature*

After confirming the presence in the PLE extract of the same analytes identified in the USAE extract, the PLE conditions were optimised. As generally reported in the literature, the parameters that most influence the PLE process are temperature and solvent composition (Pagano, Sánchez-Camargo, et al., 2018). Preliminary experiments

were carried out to determine the ranges to be used for a chemometric optimization. The results show a direct proportionality between the temperature increase and the recovery of GLSs up to a temperature of 110°C, beyond which the recovery of glucosinolates starts to decrease (Figure. 5), probably as a result of the degradation of these compounds. It is known that glucosinolates are thermosensitive molecules and the most of them are degradated over at 100°C (Hanschen, Bauer, et al., 2012; Oerlemans et al., 2006). Based on these results, the temperature range of 70-130 °C was used in the next optimization step.

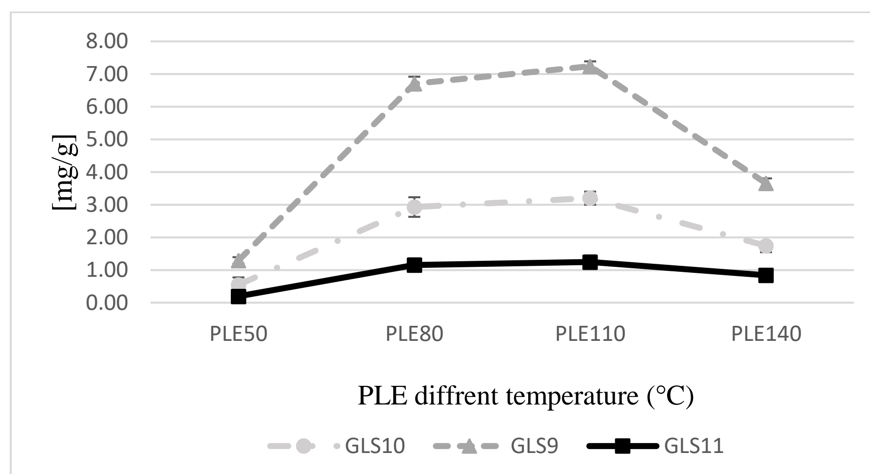


Figure 5 the behaviour of different temperatures on the recovery of the three GLSs in PLE preliminary extracts.

On the other hand, for the solvent composition, ethanol was chosen as a GRAS solvent for safe food use and for its efficiency in the recovery of GLSs, as previously demonstrated in ultrasonic extraction (Pagliari, Giustra, et al., 2022). The presence of saponins and mucilages in the *Camelina* seeds, causes the matrix to swell in contact with water, preventing the extraction cell from emptying properly. Preliminary experiment results shown that the minimum ethanol content required to ensure the proper functioning of the PLE is EtOH 60%. Following these results, the solvent composition range was set between 60% and 100% ethanol.

- *Response surface design*

After the preliminary experiments, which made it possible to establish the temperature and solvent composition ranges, the optimization of the extraction process was carried out with a chemometric approach using Response Surface Methodology (RSM) in order to maximize the recovery of the GLSs. The experimental design was set up by selecting four independent variables (temperature, number of cycles, static time and percentage of ethanol) and evaluating their impact on the extraction efficiency of the three glucosinolates (response factors) thanks to a Box-Behnken two-factor interaction design. Table 2 shows the experimental conditions for each run and the experimental values of the responses to the different experimental conditions. Quantitative analysis of GLS9, GLS10 and GLS11 was performed by UPLC-HRMS/MS.

The statistical analysis reported in Table 5 allows us to identify the significant factors and their impact on the response variables. Based on the results, the model showed a high correlation ($R2 \cong 77-79\%$), indicating a low variance of the data and a good prediction of the model with respect to all the response variables considered. From the results obtained, only the percentage of ethanol and its combination with the number of cycles have a significant influence on the efficiency of the GLS9, GLS10 and GLS11 recovery (p-value < 0.05).

Table 5 Analysis of variance of the regression model

	Sum of squares			Mean squeres		
	GLS 9	**GLS 10**	**GLS 11**	**GLS 9**	**GLS 10**	**GLS 11**
A:EtOH	3.01023E7	1.03447E8	2.07917E6	3.01023E7	1.03447E8	2.07917E6
B:cycle	2.86163E6	1.47054E7.	468470	2.86163E6	1.47054E7.	468470
C:Temperature	1.02726E6	617894.	249697	1.02726E6	617894.	249697
D:static time	122614	520833.	2610.75	122614	520833.	2610.75
A^2	1.88627E7.	8.3087E7	1.94837E6	1.88627E7.	8.3087E7	1.94837E6
B^2	534674	5.83389E6	54225.9	534674	5.83389E6	54225.9
C^2	4.5387E6	1.2012E7	133001	4.5387E6	1.2012E7	133001
D^2	107163.	5.09212E6	18934.3	107163.	5.09212E6	18934.3
AB	1.32169E7	5.40666E7	1.35956E6	1.32169E7	5.40666E7	1.35956E6
AC	463080	1.90026E6	213444	463080	1.90026E6	213444
AD	44944.0	182329.	2862.25	44944.0	182329.	2862.25
BC	576.0	1.02414E6	245025	576.0	1.02414E6	245025
BD	475410	3.51938E6	160400	475410	3.51938E6	160400
CD	2.26804E6	1.06994E7	301950	2.26804E6	1.06994E7	301950
Total error	2.3288E7	8.9001E7	2.11423E6	1.94067E6	7.41675E6	176186
Total (corr.)	1.02605E8	4.23083E8	9.94614E6			
R^2	77.3032	78.9637	78.7432			
Adj R^2	50.8237	54.4214	53.9437			
	F-value			**P-value**		
	GLS 9	**GLS 10**	**GLS 11**	**GLS 9**	**GLS 10**	**GLS 11**
A:EtOH	15.51	13.95	11.80	**0.0020**[a]	**0.0028**[a]	**0.0049**[a]
B:cycle	1.47	1.98	2.66	0.2480	0.1845	0.1284
C:Temperature	0.53	0.08	1.42	0.4808	0.7778	0.2569
D:static time	0.06	0.07	0.01	0.8058	0.7955	0.9051
A^2	9.72	11.20	11.06	**0.0089**[a]	**0.0058**[a]	**0.0060**[a]
B^2	0.28	0.79	0.31	0.6092	0.3926	0.5892
C^2	2.34	1.62	0.75	0.1521	0.2273	0.4020
D^2	0.06	0.69	0.11	0.8182	0.4235	0.7487
AB	6.81	7.29	7.72	**0.0228**[a]	**0.0193**[a]	**0.0167**[a]
AC	0.24	0.26	1.21	0.6340	0.6219	0.2926
AD	0.02	0.02	0.02	0.8816	0.8780	0.9007
BC	0.00	0.14	1.39	0.9865	0.7167	0.2611
BD	0.24	0.47	0.91	0.6296	0.5040	0.3588
CD	1.17	1.44	1.71	0.3009	0.2529	0.2150
Total error						
Total (corr.)						
R^2						
Adj R^2						

R^2 = Quadratic correlation coefficient. [a] Significant ($p < 0.05$).

As shown in the desirability plot (Figure 6), the percentage of ethanol has a linear effect on the recovery of the three glucosinolates in the range from 60 to 65%, above 65% the desired effect decreases drastically. This behavior is opposite compared to the results obtained using ultrasonic extraction techniques. This confirms the ability of PLE to use the combination of high temperature and pressure to significantly modify the chemical

properties of the extraction solvent, improving the extraction efficiency of the water and reducing the necessity to use a high concentration of organic solvent. The number of cycles linearly increases the extraction efficiency. The temperature follows a parabolic profile, indicating a peak after which recovery may decrease due to GLS structure degradation at high temperatures (MacLeod et al., 1981), while the extraction process was not affected by the static time. Temperature and static time were found to be statistically insignificant.

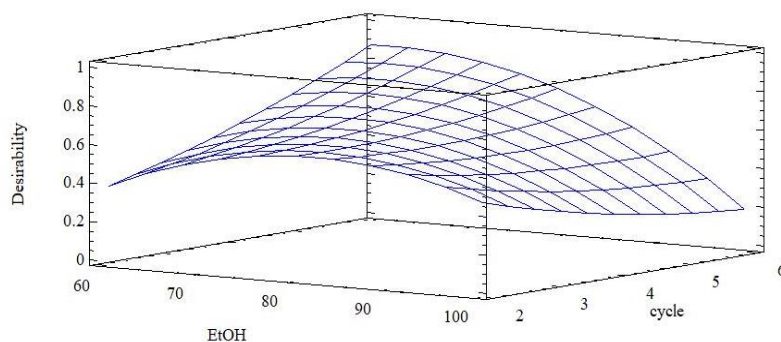


Figure 6 Desirability plot for total glucosinolate extraction as function of ethanol percentage and number of cycles.

The optimized conditions for maximizing GLSs recovery were calculated as 65% ethanol, 6 cycles, 118°C and 2 minutes static time (Opt 1), resulting in a desirability of 97.84%. It was decided to evaluate a further condition where the temperature was set to the minimum value of the range tested preliminarily since the temperature was not statistically significant (p-value > 0.05) (Opt 2). The two optimized conditions were tested and as shown in Table 6, there is no statistically significant difference (p-value > 0.05) between Opt1 and Opt2, therefore Opt2 was chosen to reduce energy consumption and to avoid the possible thermal degradation of GLSs.

Table 6 Quantitative analysis of GLS9, GLS10 and GLS11 between the optimal design condition suggested by software (Opt1) and the hypothetic optimal condition with the lowest energy consumption (Opt2).

GLSs	Opt 1 mg g^{-1} DM	Opt 2 mg g^{-1} DM
GLS 9	12.4 ± 1.25^{a}	11.6 ± 1.15^{a}
GLS 10	22.98 ± 1.16^{b}	21.37 ± 1.36^{b}
GLS 11	5.22 ± 0.41^{c}	5.01 ± 0.25^{c}

*a,b,c – means with the same letter are not significantly different

4.3.2 Comparison of extraction techniques

A comparative study between the different selected extraction techniques ISO, USAE and PLE was carried out. As shown in Table 7, the ISO procedure required a higher extraction time, volume and percentage of organic solvent than the optimized green techniques. In particular, USAE and PLE show an extraction time of 30 and 40 min respectively, lower than the 140 min of ISO method. The solvent volume is reduced from 100 mL (ISO) to 10 mL (USAE) and 15 mL (PLE). A further advantage of green techniques is the ability to improve extraction efficiency using safe solvents such as ethanol instead of methanol.

Table 7 - Optimised extraction conditions of the ISO, USAE and PLE methods

	ISO 9167-1	USAE	PLE
Quantitative of matrix	1g	1g	1g
Organic solvent (%)	100% MeOH	65% EtOH	65% EtOH
Cycles (n°)	2	2	2
Temperatures (°C)	75°C	30°C	70°C
Extraction time (min)	140min	40min	30min
Total solvent volume (mL)	100mL	10mL	15mL

Therefore, the PLE was the most efficient techniques for the recovery of glucosinolates due to its high temperature and pressure conditions, which modified the chemical-physical properties of the solvents increasing extraction capacity. With this method, the

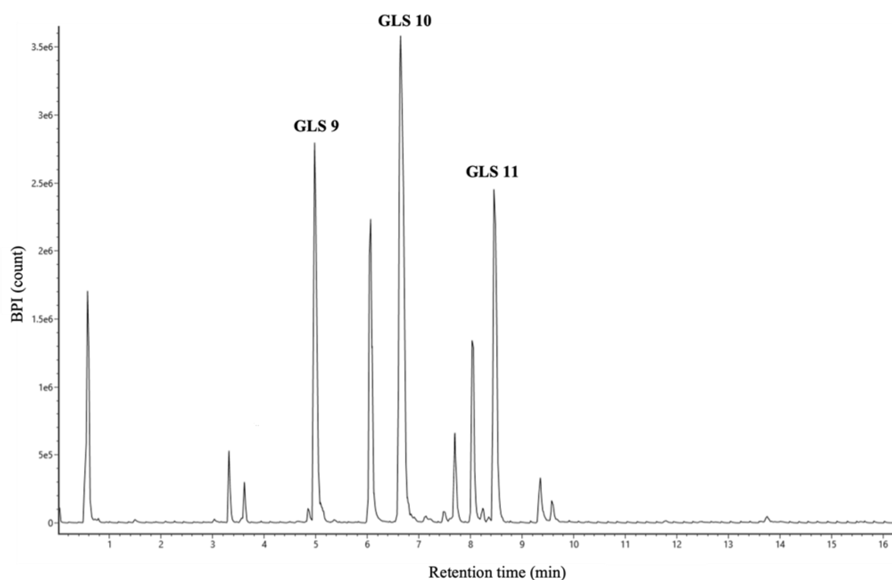
recovery of the main GLSs increases approximately of 2283% (GLS9), 2656% (GLS10) and 874% (GSL11) compared to the ISO procedure. However, the use of pressurised liquid extraction requires highly qualified personnel and a high initial equipment cost. The USAE, also improves the GLS recovery of 390% (GLS9), 200% (GLS10) and 315% (GLS11) compared to the conventional approach, and is cheaper, easier to use and slightly faster than PLE extraction. The results of the recovery of GLS from the by-product of *Camelina sativa* seeds showed a high extraction efficiency of both green techniques compared to the ISO technique. Furthermore, the analysis of the advantages and disadvantages of the PLE and USAE allows the selection of the most suitable approach according to experimental necessity.

4.3.3 Purification of the GLSs by solid phase extraction.

Considering the good contend of GLSs in *Camelina sativa* seeds by-products it was decided to purify the extract by solid phase extraction (SPE) to test the potential biological activity of GLSs. A 140 mg PLE extract was diluted in 10 mL H_2O 1% HCOOH and loaded onto a SPE column. After loading, a wash solution of 10 mL H_2O 1% HCOOH was passed through the SPE cartridge to remove non-retained compounds, while GLS was eluted with 10 mL H_2O 2% NH_4OH.

Both wash and elution fractions were collected, and each was analysed by UPLC-HRMS-DAD to detect the presence of GLS and to verify the purity of the SPE extracts. The results of the HRMS chromatographic profiling of the elution fractions, crude extract and reference standards are shown in Figure 7. The chromatographic analysis suggests that the developed SPE procedure enabled selective purification of GLS, avoiding loss of compounds of interest in the wash step. Furthermore, the presence of new minority peaks in the purified PLE extract could be detected following the de-concentration of the GLSs in the purified extract. For these reasons, a data-dependent HRMS analysis was performed to tentatively identify the compounds detected. Among all these compounds, a peak at tr 3.80 min 492.1036 m/z showed the specific fragmentation pattern of aliphatic glucosinolates according to MS/MS analysis (Fabre et al., 2007). Specifically, the 477.0805 m/z production corresponds to the loss of a methyl (-15 Da), while the 234.0807 m/z ion corresponds to the fragment remaining after the

loss of thioglucose and methyl sulfoxide. Finally, due to the presence of the 96.9596 m/z fragment corresponding to the sulphate group, it was possible to speculate that the compound studied could be glucohirsutin (GLS8).



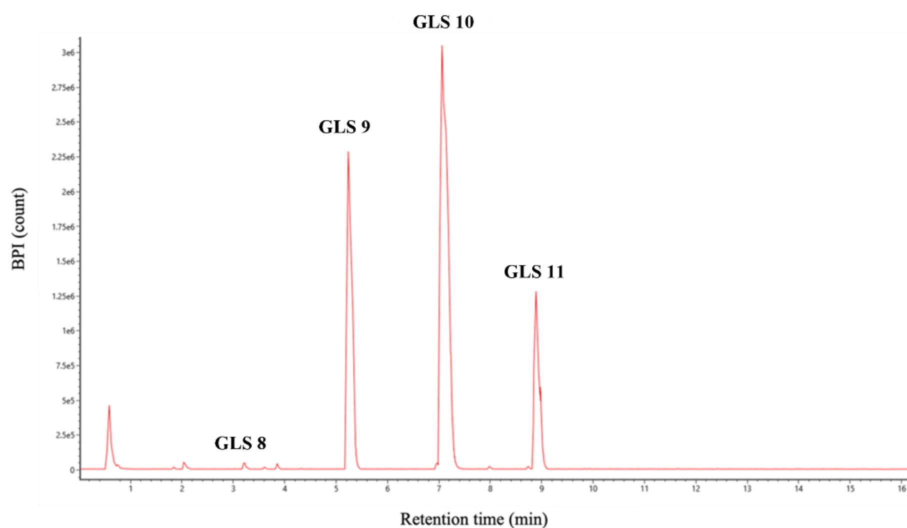


Figure 7 UPLC full MS chromatograms of pressurized liquid extraction (PLE) before (A) and after purification (B) by solid phase extraction (SPE).

4.3.4 -Predictive Bioactivities of the purified extract

The evaluation of the general biological potential of the four specific GLSs identified in C. sativa was initially investigated *in silico* using online PASS software. The analysis was obtained by entering the simplified molecular input line entry system (SMILES) strings of each molecule into the software and obtaining in response a table containing the probable activity (PA) and probable inactivity (PI) values with a related biological activity variability between 0.000 and 1.000. Biological predictions with PA > 0.700 show the greater potential biological activity.

The results reported in Table 8 show probable chemotherapeutic activity, in particular apoptosis agonist, chemopreventive and antineoplastic effects with Pa > 0.9 are predicted depending on their structure, indicating a very high probability results being reliable. This results are in agreement with the few data reported in literature regarding the activity of GLSs (Cabello-Hurtado et al., 2012). Therefore, following this analysis, the purified PLE extract was tested by spectrophotometric assays and in vitro cell analysis to explore possible biological activity of GLSs compounds.

Table 8: Main potential biologic activities predicted using Pass online based software.

Glucosinolates	Predicted bioactivities	Pa	Pi
Glucohirsutin (GLS8)	Chemopreventive	0.985	0.001
	Apoptosis agonist	0.928	0.004
	Antineoplastic	0.833	0.008
Glucoarabin (GLS9)	Chemopreventive	0.985	0.001
	Apoptosis agonist	0.928	0.004
	Antineoplastic	0.833	0.008
	Immunostimulant	0.577	0.026
Glucocamelinin (GLS10)	Chemopreventive	0.947	0.002
	Anticarcinogenic	0.907	0.002
	Apoptosis agonist	0.905	0.004
	Antineoplastic	0.782	0.014
Homoglucocamelinin (GLS11)	Chemopreventive	0.947	0.002
	Anticarcinogenic	0.907	0.002
	Apoptosis agonist	0.905	0.004
	Antineoplastic	0.782	0.014

4.3.4.1 Antiproliferative effect of purified glucosinolates extract

According to chemoprotective activity predicted by in silico analysis the potential anticancer activity was evaluated using health and cancer colon cells.

Initially, screening was performed to define the concentrations of purified extract to be used in MTT viability tests. Among the concentrations tested in the range of 100 to 1000 µg mL^{-1}, the experimental results suggested to select a concentration range of 400-800 µg mL^{-1} for further experiments. Four different cell lines were selected to perform the MTT assays and evaluate cell viability after a 48h treatment, through mitochondrial activity. The cell lines chosen were the healthy colorectal mucosa CCD841cell line and three colorectal cancer cell lines with peculiar mutations or behaviours. In particular, CaCo-2 and E705 show no hyperactivating mutations in the KRAS, NRAS, BRAF, and PIK3CA genes, with the E705 cell line carrying a silent mutation in the PIK3CA gene, whereas SW480 carries a hyperactivating mutation in exon 2 of the KRAS gene. The Caco-2 and SW480 cell lines do not respond to cetuximab, a MoAbs against EGFR, while the E705 cell line is sensitive to cetuximab (Bovio et al., 2020). A general linear model analysis on MTT assays demonstrated that the purified extract had a significantly dose-dependent antiproliferative effect on the three tumor cell lines ($p<0.001$), and that no significant effect ($p>0.07$) on the healthy cell lines was found, demonstrating a specific selectivity against tumor cell lines. The 2-way ANOVA analysis (Figure 8) using the healthy cell lines as control showed a strong effect at the 800 mg mL^{-1} concentration on the E705 and SW480 cells, where the viability dropped to 40%. Subsequently, to clarify the possible mechanisms involved, two enzymes involved in reactive oxygen species (ROS) detoxification, superoxide dismutase (SOD) and glutathione S-tranferase (GST) were assayed. SOD converts the superoxide radical ($O_2\bullet^-$) into hydrogen peroxide (H_2O_2) while GST is an enzyme that transfers glutathione to xenobiotic substrates. We hypothesize that the extract acts as a stressor element by increasing the oxidative metabolism. Upon extract addition, the healthy cells respond physiologically by increasing the activity of the enzymes, but the tumor cell lines do not. Figure 9 shows the increase in both enzymes' activity: at the highest extract concentration, SOD is increased by 4-fold ($p<0.05$) and GST by 2-fold ($p<0.05$) in healthy CCD841 cells. Caco-2 and SW480 maintain the basal level of these enzymes after treatment with both extract concentrations ($p>0.05$). E705 shows an opposite behavior: the activity of the

two enzymes is decreased by 0.5 fold already at 400 mg mL^{-1} of extract for SOD ($p<0.05$) and 800 mg mL^{-1} for GST ($p<0.05$).

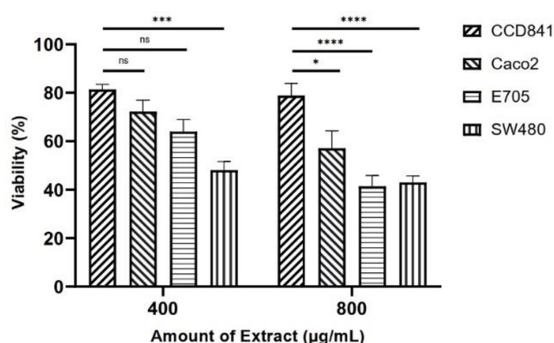


Figure 8 MTT Viability assays on four cell lines treated with *Camelina sativa* extracts at two concentrations with 2-way ANOVA statistical analysis (ns = not significant, * = p < 0.05, *** < 0.001, **** < 0.0001).

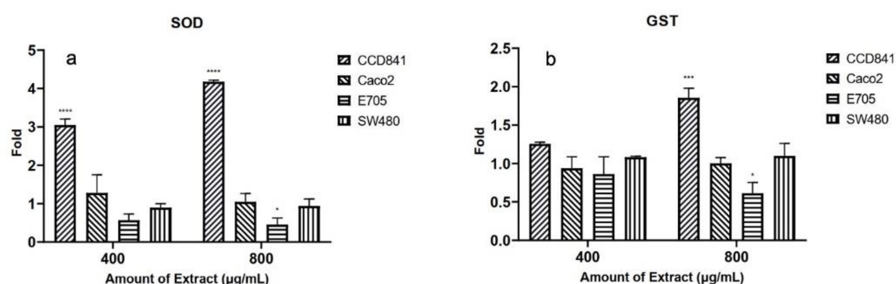


Figure 9 Fold increase in SOD (a) and GST (b) activity after 48h of treatment. The statistical analysis is a linear model against a fold control equal 1 (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$).

Considering the interesting results obtained with in vitro cellular assays and to verify that glucosinolates are unable to directly neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS). three spectrophotometric assays (ABTS, ORAC and RNS) were performed. The ABTS assay showed a TE value of 69.77±2.78 µg/mL EXT for purified camelina extract suggesting a low of capacity of the extract to neutralise ROS. This result was also confirmed by the ORAC assay that showed a higher IC50 7.75±0.22 µg/mL EXT value of percentage inactivation than that of ascorbic acid used as a positive reference (1.06±0.07 µg/mL EXT).Regarding the activity against nitric radicals, a better neutralising effect was found with an IC50 of 902.07±19.35 µg/mL

EXT much closer to that of ascorbic acid 842.18±10.25 suggesting a better capacity of the purified extract to neutralise RNS. These results confirm that GLSs contained in purified extract are unable to neutralize ROS supporting the results obtained in the in vitro cell assays.

4.3.5 Bioaccessibility and bioavailability of GLSs

In order to evaluate the possible application of the purified PLE extract rich in GLSs as an ingredient for effective dietary supplements to support human health, it was decided to assess the effects of gastrointestinal digestion on GLSs. For this purpose, the INFOGEST protocol was selected from among various static digestion models, which have a set of digestion parameters defined on the basis of human physiological knowledge and internationally agreed to adequately simulate the digestion of food or food ingredients in the adult organism. INFOGEST is widely used for bioaccessibility studies of phytochemical compounds such as polyphenols, methylxanthines and numerous secondary metabolites, as well as for observing the digestive fate of proteins, lipids and carbohydrates (Brodkorb et al., 2019; Minekus et al., 2014a).

The results obtained showed an influence of the digestive process on the degradation of the main GLSs. This finding is in line with the literature, which shows that GLSs are molecules with reduced stability, sensitive to the action of pH and enzymes. Specifically, after the intestinal digestion step, the concentrations of glucoarabin, glucocamelinin, and homoglucocamelinin remained at 32.24±2.15, 50.03±3.67 and 11.53±0.99 µg/mg EXT, respectively, with a bioaccessibility of 28% (GLS9), 26.80% (GLS10) and 15.70% (GLS11). However, in addition to the bioaccessibility of the molecules, it is also important to assess their bioavailability, or the percentage of the compound that is passively absorbed across the gastrointestinal barrier. Specifically, a bioavailability of 0%-3.45% has been calculated, indicating an inability to cross the intestinal barrier. This may be due to the structure of the molecule, which has a high molecular weight resulting in low permeability through the cell membrane. Further in-depth studies, perhaps using in vitro cellular systems to simulate the gastrointestinal barrier and evaluate its possible active absorption via transporters rather than the development of a suitable formulation to facilitate its passage, will therefore be required.

4.4 Conclusions

Two green extraction techniques (USAE and PLE) were developed to obtain high purity glucosinolates from *Camelina sativa* seed by-products. The extraction was optimised by an experimental design to evaluate the effect of the extraction parameters of each technique to increase the GLS content in the crude extract and to reduce the organic solvent. Solvent volume and ethanol percentage showed the main effect on the selected response variables for USAE extraction, while ethanol percentage and its combination with the number of cycles were the only significant factors for PLE extraction. A comparison of the different extraction techniques shows that the optimised PLE extract has the highest GLS recovery but requires stricter temperature conditions and more expensive instrumentation. However, the USAE technique can also be considered as an excellent method for GLS recovery. Although it has a lower recovery of GLS than PLE, it still guarantees a strong increase in their extraction compared to ISO extraction and requires cheaper and simpler instrumentation.

The purified extract, rich in glucoarabine, glucocamelin and homoglucocamelin, showed an interesting chemopreventive effect against several colorectal cancer cell lines, without affecting healthy cell lines. After administration of the purified GLS extract, the results showed an increase in oxidative stress in tumour cells, leading to cell death. This behaviour is also confirmed by the inability of GLS to directly neutralise ROS and RNS radicals, as verified by ORAC and ABTS assays. However, further studies are needed to investigate the mechanism of action. Finally, simulated digestion revealed a probable degradation of the compounds with reduced bioaccessibility and bioavailability; further investigations are required to assess their possibility of use in the diet as such and in formulations.

Acknowledgements

I want to thanks Professor Paola Fusi of the University of Milan-Biccoca (Italy) for the data provided on the in vitro cellular tests. Also, a big thank you to Professor Alejandro Cifuentes and all laboratory team for welcoming me at Instituto de Investigación en Ciencias de la Alimentación (CIAL), in Madrid (Spain) and to supporting me in the bioactivity and permeability studies of the extracts.

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Chapter 5

A comparative metabolomic investigation of different section of Sicilian *Citrus x limon* (L.) Osbeck, detecting potential bioactive metabolites using UHPLC-QTOF-HRMS/MS

Abstract

Citrus fruits are a diverse and economically important group of fruit crops known for their distinctive flavours and high nutritional value. Their cultivation and consumption contribute significantly to the global agricultural economy and offer a wide range of health benefits. Among the genetic diversity of citrus species, *Citrus x limon* (L.) Osbeck is particularly relevant due to its chemical composition and potential health benefits. Metabolomic profile different parts of two cultivars from the Sicily region (southern Italy) were studied and compared to select the best extract with higher potential biological activity. After multivariate analysis that suggested the most promising part of considered cultivar a detailed UHPLC-HRMS characterization revealed the occurrence of phenolics, coumarins and 3-hydroxy-3-methylglutaryl flavonoids (HMG-flavonoids). The quantification of the main metabolites contained in the selected extract was performed by UHPLC-UV analysis. The potential bioactivity of the most bioactive idroalcholic extract was analyzed in silico showing interesting antioxidant, anticancer, cardioprotective and anti-inflammatory. Finally, a preliminary study was carried out to assess the effect of the digestive process on the extracted flavonoids and their bioaccessibility to the body, to evaluate the assumption like nutraceutical and/or supplement.

Keywords: Citrus, phenolics, LC-HRMS, Metabolomic, antioxidants

5.1 Introduction

The widespread family of Rutaceae is prevalent in more than 140 countries in the world where the *Citrus* genus are considered as having an economic impact on the food chain and botanical production. In 2019, the annual production of citrus fruits was approximately 143.48 million tons (FAO, 2019) Statistics Division, Food and Agriculture Organization of the United Nations. Focusing on the lemons (*Citrus limon*) typically cultivated in the Mediterranean Area, the southern Italian region of Sicily is the main region producing cultivars protected by the label "Protected Geographical Indication" (PGI) to highlight the relevant quality of the food products related to the geographical production. In particular, the 'Femminello siracusano' (FS) and 'Femminello zagara bianca' (FZB) are the most representative cultivars typically produced in the Siracusa area, which accounts for more than 30% of the national area planted with lemons (Consortium for the Protection of the Siracusa Lemon PGI).

The large production of lemon is related to the extraction of the juice which is used in several food industry applications, while lemon peels, albedo and seeds are the main agro-industrial wastes. These waste products contain an important source of high value compounds largely used in nutraceuticals and cosmetic industries (Berk, 2016). Citrus lemons are a great source of several bioactive compounds including dietary fiber (pectin, cellulose, lignin), minerals (potassium, calcium, magnesium, selenium), organic acids (citric, oxalic, and malic acids), vitamins (vitamin C, thiamine, riboflavin), terpenes (limonene), carotenoids (lutein, β-carotene, zeaxanthin), and phenolic acids (chlorogenic, ferulic, and sinapic acids) (Gómez-Mejía et al., 2019).

Citrus lemons are also known for their high polyphenol and flavonoid content (hesperidin, narirutin, hesperetin, diosmin) (Imeneo et al., 2022). These compounds exert many, in vitro and in vivo, biological effects such as the decrease of the expression of some inflammatory mediators (Cirmi et al., 2021; de Souza et al., 2022) and reduction oxidative stress (Bussmann et al., 2022; Montalbano, Mhalhel, et al., 2021). In addition, flavonoids, such as hesperetin, affect the expression of antioxidant enzymes such as GSH and superoxide dismutase (SOD) by modulating the nuclear factor erythroid 2-related factor 2 (Nrf2)-ARE pathway (Zhu et al., 2017). Thus, they prevent pathological processes such as aging and age-related diseases (Burton et al., 2016; Yousefzadeh et al.,

2018), cancer (Tuli et al., 2023), obesity (Montalbano et al., 2019; Montalbano, Maugeri, et al., 2021) and cardiovascular diseases (Liu et al., 2022). Furthermore, they have attracted great interest in combating neurodegenerative disorders due to their anti-apoptotic mechanisms and enhancing neuronal plasticity (Hua et al., 2016; Mhalhel et al., 2023). The phytochemical compounds have been also shown the capability to reduce neuroinflammation (Calis et al., 2019; Yarim et al., 2022) and modulate the activity of several enzymes involved in oxidative stress, such as superoxide dismutase (SOD), cyclooxygenase-1 (COX-1), and cyclooxygenase-2 (COX-2) (Park & Song, 2019). In addition, numerous studies have shown that some flavonoids can interfere with the formation and accumulation of neurotoxic proteins, such as amyloid β-protein 42 (Aβ42) (Ali et al., 2019) and α-synuclein (Jia et al., 2019), which are responsible for the progression of neurodegenerative diseases.

In line with high bioactive properties reported for Citrus, this study aimed to compare the phenolic profiles of FS and FZB cultivar harvested in Sicily. Their different parts (peel, pulp, seeds, and albedo) in two different stages of ripening (green and yellow lemons) were considered to select the best part of the fruit and riping stage providing the higher recovery of the phenolic fraction with biological activities. The screening of all the hydroalcoholic extracts, aimed to select the extract with higher phenolic content with a promising biological activity, was performed by the determination of total polyphenol content (TPC), and related capacity to quenching DPPH and ABTS radicals evaluated by spectrophotometric assays. Once obtained the detailed characterization by HPLC-UV/HRMS analysis and evaluated the preliminary antioxidant assays, the peels in the early ripening stage (green lemon) of FZB were selected as representative for its higher phenol content and complex profile for further investigation. Moreover, the semi-quantitative analysis of the most representative secondary metabolites occurring in the selected extract was evaluated by UHPLC-UV. Finally, a preliminary study was carried out to assess the effect of the digestive process on the extracted flavonoids and their bioaccessibility to the body, with a view to possible application as a food supplement.

5.2 Materials and Methods

5.2.1 Chemical and reagents

MS-grade acetonitrile (ACN) and water were supplied by Romil (Cambridge, UK). Ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). Analytical-grade methanol (MeOH) and ethanol (EtOH), MS-grade formic acid (HCOOH). Standards of hesperetin (≥ 98 % HPLC), luteolin (≥ 98 % HPLC), eriocitrin (≥ 98 % HPLC), rutin (≥ 98 % HPLC), apigenin (≥ 98 % HPLC), naringenin (≥ 98 % HPLC) and coumarin (≥ 98 % HPLC) were obtained from Sigma-Aldrich (Milan, Italy).

5.2.2 Samples collection

The two different Sicilian cultivars of Citrus limon FS and FZB, were provided by "Vasile Sebastiano farm" (Siracusa). Samples were collected at two different stages of ripeness in the October and November period (green and yellow stage). All lemons were grown under equal agronomic and environmental conditions. After collection of the samples, they were stored at +4°C for 1 night.

5.2.3 Extraction and sample preparation

Hydroalcoholic extraction of all parts (peel, pulp, seeds, and albedo) of the lemon of both FS and FZB cultivars was performed as reported in our paper with slight modification (Cannavacciuolo et al., 2023). The matrices were dried in an oven at a controlled temperature of 40 °C and then, reduced to powder by mechanical grinding. For each powder 250 mg was weighed and 5 mL of a solution EtOH-H_2O (50 % v/v) was added. The ultrasonic bath was set at room temperature and 3 cycles of sonication were performed for a time of 10 min. The supernatants were recovered by a centrifugation at 6000 rpm for 5 min. The supernatants obtained from the 3 cycles (15 mL) were transferred to a glass flask and the organic solvent was removed via Rotavapor. The aqueous samples were stored at -80 °C overnight and then subjected to freeze-drying until the dry extract was obtained.

5.2.4 Determination of total phenol content

Determination of the total phenolic content (TPC) of the individual parts of each cultivar at the two different stages of ripening was carried out according to the Folin-Ciocalteu method using gallic acid as the calibration standard (Pagliari, Cannavacciuolo, et al., 2022). Briefly, 20 µL of diluted extract (0.3 mg mL-1) and 5 µL of Folin-Ciocalteu reagent were diluted with 145 µL of reagent water and mixed with 30 µL of Na2CO3 (20 % v/v) in a 96-well microplate. After 45 min at 25 °C, absorbance was read at 765 nm with a Multiskan Go spectrophotometer (Thermo Fischer Scientific). Results were expressed as mg of gallic acid equivalent (GAE) contained in 1 g of extract (mg GAE/g) obtained by the mean of three independent measurements considering the standard deviation (SD) (Table 1).

5.2.5 Measurement of antioxidant capacity (ABTS and DPPH assays)

The radical scavenging activity of extracts were determined using the 1,1- diphenyl-2-picrylhydrazyl radical (DPPH•) and 2,2-azinobis-(3-ethylbenzothiazoline-6- sulfonate radical cation) (ABTS•+) by the slightly modified methods previously described (Cannavacciuolo et al., 2023). Briefly, an aliquot (50 µL) of the extract (1 mg mL^{-1}) or standard solution (2.5–10 µg mL−1) was added to 950 µL of prepared DPPH• radical solution (0.14 mM). After darkness incubation for 30 min at room temperature, samples were read by spectrophotometer at a wavelength of 515 nm. For the ABTS•+ scavenging activity, the reaction was initiated by the addition of 50 µL of extract in 950 µL of diluted ABTS (14 mM) of each sample solution. The spectrophotometer was set with a wavelength of 734 nm. Determinations were repeated three times. The absorbance was calculated for each concentration relative to a blank absorbance (methanol) and was plotted as a function of the concentration of compound or standard, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The antioxidant activity was expressed as Trolox equivalents (TE) value representing the µmol of a standard Trolox solution for g of dry matrix (DW).

5.2.6 UHPLC-UV/HRMS/MS profile of citrus extracts

UHPLC-UV/HRMS analysis were performed using an electrospray ionization coupled liquid chromatography (ESI) and high-resolution mass spectrometry (UHPLC-

ESI/HRMS) system, a Waters ACQUITY UPLC system coupled to a Waters Xevo G2-XS QTof mass spectrometer (Waters Corp., Milford, MA, USA), operating in negative and positive ionization modes. Chromatographic separation was performed using a Biphenyl column (100 mm × 2.1 mm, 2.6 µm; Phenomenex, Torrance, CA, USA), and the mobile phase consisting of 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in acetonitrile (B), at flow rate of 0.4 mL min^{-1}. A linear gradient from 5 to10 % B held for 2 min; up to 80 % B from 2.0-17.0 min; up to 95 % B for 1 min. A column wash at 95 % B was held for 5 min and a column equilibration for 3-min (5 %B) before the next injection subsequent analysis. The autosampler was set to inject 5 µL of each sample at a concentration of 0.5 mg mL^{-1}. For the ESI source, the following experimental conditions were adopted: electrospray capillary voltage of 2.5 kV, source temperature of 150°C, and desolvation temperature of 500 °C. MS spectra were acquired by full-range acquisition, covering a mass range of 50 to 1200 m/z. HRMS/MS analysis was performed by data-dependent scanning (DDA) experiments, selecting the first and second most intense ion from the HRMS scan event and subjecting them to collision-induced dissociation (CID) by applying the following conditions: the minimum signal threshold at 250, isolation width at 2.0 and normalized collision energy at 30 %. In both full scan and MS/MS modes, a resolving power of 30000 was used. Chemical deconvolution was attributed using UNIFI software, matching MS/MS spectra with Chemspider database along with a self-made library of phenolic compounds and confirmation with literature reports.

5.2.7 Semi-quantitative analysis by UHPLC-UV

For quantitative analysis, stock solutions (1 mg mL^{-1}) of the compounds used as external standards were prepared by dissolving each compound in a methanol/water solution (50 % v/v). Increasing solution concentrations (0.1, 0.5, 1, 5, 10, and 50 µg mL^{-1}) were prepared for calibration curve construction by UHPLC-UV analysis, with wavelength set at 280 nm and 330 nm. Specifically, 5 µL of each standard solution of each concentration was injected into a technical triplicate. Calibration curves were obtained by linear regression using Excel 2016 software, considering the area of the external standards versus the known concentration of each compound. The obtained calibration curves showed good linearity with correlation coefficients (R^2) ranging from 0.9969 to

0.9999. Quantitative data are expressed as mg g^{-1} dry extract (EXT). MassLynx software (version 4.2) was used for instrument control, data acquisition, and processing.

5.2.8 Multivariate statistical analysis

A complex dataset containing UPLC-HRMS data of all investigated compounds was organized containing 27 variables (metabolites tentatively identified) and 16 observations (investigated extracts). To observe the comprehensive distribution of the metabolites in four the samples, the integrations (AUC) of LC-HRMS signals were selected in the matrices and normalized by average function. The obtained dataset was processed by SIMCA®17 (Sartorius, Goettingen, Germany) for statistical analysis and modelling of score and loading plots. PCA and PLS models were described by two main principal components, scaled by Pareto mode and Autofit function. For reproducibility of the analysis, three extraction replicates and three replicate injections per sample extract were provided in a random way. Periodical injections of extraction solvent (blank) and a sample mix containing all the samples (QC) were analysed each five-acquisition batch. The results of QC were centered ensuring the reproducibility, the stability of the MS signals during the full acquisition batch and the robustness of the chemometric results.

5.2.9 In vitro gastrointestinal digestion by INFOGEST protocol

Gastrointestinal digestion was simulated following the INFOGEST protocol described by Minekus (Minekus et al., 2014b). Briefly, the oral, gastric and intestinal phases were simulated by mimicking the salt, enzyme composition, pH, time and temperature of each phase of the digestive process. At the end of the intestinal phase, the digested extracts were analyzed by UPLC-HRMS in order to quantify the remaining compounds. Bioaccessibility and bioavailability was calculated as reported by Canas (Canas et al., 2022):

$$\% \, bioaccessibility = \frac{digested\ fraction}{non - digested\ fraction} * 100$$

Where non-digested fraction and digested fractions are the concentration of compound before and after digestion simulation, respectively.

$$\% \, bioavailaility = \frac{digested \, fraction * absorption}{non - digested \, fraction} * 100$$

The absorption was estimated in silico using pkCSM-pharmacokinetics (http://biosig.unimelb.edu.au/pkcsm/, consulted on 5 September 2023) e ADMETlab (https://admet.scbdd.com/, consulted on 5 September 2023). Chemoinformatic free software.

5.2.10 Statistical analysis

UHPLC-UV/HRMS experiments were performed in technical triplicate and biological duplicate. one-way ANOVA assessed the variance between groups evaluated considering to be significant at $p \leq 0.05$ on Microsoft Excel 2016. DPPH and ABTS radical scavenging tests were performed in triplicate and the results were expressed as mean ± standard deviation (SD). The Principal Component Analysis (PCA) and Partial Least Square (PLS) were considered as imaging techniques operating the linear regressions on the mean-scaled dataset to select the two main principal components in a Multivariate data analysis approach. The choice of principal components was established based on the fitting (R2X, 0.860) and predictive (Q2X, 0.376) values. For PCA models, the chosen PC1 and PC2 explained 56.4% and 15.5% of the variance, respectively, for a total explained variance of 71.9%. The PLS analysis showed a distinct separation (R2Y, 0.872) and good predictability (Q2, 0.741). No outliers were detected at a confidence level of 95% based on Hotelling's T2 and Q residual statistical test.

5.3 Results and Discussion

5.3.1 Preliminary antioxidant evaluations

The chromatographic profiles (UV280 nm) of the ethanolic extracts 50% of all studied samples showed some differences. With the aim to select the extract with higher phenolic compounds content and consequently the higher radical scavenging, preliminary spectrophotometric assays were performed. All the obtained extracts were tested by Folin–Ciocalteu, ABTS and DPPH assays followed by LC-HRMS analysis for a deep chemical characterisation.

The comparison of the TPC, DPPH, and ABTS data reported in Table 1, suggested that the peels showed the higher antioxidant capacity than pulp, albedo, and seeds, which were not showing considerable variability. Based on previous results, the differences between green or yellow ripening stages of both FS and FZB, were considered. In particular, green peels showed higher TPC, with values of 18.27 ± 1.35 and 25.03 ± 1.50 µg GAE/g DW for FS and FZB respectively, than yellow peels characterised by values of 20.47 ± 1.37 and 16.11 ± 2.03 µg GAE/g DW for FS and FZB respectively (Table 1). The general overview of preliminary antioxidant assays reveals the peels of FZB in green stage provided the most representative content of phenols 25.03 ± 1.50 µg GAE/g DW also characterised by the highest preliminary activity in quenching ABTS and DPPH radicals 89.42 ± 2.53 and 44.40 ± 2.88 µM Trolox/g DW, respectively (Table 1).

Table 1. Means of total phenolic content and antioxidant activity of different parts of citrus extract

Sample	TPC (µg GAE/g DW)	ABTS (µmol Trolox/g DW)	DPPH (µmol Trolox/g DW)
FS green peel	18.27 ± 1.35	61.69 ± 1.27	22.83 ± 1.15
FS yellow peel	20.47 ± 1.37	66.57 ± 2.32	26.81 ± 2.72
FZB green peel	25.03 ± 1.50	89.42 ± 2.53	44.40 ± 2.88
FZB yellow peel	16.11 ± 2.03	83.53 ± 4.25	38.22 ± 1.30
FS green albedo	6.72 ± 0.23	23.73 ± 1.84	11.57 ± 4.69
FS yellow albedo	8.83 ± 1.29	46.91 ± 2.88	31.27 ± 2.15
FZB green albedo	9.26 ± 1.22	56.21 ± 2.81	18.15 ±10.17
FZB yellow albedo	9.43 ± 1.57	37.22 ± 1.59	27.92 ± 3.14
FS green pulp	3.58 ± 0.70	53.62 ± 2.17	33.08 ± 3.60
FS yellow pulp	6.41 ± 1.46	40.11 ± 1.40	33.36 ± 3.02
FZB green pulp	4.75 ± 0.48	35.97 ± 2.85	26.12 ± 0.75
FZB yellow pulp	3.95 ± 0.91	44.39 ± 2.78	38.23 ± 3.33
FS green seeds	4.79 ± 0.42	15.35 ± 0.76	10.95 ± 1.20
FS yellow seeds	3.14 ± 0.59	9.76 ± 0.46	7.61 ± 0.57
FZB green seeds	3.04 ± 0.53	15.05 ± 1.19	9.02 ± 0.86
FZB yellow seeds	0.81 ± 0.20	8.99 ± 0.27	5.42 ± 0.36

5.3.2 Chemical characterization by using UHPLC-HRMS/MS analysis

The obtained citrus extracts were analysed by UHPLC-UV-HRMS/MS to investigate their qualitative profile and in Figure 1 is shown a representative UHPLC chromatogram at 280 nm of FZB in green stage extract. A total of 36 peaks were tentatively identified by comparing retention time, UV/Vis spectra and MS data (accurate mass and MS fragment of the compounds detected with standard compounds, whenever available, and compounds reported in the literature and databases. The identities, retention times, and MS data of each compound are listed in Table 2.

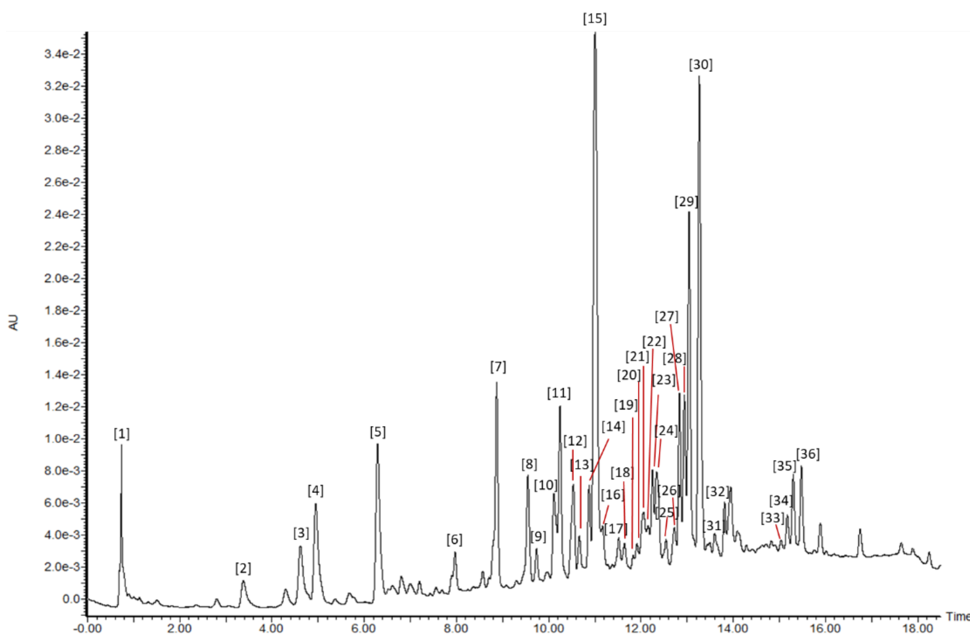


Figure 1 UPLC-UV-Chromatograms of FZB in green stage extract recorded at 280 nm

5.3.2.1 Identification of flavonoids

Flavonoids are a heterogeneous group of polyphenols characterized by high antioxidant activity widely distributed in *Citrus*. Their different occurrence among the different species and varieties influences the appearance, the taste and nutritional value of the fruit. Generally, the flavonoids in plants could be in both aglycone or glycosylated form

with sugar moieties of hexoses, deoxyhexoses or pentoses. Depending on the linkage of sugar moieties to the flavonoid aglycon, C-glycosides and O-glycosides are easily distinguishable by unambiguous MS/MS spectra depending on the nature of the sugar unit. In particular compounds 6,7,8,11,18, 21 and 22 (table 2) belong to C-glycosides generating MS/MS neutral losses of 30 Da, 90 Da and 120 Da in the case of hexose sugars, 74 Da and 104 Da in the case of deoxyhexose sugars, and 60 Da for pentose sugars (Table 2). Compounds 13, 14, 15, 16, 20, 23, 25, 26 and 30 (table 2) are characterised by O-glycosylation pattern in MS/MS spectra due to the neutral loss of 162 Da (hexose sugar), 146 Da (deoxyhexose sugar) and 132 Da (pentose sugar) (Park & Song, 2019). Compounds 17, 28, 31, 33-36 are aglycons scaffold flavonoids occurring in the final part of LC-HRMS chromatogram (Table 2). The compound 6 and 13 showed a similar $[M-H]^-$ parent ion at m/z 609.1463 and m/z 609.1458 respectively, with a molecular formula $C_{27}H_{30}O_{16}$. The retention times and MS/MS spectra reveal their different chemical behaviour. In fact, the detailed analysis of HRMS/MS spectra assigned the compound 6 to lucenin 2 and compound 13 to rutin due to the characteristic fragmentation patterns. In particular, the MS/MS spectrum of compound 6 show the m/z at 519.1145 and m/z 489.1039 related to the loss of the first C-glycoside residue along with the presence of m/z 399.0719 and 369.0612 attributed to the loss of the second C-glycoside residue. The match with MS/MS spectrum is related to the lucenin 2 (Cannavacciuolo et al., 2023). The product ion 300.1458 $[M-(C_{12}H_{22}O_{11})-H]^-$ in MS/MS spectrum of compound 13 corresponds to the neutral loss of a rutinoside unit formed by a deoxyhexose sugar (146 Da) and hexose moiety (162 Da) linked via O-glycosylation to the scaffold. The resulting in the aglycone with m/z 300.0267 identified as quercetin due to the formation of a characteristic fragment of m/z 271.0241 (Śliwka-Kaszyńska et al., 2022).

5.3.2.2 Identification of HMG-Flavonoids

Compound 27, 29 and 32 (Table 2) showed a typical MS/HRMS signals of 3-hydroxy-3-methylglutaryl (HMG) moiety linked to phenolic residues as the loss of 144 Da ($C_6H_8O_4$) (Table 2). The MS/MS spectrum of compound 29 (m/z 681.1729) showed a ion $[M-144-H]^-$ at m/z 537.1250 along with the fragment $[M-162- 144-H]^-$ at m/z

375.0719 suggesting the presence of 3-hydroxy-3-methylglutaryl (HMG) moiety and the glycosyl group attached to the aglycone as the identitied of limocitrol-HMG-*O*-hexoside (El-Sayed et al., 2017).

5.3.2.3 Identification of coumarins

The extracts show the occurrence of coumarins investigated with a good MS response in positive mode (Table 2). Generally, coumarins exhibited a distinct fragment ion $[M-CO_2+H]^+$ from the pyran-2-one scaffold. The described fragmentation pattern could be diagnostic for the characterization of coumarins in the fruit, as in the case of the peaks 9, 12, 19 and 24 (Table 2). In particular, compound 19 showed a molecular formula of $C_{10}H_8O_4$ calculated for $[M+H]^+$ ion at m/z 193.0492. The $[M-CO_2+H]^+$ ion at m/z 150.0306 allowed the identification of scopoletin (Yang et al., 2010; Zhang et al., 2022).

Table 2. List of metabolites putatively identified Compounds identified by UHPLC-QTOF-HRMS/MS analysis.

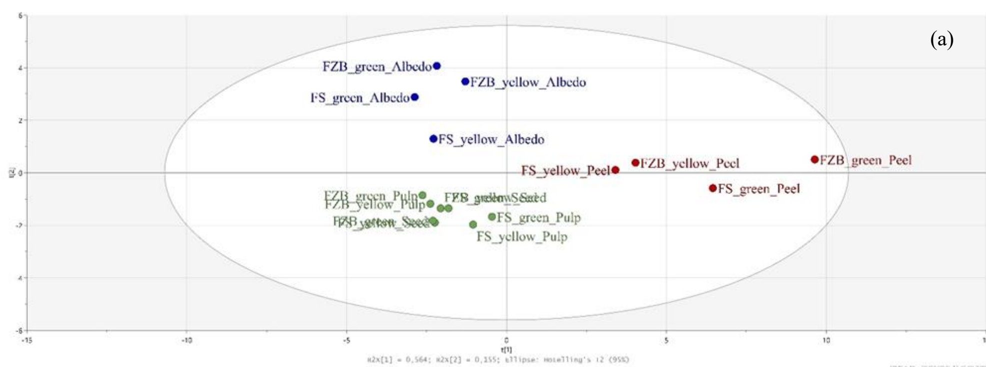
n	Rt (min)	[M-H]- / [M+H]+	Compound	Molecular formula	ppm	MS/MS	Ref.
1	0.59	191.0554^-	quinic acid	$C_7H_{12}O_6$	-3,8	173,0441/127,0389/ 71,0130	chemspider
2	3.42	355.0669^-	p-coumaroyl aldaric acid isomer 1	$C_{15}H_{16}O_{10}$	-0.4	265.0346/191.0190/ 173.0080/163.0391/ 147.0287/119.0492	(Dueñas et al., 2015)
3	4.95	355.0669^-	p-coumaroyl aldaric acid isomer 2	$C_{15}H_{16}O_{10}$	-0.9	209.0293/191.0190/ 173.0080/163.0391/ 147.0287/119.0492	(Dueñas et al., 2015)
4	5.05	355.0669^-	p-coumaroyl aldaric acid isomer 3	$C_{15}H_{16}O_{10}$	-0.7	209.0293/191.0190/ 173.0080/163.0391/ 147.0287/119.0492	(Dueñas et al., 2015)
5	6.28	365.1450^-	propyl-HMG-hexoside	$C_{15}H_{26}O_{10}$	-0.9	221.1020/161.0446/ 125.0235/101.0234	(Zhang et al., 2022)
6	7.99	609.1463^-	lucenin 2	$C_{27}H_{30}O_{16}$	0.3	519.1145/489.1039/ 399.0719/369.0612/ 177.0184	(Otify et al., 2015)
7	8.99	$593,1519^-$	apigenin 6,8-di-*C*-hexoside	$C_{27}H_{30}O_{15}$	1.2	503,1090/473,0950/ 383,0639/353,0512	(Otify et al., 2015)
8	9.55	447.0925^-	orientin	$C_{21}H_{20}O_{11}$	-1.7	357.0603/327.0499/ 297.0393/133.0285	(Pereira et al., 2012)
9	9.72	193.0493^+	isoscopoletin	$C_{10}H_8O_4$	-1.4	178.0255/150.0306/ 133.0279/122.0355	chemspider
10	10.30	565.1552^+	lucidin primeveroside	$C_{26}H_{28}O_{14}$	0	433.1126/415.1018/ 313.0700/283.0595/ 271.0594	chemspider
11	10.39	623.1616^-	chrysoeriol 6,8-di-C-glucoside	$C_{28}H_{32}O_{16}$	-0.3	503,1044/413,0718/ 383,0598/191.0335	(Gattuso et al., 2006)

12	10.66	163.0386^{+}	3-hydroxycoumarin	$C_9H_6O_3$	-2.5	107.0487	chemspider
13	10.68	609.1458^{-}	rutin	$C_{27}H_{30}O_{16}$	1.9	300.0267/271.0241	Std
14	10.84	431.0978^{-}	apigenin-7-*O*-glucoside	$C_{21}H_{20}O_{10}$	1.2	341.0656/311.0551/ 283.0601/269.0442	(March et al., 2006)
15	11.08	595.1688^{-}	eriocitrin	$C_{27}H_{32}O_{15}$	3.3	287.0552/151.0029	Std
16	11.10	435.1281^{+}	naringenin-7-*O*-glucoside	$C_{21}H_{22}O_{10}$	-1.0	381.0698/289.0698/ 263.0543/245.0436/ 219.0280/195.0281/ 165.0175/153.0175	(Gao et al., 2022)
17	11.17	287.0547^{-}	kaempherol	$C_{15}H_{10}O_6$	-1.1	163.0382/153.0178	chemspider
18	11.22	607.1667^{-}	zivulgarin	$C_{28}H_{32}O_{15}$	-0.3	443.0970/323.0552/ 101.0242	chemspider
19	11.35	193.0492^{+}	scopoletin	$C_{10}H_8O_4$	-1.9	178.0255/150.0306/ 133.0279/122.0355	(Yang et al., 2010; Zhang et al., 2022)
20	11.87	623.1622^{-}	isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	0.8	315.0497/300.0260/ 151.0026	(El-Zahar et al., 2022)
21	12.09	461.1082^{-}	scoparin	$C_{22}H_{22}O_{11}$	-1.7	371.0761/341.0657/ 298.0472	(Gattuso et al., 2006)
22	12.15	463.1235^{+}	diosmetin-C-glucoside	$C_{22}H_{22}O_{11}$	0.1	379.0810/367.0807/ 343.0810/313.0703/ 298.0467	(Fu et al., 2016)
23	12.28	579.1704^{-}	naringenin-7-O-rutinoside	$C_{27}H_{32}O_{14}$	-2.6	459.1144/271.0606/ 151.0029	(Mullen et al., n.d.)
24	12.41	147.0439^{+}	coumarin	$C_9H_6O_2$	-1.3	119.0482/101.0385	chemspider
25	12.60	607.1671^{-}	diosmin	$C_{28}H_{32}O_{15}$	0.4	299.0553/284.0317/ 161.0231	(Zhang et al., 2022)
26	12.78	609.1824^{-}	hesperidin	$C_{28}H_{34}O_{15}$	-0.1	301.0711	(Mullen et al., n.d.)
27	12.87	651.1567^{-}	tetrahydroxydimethoxy flavone HMG-hexoside	$C_{29}H_{32}O_{17}$	0.5	607.1673; 589.1590 549.1228; 507.1143 345.0618	(Fu et al., 2016)
28	12.95	287.0561^{-}	eriocytrol	$C_{15}H_{12}O_6$	-0.2	151.0029/135.0444	(Santos et al., 2010)
29	13.04	681.1729^{-}	limocitrol-HMG-*O*-hexoside	$C_{30}H_{34}O_{18}$	1.2	619.1671/579.1359/ 537.1250/375.0719/ 360.0487	(El-Sayed et al., 2017)
30	13.27	609.1817^{-}	chrysoeriol 7-*O*-rutinoside	$C_{28}H_{32}O_{15}$	0.4	301.0702/286.0465	(Min Vivian Goh et al., 2022)
31	13.50	287.0549^{+}	luteolin	$C_{15}H_{10}O_6$	-0.6	153.0178	Std
32	13.70	765.1889^{-}	isorhamnetin-di-HMG-*O*-glycoside	$C_{34}H_{38}O_{20}$	0.7	503.1196/473.1090/ 383.0770/353.0663	(Cannavacciuolo et al., 2023)
33	15.09	299.0556	diosmetin	$C_{16}H_{12}O_6$	-1.7	284.0319/256.0368	(Hamdan et al., 2020)
34	15.19	345.0612^{-}	limocitrin	$C_{17}H_{14}O_8$	-1.3	330.0371/315.0136	chemspider
35	15.37	301.0716^{-}	hesperitin	$C_{16}H_{14}O_6$	-0.7	285.0394/164.0105/ 136.0157/1088.0204	Std
36	15.50	375.0730^{-}	limocitrol	$C_{18}H_{16}O_9$	2.3	360.0481/317.0298/ 302.0062/274.0111	(El-Sayed et al., 2017)

5.3.3 Principal Component Analysis

The different ripening stages (green and yellow respectively), along with the parts of the lemon fruit (peels, pulp, albedo and seeds) of both FS and FZB cultivars were considered as main variables in the modelled samples discrimination.

The distribution of the samples was performed by PCA whose the score plot show that the first principal component (PC1) divided the peels of FS and FZB, in both green and yellow growth stage, on the right part of the PCA score plot (PC1 > 0) highlighted in red cluster (Figure 2a). The incidence of the metabolites on the following clusterization is related to their distance from the origin in loadings plot (Figure 2b). In particular, compounds 1 and 4, (quinic acid and p-coumaroyl aldaric acid isomer, respectively) along with the C-glycosides flavonoids (compounds 6 and 7) and HMG-flavonoids (compounds 29 and 32) and the aglycon (compound 34) positively affecting the cluster discrimination with higher incidence (Figure 2b). The blue cluster on the upper left quadrant of the score plot (PC1 > 0; PC2 < 0) contained albedo samples of both FS and FZB in both green and yellow stage (Figure 2a). This cluster is discriminated for the occurrence of compounds 3, 14 and 23 mainly affecting the distance from the origin of the loading plot (Figure 2b). The samples of seeds and pulp samples were plotted without significant separation in the bottom left quadrant with PC1 < 0 and PC2 < 0 as showed by the green cluster in PCA score plot (Figure 2a).



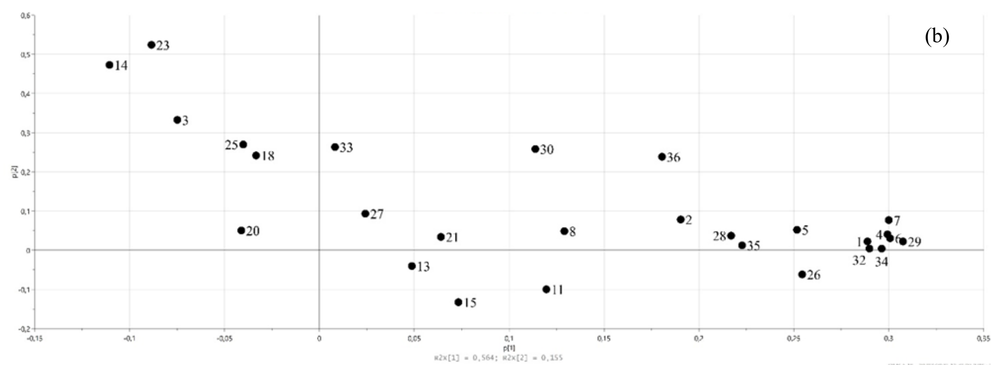


Figure 2. PCA score plot (a) and Loading plot (b) performed on the peels (red cluster), albedo (blue cluster), pulp and seeds (green cluster).

5.3.4 Partial Least Square analysis

The correlation between the large set of samples and preliminary antioxidant activity was provided by PLS analysis with the aim to select the extract with more interesting activity for further *in vitro* investigation. In particular, PLS was performed for the attribution of metabolites to the hydroalcoholic extracts based on the different capacities to quench DPPH and ABTS radicals among the samples of peel, pulp, and albedo in both green and yellow growing stage. The first principal component (PC1) of PLS scores plot (Figure 3a) separate the peel (PC1 > 0) on the right part of the plot against pulp, seeds and albedo on the left part of the plot (PC1 < 0), while the second principal component (PC2) separated the FZB cultivar (PC2 < 0) from FS cultivar (PC2 > 0) (Figure 3a). The loading plot shows metabolites that significantly affected the discrimination of samples observed in the score scatter plot (Figures 3b). The incidence of compounds 1, 4, 6, 7, 29 and 34 (Table 2) determined the distribution of the peels characterised by the higher activity in both DPPH and ABTS assays. Among the peels of investigated lemons fruits, the FZB in the early stage of growth (green) was the more positively affected by the PLS discrimination far away the origin of the score plot (Figure 3a).

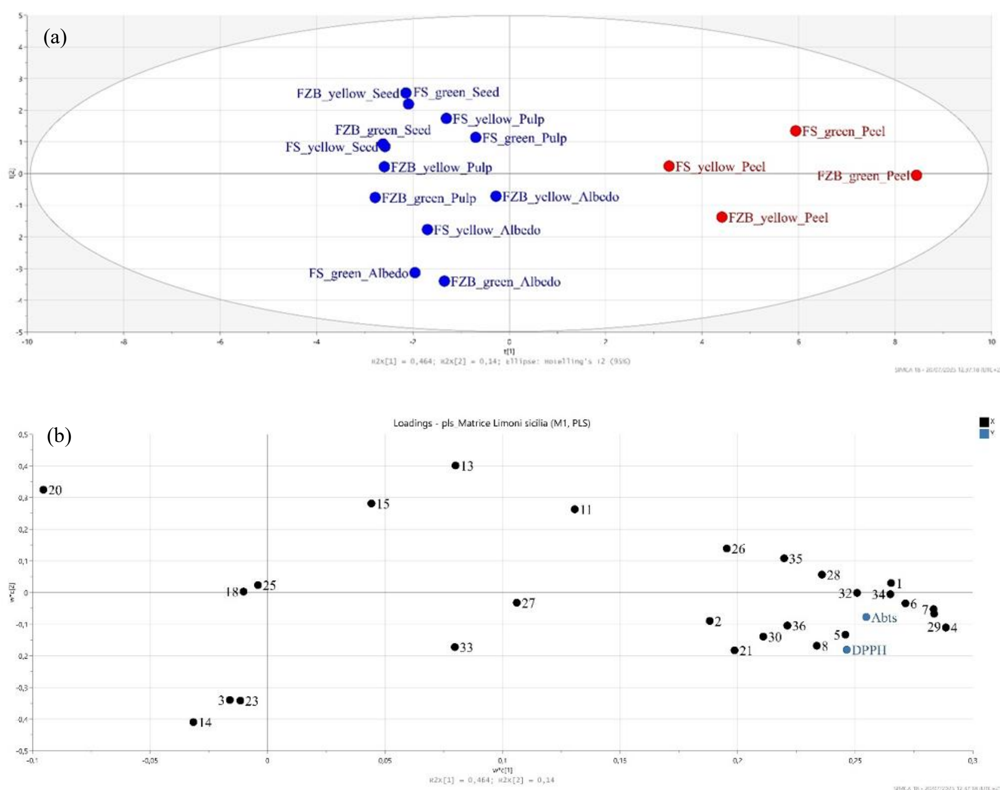


Figure 3. PLS score plot (a) and Loading plot (b) performed on the peels (red cluster), albedo, pulp and seeds (blue cluster).

5.3.5 Target analysis

The metabolomic workflow described guided the selection of the extract with the highest activity in terms of the compounds, thus a semi-quantitative analysis of the major phenolic compounds (flavonoids and coumarins) present in the selected extract was performed. Quantification was performed by UHPLC-UV analysis at a wavelength of 280 nm using calibration curves of standards available in the range 0.1-50 µg mL^{-1} (Table 3). Compounds for which no reference standards were available were quantified in standard equivalents using the most chemically related standard available. The highest phenolic compounds present in the peel extract were the C-glycoside orietin (compound 8) at 1104.39 ± 46.76 µg/g EXT, followed by the O-glycoside eriocitrin (compound 15) at 1041.17 ± 29.64 µg/g EXT. Among the coumarins, compound 24 (coumarin) showed the highest amount with 339.69 ± 16.22 µg/g EXT, confirming that coumarins are minority compounds of this extract present in low concentration.

Table 3. Quantitative analysis of main compounds of FZB peel green stage.

n	Compounds	std	*µg/g EXT
6	lucenin 2	luteolin	1012.07 ± 58.25
7	apigenin 6,8-di-C-hexoside	apigenin	608.96 ± 22.16
8	orientin	luteolin	1104.39 ± 46.76
9	isoscopoletin	cumarin	294.82 ± 35.36
12	3-hydroxycoumarin	cumarin	333.44 ± 31.50
13	rutin	rutin	547.65 ± 12.87
14	apigenin-7-O-glucoside	apigenin	495.73 ± 32.01
15	eriocitrin	eriocitrin	1041.17 ± 29.64
16	naringenin-7-glucoside	naringenin	303.04 ± 22.28
19	scopoletin	coumarin	286.61 ± 17.67
23	naringenin-7-O-rutinoside	naringenin	329.44 ± 21.25
24	coumarin	coumarin	339.69 ± 16.22
26	hesperidin	hesperitin	493.01 ± 20.05
28	eriocytrol	eriocitrin	700.20 ± 48.91
30	chrysoeriol 7-*O*-rutinoside	chrysoeriol	928.18 ± 36.82
31	luteolin	luteolin	971.16 ± 55.31
35	hesperitin	hesperitin	517.80 ± 33.08

* Data expressed as µg std equivalent/g dried extract (EXT)

5.3.6 In silico PASS software approach for predicting biological activities.

Flavonoids and coumarins found in FZB green stage at higher amount or representative for a class of metabolites were tested in silico using Online Pass software to assess the health benefits associated with the extract and to guide future in vitro studies. The analysis was obtained by entering the simplified molecular input line entry system (SMILES) strings of each molecule into the software and obtaining in response a table containing the probable activity (PA) and probable inactivity (PI) values with a related biological activity variability between 0.000 and 1.000. Biological predictions with PA > 0.700 show the greater potential biological activity.

The final predictive results revealed numerous therapeutic properties associated with these analytes. In particular, an interesting antioxidant, free radical scavenging and oxidative peroxidase inhibiting power with a Pa value (Pa > 0.9) was obtained for almost all compounds. These data confirm the strong antioxidant activity of the peels observed in the preliminary ABTS and DPPH assays. Furthermore, the analytes present in FZB green stage showed a high probability of chemopreventive, antineoplastic, antidiabetic and cardioprotective activity with Pa values (Pa > 0.7). The results of target analysis are resumed in Table 4. In addition to considering the therapeutic activities and/or targets of individual molecules in the extract, it can also be hypothesised that the overall consumption of the extract may synergistically enhance all biological processes. Numerous research papers have well documented the biological actions of citrus metabolites and their formulations (Dadwal & Gupta, 2021), strongly supporting these predictions.

Table 4: Main potential biologic activities predicted using Pass online based software.

glucosinolates	predicted bioactivities	Pa	Pi
lucenin 2	Cardioprotectant	0.950	0.002
	Free radical scavenger	0.899	0.002
	Chemopreventive	0.876	0.003
	Antioxidant	0.847	0.004
	Antineoplastic	0.835	0.008
	Antidiabetic	0.815	0.005
Apigenin 6,8-di-C-hexoside	Cardioprotectant	0.948	0.002
	Hepatoprotectant	0.916	0.002
	Free radical scavenger	0.877	0.002
	Chemiopreventive	0.859	0.003
	Antioxidant	0.837	0.003
	Antineoplastic	0.833	0.008
	Antidiabetic	0.822	0.005
orientin	Free radical scavenger	0.955	0.001
	Cardioprotectant	0.952	0.002
	Hepatoprotectant	0.927	0.002
	Chemopreventive	0.881	0.003
	Antioxidant	0.828	0.003
isoscopoletin	Antimutagenic	0.898	0.002
	Peroxidase inhibitor	0.811	0.004
	Apoptosis agonist	0.750	0.011
	Free radical scavenger	0.742	0.003
3-hydroxycoumarin	Peroxidase inhibitor	0.830	0.004
	Vasoprotector	0.807	0.005

	Antimutagenic	0.739	0.005
rutin	Free radical scavenger	0.988	0.001
	Cardioprotectant	0.988	0.001
	Anticarcinogenic	0.983	0.001
	Vasoprotector	0.980	0.001
	Hepatoprotectant	0.968	0.001
	Chemopreventive	0.968	0.001
	Antioxidant	0.923	0.003
	Antifungal	0.784	0.006
Apigenin-7-O-glucoside	Hemostatic	0.976	0.001
	Cardioprotectant	0.971	0.001
	Vasoprotector	0.969	0.001
	Free radical scavenger	0.949	0.001
	Antihypercholesterolemic	0.929	0.003
	Lipid peroxidase inhibitor	0.926	0.002
	Anticarcinogenic	0.926	0.002
eriocitrin	Lipid peroxidase inhibitor	0.989	0.001
	Free radical scavenger	0.986	0.001
	Anticarcinogenic	0.981	0.001
	Chemopreventive	0.970	0.001
	Hepatoprotectant	0.970	0.001
	Antihypercholesterolemic	0.963	0.001
Naringenin-7-glucoside	Lipid peroxidase inhibitor	0.815	0.003
	Antihypercholesterolemic	0.816	0.005
	Antioxidant	0.794	0.003
	Free radical scavenger	0.794	0.003
	Antineoplastic	0.751	0.018
scopoletin	Antimutagenic	0.898	0.002
	Peroxidase inhibitor	0.811	0.004
	Apoptosis agonist	0.750	0.011
	Free radical scavenger	0.742	0.003
Naringenin-7-O-rutinoside	Lipid peroxidase inhibitor	0.986	0.001
	Free radical scavenger	0.983	0.001
	Anticarcinogenic	0.979	0.001
	Vasoprotector	0.970	0.001
	Chemopreventive	0.967	0.001
	Hepatoprotectant	0.964	0.001
	Antioxidant	0.880	0.003
Coumarin	Antimutagenic	0.767	0.004
	Membrane permeability inhibitor	0.734	0.026
Hesperidin	Lipid peroxidase inhibitor	0.991	0.001
	Free radical scavenger	0.989	0.001
	Anticarcinogenic	0.982	0.001
	Chemopreventive	0.975	0.001
	Hemostatic	0.963	0.001
Eriocytrol	Lipid peroxidase inhibitor	0.989	0.001
	Free radical scavenger	0.986	0.001

	Anticarcinogenic	0.981	0.001
	Vasoprotector	0.976	0.001
	Chemopreventive	0.970	0.001
	Cardioprotectant	0.941	0.002
	antioxidant	0.879	0.003
chrysoeriol 7-*O*-rutinoside	Hemostatic	0.988	0.001
	Free radical scavenger	0.986	0.001
	Cardioprotectant	0.984	0.001
	Anticarcinogenic	0.968	0.001
	Lipid peroxidase inhibitor	0.962	0.002
Luteolin	Antimutagenic	0.940	0.001
	Peroxidase inhibitor	0.936	0.002
	Vasoprotector	0.901	0.003
	Apoptosis agonist	0.866	0.005
	Antioxidant	0.775	0.004
hesperetin	Lipid peroxidase inhibitor	0.885	0.003
	Antimutagenic	0.881	0.002
	Free radical scavenger	0.878	0.002
	Chemopreventive	0.815	0.004
	Antioxidant	0.746	0.004

5.3.7 Bioaccessibility and bioavailability of flavonoids and coumarins

The importance of dietary antioxidants such as polyphenols and flavonoids is well established. It has been widely demonstrated that these molecules can counteract adverse oxidative effects by preventing the onset of even life-threatening pathological conditions (Gómez-Mejía et al., 2019; Khan et al., 2014). However, oral ingestion of a product involves its digestion, it will therefore be important for future evaluations to take into account the effect of gastrointestinal digestion, in terms of environmental conditions and enzymes, on the molecules present in the FZB green stage extract selected as the richest in bioactive molecules after multivariate analysis. INFOGEST protocol was selected from among various static digestion models, which have a set of digestion parameters defined on the basis of human physiological knowledge and internationally agreed to adequately simulate the digestion of food or food ingredients in the adult organism. INFOGEST is widely used for bioaccessibility of polyphenols, as well as for observing the digestive fate of proteins, lipids and carbohydrates (Brodkorb et al., 2019; Minekus et al., 2014b).

There are numerous studies in the literature on the simulation of the digestion process of polyphenol-rich exacts (Bouayed et al., 2011; Ferreira-Santos et al., 2023; Goh & Barlow, 2004; Peña-Vázquez et al., 2022), but a class of molecules is the most abundant and heterogeneous group of secondary metabolites in nature, the results are ambiguous and it is difficult to establish a general behavior. The bioaccessibility of these compounds is influenced by many factors, including their structure (location of the hydroxyl groups), molecules size, solubility, glycosylation, conjugation and methylation. For example, it has been observed that interactions of phenolic compounds with sugars or other analytes can protect them from enzymatic hydrolysis, affect their bioavailability (Bouayed et al., 2011; Gonzales et al., 2015; Rasera et al., 2023). In general, the acidic environment of the stomach can favour an increase the release of polyphenol from matrix, whereas the alkaline environment of the intestine can involve in a degradation (Bouayed et al., 2011; Gonzales et al., 2015; Rasera et al., 2023).

During the simulation of the gastrointestinal digestion process the fate of the flavonoids, coumarin and phenolic acid present in FZB peel green stage extract were evaluated. The result obtained after digestion process show a good resistance of most of the flavonoids and coumarins present in the FZB peel green stage extract to the enzymatic action and to the pH variation of each phase (Table 5). This date is agree with Goh's study (Goh & Barlow, 2004) who reported a degradation of flavonoids after digestion process of around 5%. However, the bioaccessibility of more apolar aglycon flavonoids such as diosmetin, limocitrin, limocitrol and hesperetin were drastically reduced to about 90-95 %, This may be due to the ring opening (Jang et al., 2021). However, an interesting finding is the increase in bioaccessibility observed for some glycosylated flavonoids and phenolic acids. Other studies conducted on food or its extracts observed an increase in the bioavailability of flavonoids (Peña-Vázquez et al., 2022). The increasing in flavonoid content may be due to the release of phenolic compounds bound to other matrix components and/or the degradation of polymer or conjugate of polyphenols into their respective derivatives. Enzymes, pH and bile salts in the intestinal environment can cause changes in the chemical structure of phenolic compounds, resulting in molecules with different bioaccessibility (Ferreira-Santos et al., 2023). However, further studies will be needed to investigate and better understand the origin of the observed increase.

In addition to the bioaccessibility, it is also important to assess the absorption capacity to complete the information on their bioavailability. Using the pkCSM software, it was possible to predict in silico the passive transport of most of the analytes identified in FZB peel green stage across the membrane of enterocytes in adult organisms. The data in Table 4, suggest that most glycosylated flavonoids have an intestinal absorption of 13-30%, resulting in reduced bioavailability.

This results are in agreement with literature data that shown as the glycosylated flavonoids have low bioavailability in the small intestine about 5-10% while the most part of the ingested phenols reaching the colon where they are metabolized by microorganisms (Ferreira-Santos et al., 2023). However, some glycosylated flavonoids could be absorbed in the small intestine through active transport, by using the Na^+-dependent glucose transporter (SGLT1) and/or the lactase floridzinn hydrolase (LPH), improving bioavailability (Hollman, 2004). Given this complex mechanism of active and/or passive absorption further studies are required to define the effective bioavailability of the bioactive compounds in the extract.

Table 5 - The most important potential biological activities of the main compounds found in the peel green stage of FZB were predicted using the Pass online based software.

n	compounds	Bioacc. (%)	Bioava. (%)	% Absorbtion (human)	LogP
1	quinic acid	115.30	40.16	14.75	-1.77
2	p-coumaroyl aldaric acid isomer 1	108.10	13.70	12.67	-2.44
3	p-coumaroyl aldaric acid isomer 2	105.63	-	-	-
4	p-coumaroyl aldaric acid isomer 3	96.78	-	-	-
5	propyl-HMG-hexoside	112.27	-	-	-
6	lucenin 2	116.45	13.55	11.64	-2.88
7	apigenin 6,8-di-*C*-hexoside	108.92	15.97	14.66	-2.84
8	orientin	99.29	43.42	43.73	-2.91
9	isoscopoletin	94.11	89.92	95.55	-2.54
10	lucidin primeveroside	81.53	-	-	-
11	chrysoeriol 6,8-di-C-glucoside	109.94	14.61	13.29	-2.86
12	3-hydroxycoumarin	83.18	78.65	94.55	-2.13

13	rutin	82.41	19.32	23.45	-2.89
14	apigenin-7-*O*-glucoside	96.47	36.28	37.61	-2.56
15	eriocitrin	73.99	17.85	24.12	-2.90
16	naringenin-7- *O*-glucoside	39.02	14.06	36.04	-2.89
17	kaempherol	71.05	52.78	74.29	-3.04
18	zivulgarin	106.17	41.90	39.47	-2.83
19	scopoletin	94.11	89.67	95.28	-2.50
20	isorhamnetin-3-O-rutinoside	94.97	28.59	30.10	-2.90
21	scoparin	93.84	42.60	45.39	-2.88
22	diosmetin-C-glucoside	72.35	-	-	-
23	naringenin-7-O-rutinoside	107.52	43.05	36.63	-3.04
24	coumarin	84.77	82.05	96.79	-1.51
25	diosmin	106.20	31.14	29.32	-2.93
26	hesperidin	110.12	30.76	27.93	-2.90
27	tetrahydroxydimethoxy flavone HMG-hexoside	101.43	-	-	-
28	eriocytrol	65.69	-	-	-
29	limocitrol-HMG-*O*-hexoside	101.40	-	-	-
30	chrysoeriol 7-*O*-rutinoside	118.22	37.52	31.74	-2.93
31	luteolin	9.00	0.00	81.13	-3.09
32	isorhamnetin-di-HMG-*O*-glycoside	82.96	-	-	-
33	diosmetin	9.45	7.55	79.90	-3.24
34	limocitrin	5.32	4.46	83.84	-2.89
35	hesperitin	9.19	7.69	83.73	-2.89
36	limocitrol	3.54	2.89	81.68	-2.89

5.4 Conclusions

Citrus fruits are a widely cultivated food source and consequently produce a wide variety of by-products. In particular, in this work the industrial by-products (peel, pulp, albedo and seeds) of two specific cultivar, commonly called 'Femminello siracusano' (FS) and 'Femminello zagara bianca' (FZB), grown in southern Italy (Sicily region), were analysed and compared. UHPLC-ESI-HRMS/MS analysis of the hydroalcoholic extract of the different by-products revealed different phenolic profiles, identifying the presence of 36 compounds belonging to the classes of flavonoids, coumarins and phenolic acids.

Multivariate PCA and PLS analyses were also used to compare the metabolic profile with the antioxidant activity tested in vitro. The results allowed the identification of

metabolites 1, 4, 6, 7, 29 and 34 as the most influential in the selection of the peels, especially that of FZB green stage as the richest in bioactive polyphenols. The main metabolites of the peel FZB green stage extract were quantified and analysed by in silico analysis to evaluate the potential bioactivities of the extract, confirming an antioxidant activity together with antidiabetic, chemopreventive and cardio preventive properties. Finally, simulation of in vitro gastrointestinal digestion showed excellent bioaccessibility of phenolic compounds in lemon peel. Bioavailability was measured in silico and showed low intestinal permeability by passive transport, but active membrane transport must be considered. In conclusion, lemon peel, especially the immature white Zagra variety, can be considered as a promising source of antioxidants for the maintenance of well-being.

Acknowledgements

I want to thanks Professor Nicola Cicero of the University of Messina (Italy) for the *Citrus limon* by-products provided for the study. Also, a big thank you to Professor Alejandro Cifuentes and all laboratory team for welcoming me at Instituto de Investigación en Ciencias de la Alimentación (CIAL), in Madrid (Spain) and to supporting me in the bioactivity and permeability studies of the extracts.

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Outlooks

This project demonstrated how the study of metabolomic applied to agro-food wastes allows the identification of metabolites potentially bioactive against NCDs, nowadays the cause of 70% of deaths worldwide.

By studying the metabolome of three different agro-industrial by-products, it was possible to identify alternative sources of bioactive molecules compared to the commonly used edible parts. In particular, the cocoa bean shells discarted after roasting were found to be an excellent source of methylxanthines (theobromine and caffeine), known and studied mainly as neuroprotectants and neurostimulants. The *Camelina sativa* seed pressed-cake by-product has proved to be a good source for the recovery of glucosinolates manly glucocamelinin, glucoarabinin and homoglucocamelinin, and some probable minority GLSs such as glucohirsutin, identified for the first time in *Camelina sativa,* all with promising chemopreventive activities. Finally, lemon peel, one of the main by-products of citrus fruits, can be an excellent source of phenolic compounds (flavonoids, coumarins and phenolic acids) whose antioxidant, anti-tumour, anti-diabetic and cardioprotective activities have been widely studied. The high metabolic variability of agro-industrial by-products and the need to continue studying them looking for beneficial molecules and/or applications has been demonstrated.

Furthermore, this work highlighted the importance of extraction process innovation and optimisation over the conventional methods that use toxic organic solvents that raise concerns about environmental pollution, worker safety and potential health risk to consumers. In particular, two different eco-friendly extraction techniques, ultrasound-assisted extraction and pressurised liquid extraction, have been developed and optimised in order to investigate the factors that more impact on the process and to find the optimal extraction conditions. This allowed us to improve the extraction efficiency while making the process more sustainable by reducing the use of organic solvents, time. Finally, this project highlights the importance in food science research to assess the bioaccessibility of bioactive compounds in the extracts by an in vitro digestion experiment that mimic the conditions of the human gastrointestinal tract called INFOGEST protocol. By using this standardized method, the investigation of how extract and their components behave

during digestion (stability and degradation) can be obtained, providing a better understanding of their role in disease prevention and health promotion. This information can also guide to the development of better formulation to avoid degradation, improving the health benefits associated with these compounds.

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- Stefania Pagliari, Mirea Sicari, Lidia Pansera, Werther Guidi Nissim, Kamel Mhalhel, Sepand Rastegar, Antonino Germanà, Nicola Cicero, Massimo Labra, Ciro Cannavacciuolo, Giuseppe Montalbano, Luca Campone. "A comparative metabolomic investigation of different sections of Sicilian *Citrus limon*, characterization of bioactive metabolites and evaluation of in-vivo toxicity on zebrafish embryo" **submitted to Journal of Food Science through ScholarOne Manuscripts on 23 October 2023.** Manuscript number: JFDS-2023-1648
- Ciro Cannavacciuolo; Stefania Pagliari; Rita Celano; Luca Rastrelli, Luca Campone. “Critical analysis of green extraction techniques used for botanicals: Trends, priorities, and optimization strategies -A review.” **submitted to Trends in Analytical Chemistry Manuscripts on 30 July 2023.** Manuscript number: TRAC-D-23-00473

Other Publications during the PhD years

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