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DEVELOPMENT OF GREEN EXTRACTION TECHNIQUES FOR THE DETERMINATION OF BIOACTIVE MOLECULES FROM PLANT MATRICES

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Abstract

In recent years, interest in developing green extraction methods for recovering bioactive compounds from natural sources has grown significantly. This interest is driven both by the need to reduce the environmental impact of traditional chemical processes and by the urgency to valorize by-products and waste from the agri-food and industrial sectors, in line with the principles of green chemistry and the circular bioeconomy.

In this context, the aim of this doctoral research was to develop and optimize analytical methods based on new "green" extraction techniques for the identification of bioactive molecules from plant matrices. Attention was focused on phenolic compounds, flavonoids, and protein pigments present in plant and microalgal sources.

The research focused on developing new sustainable extraction methods, using innovative and environmentally friendly solvents such as Deep Eutectic Solvents (DESs) and their supramolecular analogues (SUPRADESs), which are considered among the most promising green solvents for applications in analytical chemistry, pharmaceuticals, and nutraceuticals.

DESs are binary or ternary mixtures composed of a Hydrogen Bond Donor (HBD) and a Hydrogen Bond Acceptor (HBA), which form deep eutectic mixtures through hydrogen bonding. These solvents have unique characteristics, such as low vapor pressure, non-volatility, biodegradability, low toxicity, and adjustable polarity, making them suitable for replacing traditional organic solvents. When both components forming the DES are of natural origin, they are called Natural Deep Eutectic Solvents (NADESs).

An initial research project focused on developing a "green" extraction method using DESs to extract phenolic compounds and phycocyanin (PC) from Spirulina. Optimizing the extraction parameters—including the solid/liquid ratio, temperature, and extraction time—allowed for maximizing the yield, obtaining extracts with high content of both phenolic compounds and PC through a process completely free of toxic organic solvents. Specifically, the optimized extraction procedure involved the use of a betaine-glucose NADES under the following conditions: a matrix/solvent ratio of 1:20

(w/w), a temperature of 50°C, and an extraction time of one hour. The developed procedure ensured the recovery of phenolic compounds and PC equal to 11.77 ± 1.23 mg of gallic acid equivalents (GAE) and 27.56 ± 2.46 mg per g of Spirulina powder, respectively. The PC in the NADES extract showed a slower degradation compared to the aqueous extract. Finally, the phenolic compound profile of the extract was determined by High Performance Liquid Chromatography (HPLC) coupled to mass spectrometry (MS) analysis, which allowed us to identify four phenolic compounds and two organic acids.

The second research line focused on the use of SUPRADES to extract poorly soluble phenolic compounds, particularly quercetin. This flavonoid is of great interest due to its antioxidant, anti-inflammatory and anti-cancer properties. SUPRADESs are a new class of supramolecular eutectic solvents characterized by the presence of cyclodextrins (CDs). CDs are cyclic oligosaccharides known for their ability to form inclusion complexes with various guest molecules, improving their solubility, stability, and bioavailability.

The experiments conducted demonstrated that SUPRADES containing β-CD can be used as extraction solvents, particularly for poorly water-soluble molecules such as quercetin. The extract was analyzed using HPLC with a Diode Array Detector (DAD). However, to evaluate the extraction capacity of these solvents, it is necessary to release the analyte from the SUPRADES. Various solvents for analyte release were tested, and the use of ethanol (EtOH) as a release solvent proved to be an ideal compromise between recovery efficiency (approximately 70%) and environmental sustainability.

For both research projects, it was essential to work within a circular economy framework and in accordance with the principles of green chemistry.

From a sustainability perspective, the entire proposed approach fully complies with the principles of green chemistry, as it includes:

• the use of non-toxic and biodegradable solvents;

• the use of low process temperatures;

• the reduction of energy consumption and solvent volumes;

The evidence gathered during the PhD period confirms that DES and SUPRADES represent a valid alternative to traditional organic solvents, combining analytical efficiency, selectivity, and environmental sustainability. Furthermore, the ability of DES to stabilize complex bioactive molecules, such as PC, offers the possibility to develop new formulations with extended shelf life, applicable in the pharmaceutical and nutraceutical fields.

In conclusion, this research has contributed to the advancement of analytical chemistry by adhering to the principles of green chemistry and developing innovative extraction strategies that can be applied on an industrial scale.

Future developments may include extending these extraction methods to new classes of bioactive molecules, further studies on the stability of SUPRADES–host molecule complexes, and the application of these SUPRADES as extraction solvents for plant matrices.

Acknowledgements

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Table of Content

List of Figures

List of Table

List of Abbreviations

2,2-diphenyl-1-picrylhydrazyl	DPPH
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)	ABTS
2,6-di-O-methyl-β-cyclodextrin	DM-β-CD
Allophycocyanin	APC
Alternating Current	AC
Benzyltriethylammonium chloride	BTAC
Chemical Ionization	CI
Chloride Anion	Cl^-
Choline Chloride	ChCl
Cyclodextrin	CD
Deep Eutectic Solvent	DES
Diethylene Glycol	DEG
Dimethyl Sulfoxide	DMSO
Diode Array Detector	DAD
Direct Current	DC
Dispersive Liquid Liquid Microextraction	DLLME
Electron Impact	EI
Electrospray Ionization	ESI
Ethylene glycol	EG
Ferric Reducing Antioxidant Power	FRAP
Fourier Transform Infrared Spectroscopy	FTIR
Gallic Acid Equivalents	GAE
Gas Chromatography	GC
Glycerol	Gly
Height Equivalent to a Theoretical Plate	HETP
High Performance Liquid Chromatography	HPLC
High Performance Thin Layer Chromatography	HPTLC
High Pressure Temperature Extraction	HPTE
Hollow Fiber Liquid Phase Microextraction	HF-LPME
Hydrogen Bond Acceptor	HBA
Hydrogen Bond Donor	HBD

Hydroxyethyl-β-cyclodextrin	HE-β-CD
Hydroxyl Groups	OH
Hydroxypropyl-β-cyclodextrin	HP-β-CD
Ion Exchange Chromatography	IEC
Ionic Liquids	ILs
Isoprene	IPR
Lactic Acid	LA
Liquid Chromatography	LC
Mass Spectrometry	MS
Matrix Assisted Laser Desorption/Ionization	MALDI
Matrix Solid Phase Dispersion	MSPD
Methylated-β-cyclodextrin	M-β-CD
Microextraction By Packed Sorbent	MEPS
Microwave Assisted Extraction	MAE
N,N- dimethylcyclohexylamine	DMCA
N,N-Dimethylbenzylamine	DMBA
Natural Deep Eutectic Solvents	NADESs
Normal Phase	NP
Number of theoretical plates	N
Octanoic Acid	OA
One Factor At Time	OFAT
Oxygen Radical Absorbance Capacity	ORAC
Phycobiliprotein	PBP
Phycobilisomes	PBS
Phycocyanin	PC
Phycocyanobilin	PCB
Phycoerythrin	PE
Phycoerythrobilin	PEB
Phycoerythrocyanin	PEC
Phycourobilin	PUB
Phycoviolobilin	PVB
Pressurized Liquid Extraction	PLE
Pulsed Electric Fields	PEFs
Randomly methylated-β-cyclodextrin;	RAME-β-CD

Refractive Index	RI
Reversed Phase	RP
Selected Ion Monitoring	SIM
Single Drop Microextraction	SDME
Solid Liquid Extraction	SLE
Solid Phase Extraction	SPE
Solid Phase Microextraction	SPME
Stir Bar Sorptive Extraction	SBSE
Sulfated-β-cyclodextrin;	S-β-CD
Sulfobutylether-β-cyclodextrin	SBE-β-CD
Supercritical Extraction	SC
Supramolecular Deep Eutectic Solvent	SUPRADES
Tetrabutylammonium Chloride	TBAC
Thin Layer Chromatography	TLC
Time Of Flight	TOF
Total Phenolic Content	TPC
Trolox Equivalent Antioxidant Capacity	TEAC
Ultra High Performance Liquid Chromatography	UHPLC
Ultrasound Assisted Extraction	UAE
Ultraviolet	UV
Volatile Organic Compounds	VOCs

STATE OF THE ART

CHAPTER 1

1. Bioactive molecules

Bioactive molecules are compounds of natural or synthetic origin that have a beneficial effect on the health of living organisms. They are able to interact with biological systems and influence various metabolic, physiological and cellular processes by activating or inhibiting enzymes, modulating membrane receptors, regulating gene expression and influencing intracellular signalling pathways [1].

These compounds are widespread in nature and can be found in plants, animals and microorganisms. However, it is essential to differentiate between bioactive molecules and essential nutrients [2].

Essential nutrients, such as carbohydrates, proteins, lipids, vitamins and minerals, play a fundamental role in survival and they must be taken in through food as the body does not produce them in sufficient quantities. If these nutrients provide energy and ensure the proper functioning of vital processes, bioactive molecules are not necessary for survival. However, the integration of these compounds into dietary habits has been shown to have a positive effect on overall well-being, as they appear to play a role in activating protective, adaptive, and preventive mechanisms against pathological conditions [3].

Indeed, consuming these molecules through diet or specific supplements has been demonstrated to help reduce the risk of developing chronic degenerative diseases [4].

Known biological activities include antioxidant, anti-inflammatory, antimicrobial, immunomodulatory and cardioprotective effects. Some bioactive molecules have also demonstrated neuroprotective and anticarcinogenic properties. Their properties depend on their chemical structure, dosage and bioavailability [5].

Bioactive molecules found in plants can be classified into three main groups: alkaloids, terpenes and phenolic compounds [6]. Alkaloids are characterized by the presence of nitrogen and an alkaline pH, generally featuring a heterocyclic structure [7]. The most widely employed classification of alkaloids is as follows: the first category comprises true alkaloids, including atropine, nicotine, and morphine; secondly, the term "protoalkaloids" is defined as adrenaline and ephedrine; thirdly, the term

"pseudoalkaloids" refers to caffeine, theobromine, and theacrine [8]. Alkaloids are commonly used in therapy and pharmacology, as they possess various biological properties, including anti-inflammatory, antidepressant, anticancer, antiviral, and antihypertensive activities, as well as antimicrobial and antimalarial effects [9].

Another class of bioactive molecules found mainly in plants is terpenes. They can be classified into different categories based on the number of carbon atoms and the presence of isoprene (IPR) units [10].

The most prominent class of compounds within this category are carotenoids and oxocarotenoids (xanthophylls), which are classified as tetraterpenes are comprised of 40 carbon atoms or eight IPR units. Carotenoids, such as β-carotene, lutein, lycopene and zeaxanthin, are fat-soluble coloured pigments found in vegetables and fruit, giving them a yellow, orange and even red colour [11].

Phenolic compounds are a large class of bioactive molecules mainly found in plants, characterized by the presence of one or more aromatic rings linked to hydroxyl groups (-OH). These structures give phenolic compounds strong antioxidant activity and the ability to interact with various biological targets [12]. In fact, their structural diversity gives these compounds different biological effects. In plants, they serve defense functions against environmental stress; in humans, they are associated with the modulation of inflammatory processes, protection from oxidative stress, and support for cardiovascular health. These effects make such compounds particularly relevant for nutraceuticals [13].

In addition to producing bioactive molecules such as alkaloids, terpenes and phenolic compounds, plants synthesise a wide range of secondary metabolites. Most of these compounds are not essential for plant survival but play fundamental ecological roles, such as defence against pathogens, attraction of pollinators and environmental adaptation, and can have significant effects on human health, contributing both to the prevention and treatment of various diseases [14].

These metabolites include glucosinolates, saponins and pigments. Pigments can be divided into two main groups: fat-soluble (see section on terpenes) and photosynthetic ones [15].

Photosynthetic pigments are molecules that capture light energy and convert it into chemical energy [16]. The main pigments are chlorophylls and carotenoids. The former ones absorb blue and red light and reflect green light, while carotenoids (carotenes and xanthophylls) absorb other wavelengths and protect against photo-oxidation. In some organisms, such as red algae and cyanobacteria, phycobilins (phycocyanin (PC) and phycoerythrin) are also found [17].

They forward the energy of the harvested light to chlorophylls for photosynthesis. Therefore, they serve as "secondary light harvesting pigments" [18]. Scientific interest in these pigments is constantly growing thanks to their recognised biological activities with beneficial effects on human health [19]. PC is a phycobiliprotein (PBP) found mainly in Spirulina and other microalgae, and is considered a bioactive molecule with several potential benefits for human health, like antioxidant, anti-inflammatory, antimicrobial, antimelanogenic and anti-cancer properties [20].

1.1 Phenolic Compounds

Phenolic compounds represent the most widespread class of secondary metabolites in the plant kingdom. They are mainly produced via the shikimate pathway from L-phenylalanine and L-tyrosine and contain one or more hydroxyl groups directly attached to the aromatic ring. Secondary metabolites originate from primary metabolites, such as carbohydrates, amino acids and lipids. They serve protective functions both against ultraviolet (UV) rays and against pathogens such as viruses, bacteria, and insects, and are responsible for the smell, color, and taste of plant products [21]. In recent years, the multiple biological activities of phenolic compounds against various diseases and disorders have received particular attention. The several health benefits of phenolic compounds include anti-cancer, antithrombotic, antiulcer, antiarteriogenic, antiallergic, anti-inflammatory, antioxidant, immunomodulatory, antimicrobial, cardioprotective, and analgesic effects [22].

Chemically, phenolic compounds can be classified according to the number of carbon atoms in their molecules [23], as shown in Table 1.

Structure	Class
C_6	simple phenolics
C_6 - C_1	phenolic acids and aldehydes
C_6 - C_2	acetophenones and phenylacetic acids
C_6 - C_3	cinnamic acids, coumarins
C_{15}	Flavonoids
C_{30}	biflavonyls
C_6-C_1-C_6, C_6-C_2-C_6	benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	quinones
C_{18}	betacyanins
Lignin	polymers
Tannins	oligomers or polymers

Table 1: Phenolic compounds classification according to literature [23]

Phenolic compounds can also be classified based on their origin, as synthetic or natural [24]. A more detailed description is reported in Fig. 1.

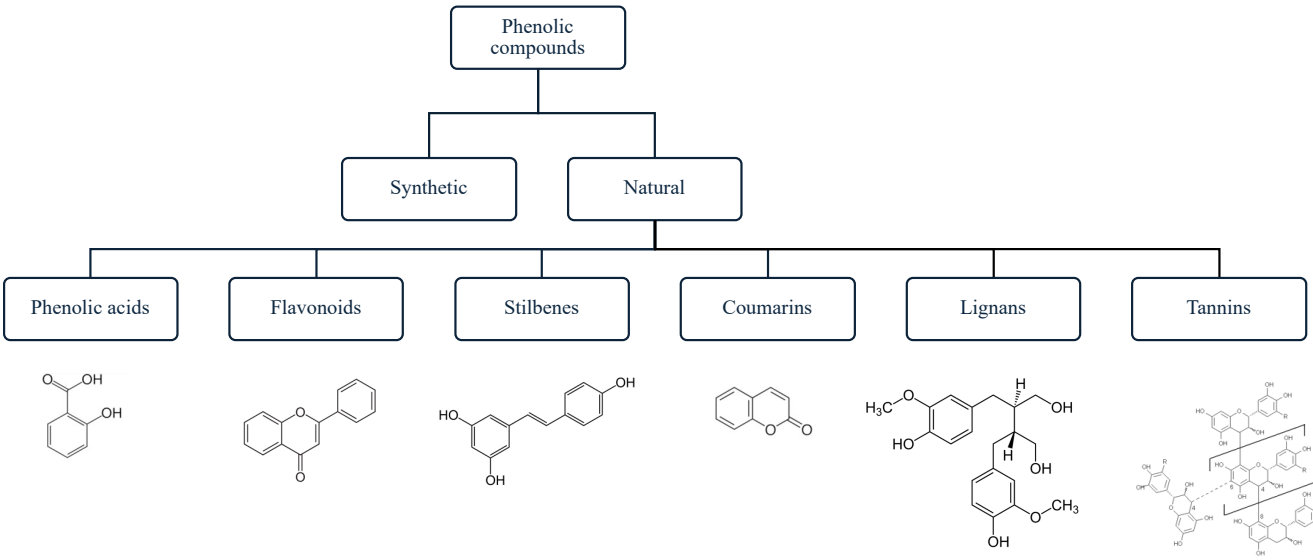

Fig. 1: Classification of phenolic compounds

Phenolic acids: The term "phenolic acids" generally describes phenolic compounds that have a carboxylic acid group. Phenolic acids are one of the main classes of plant phenolic compounds and are mainly divided into two subgroups: hydroxybenzoic acid (Fig.2) and hydroxycinnamic acid (Fig.3) [25].

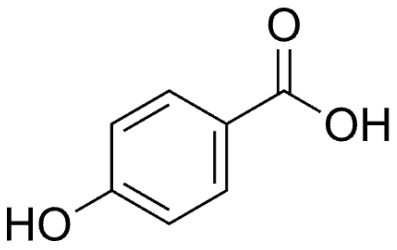

Fig. 2: Hydroxybenzoic acid

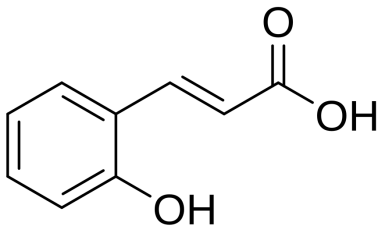

Fig. 3: Hydroxycinnamic acid

Flavonoids: They belong to a class of low-molecular-weight phenolic compounds that are widely distributed throughout the plant kingdom. Most floral pigments in angiosperm families belong to this class. However, flavonoids are not found only in flowers, but also in all parts of the plants [26]. They are known for their antioxidant, anti-inflammatory, antimutagenic, and anticancer properties. They are also capable of modulating key cellular enzymatic functions. Flavonoids can be divided into several subgroups depending on the carbon of the C ring to which the B ring is attached, the degree of unsaturation, and the oxidation of the C ring. Flavonoids in which the B ring is attached at position 3 of the C ring are called isoflavones. Those in which ring B is bonded at position 4 are called neoflavonoids, while those in which ring B is bonded at position 2 can be further divided into several subgroups based on the structural characteristics of ring C. These subgroups are: flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalcones (see Fig. 4) [27].

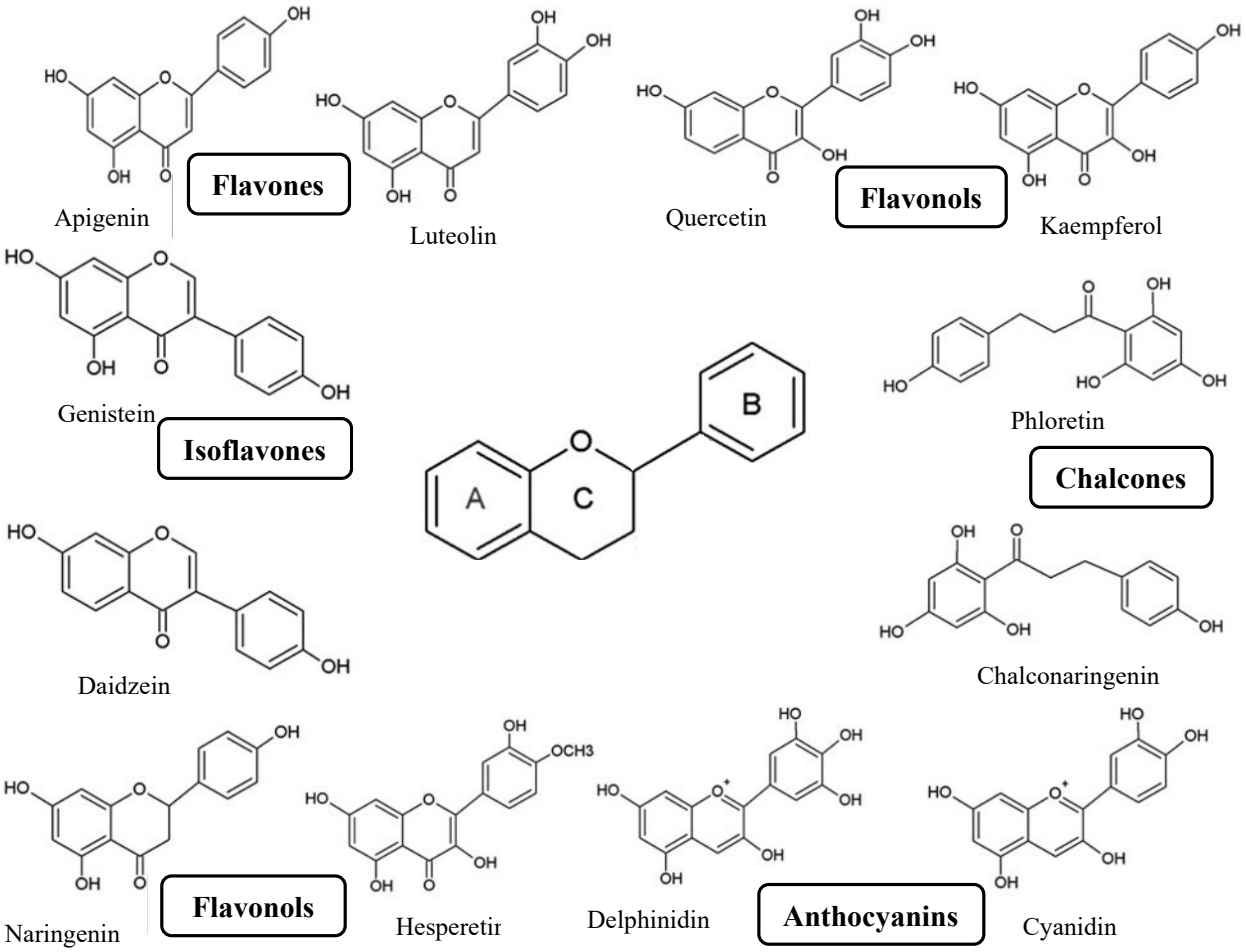

Fig. 4: Classification of flavonoids

Stilbenes: Plants synthesize polyphenolic substances called stilbenes to defend themselves against pathogens, bacterial and fungal proliferation, and to protect themselves from the harmful effects of UV rays. They are characterized by a carbon skeleton of 1,2-diphenylethylene (C6–C2–C6), consisting of an ethylene group at the center of two benzene rings [28]. Among stilbenes, the best known compound is resveratrol, which is particularly valued for its anti-inflammatory medicinal properties [29]. Resveratrol structure is reported in Fig. 5.

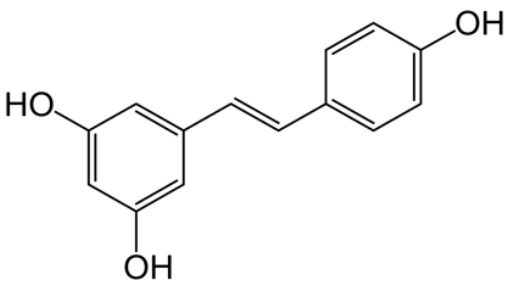

Fig. 5: Classification of flavonoids

Coumarins: These are secondary metabolites that occur naturally in various plant families and essential oils. The basic nucleus of coumarin corresponds to the compound benzo-a-pyron (2H-1-benzopyran-2-one) (Fig. 6). Among the various therapeutic effects that have been identified, it has been discovered that they have antimicrobial, antiarrhythmic, anti-HIV and antineoplastic activity [30].

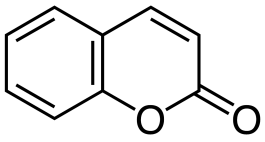

Fig. 6: 2H-1-benzopyran-2-one structure

Lignans: They consist of two propylbenzene units linked by a β, β bond [31]. Lignans can be classified into eight structural subgroups based on how oxygen is incorporated and the cyclization pattern. These subgroups include dibenzylbutyrolactol, dibenzocyclooctadiene, dibenylbutyrolactone, dibenzylbutane, arylnaphthalene, aryltetralin, furan and furofuran (Fig. 7) [32]. Lignans, like most phenolic compounds, also exhibit various biological properties, including anti-inflammatory, antioxidant, and anti-cancer activities. In addition, some studies have highlighted the ability of these compounds to reduce the risk of cardiovascular diseases [33].

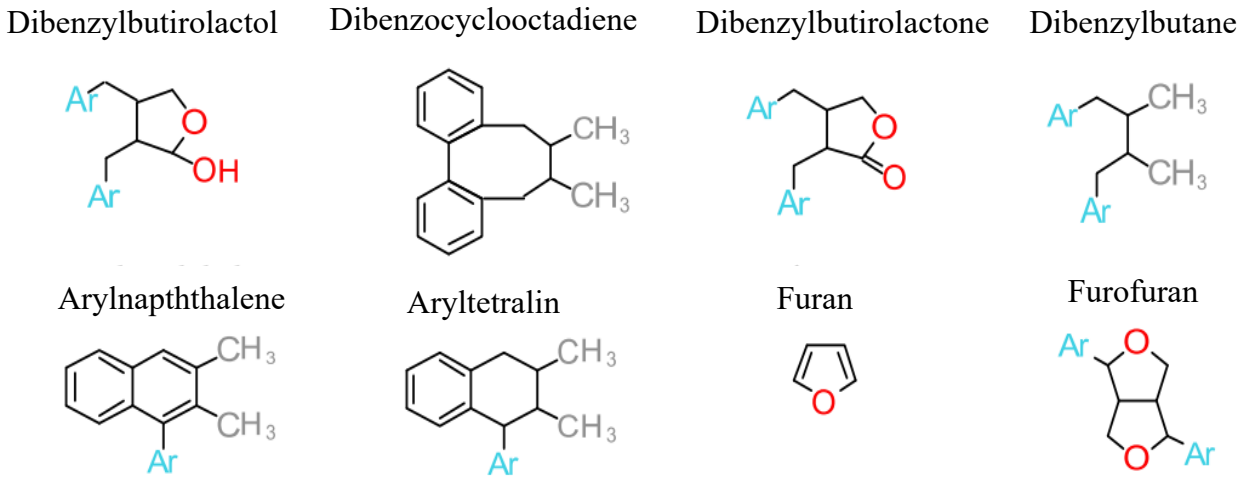

Fig. 7: Classification of Lignans

Tannins: they have been defined as water-soluble phenolic compounds with a molecular weight ranging from 500 to 3000 Da [34]. Indeed, tannins are to be classified into two distinct categories: hydrolysable and non-hydrolysable (see Fig. 8). Hydrolysable tannins contain a central nucleus of polyvalent alcohol such as glucose and hydroxyl groups, which are partially or totally esterified by gallic acid (gallotannins) or hexahydroxyphenyl acid (ellagitannins). Condensed (non-hydrolysable) tannins are structurally more complex than the hydrolysable ones; their complete structure has yet to be determined. They are mainly polymerized products of flavan-3-ols and flavan-3,4-diols, or a mixture of both of them [35].

From a nutritional point of view, tannins have been considered "antinutrients," meaning they are poorly utilized by the body. In fact, tannis form complexes with proteins, starch, and digestive enzymes, leading to a reduction in the nutritional value of foods [36].

These compounds are still used in the pharmaceutical field due to their bactericidal properties. They react irreversibly with proteins, forming complexes within bacterial membranes and neutralizing their activity. Tannin-based drugs for the treatment of intestinal infections have been available on the market for quite some time [37].

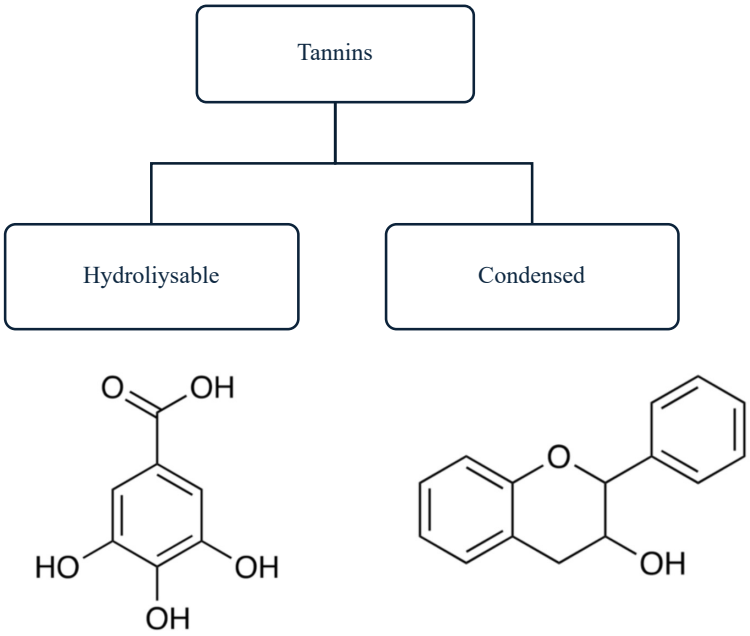

Fig. 8: Classification of tannins

1.2 Phycocyanin

PC is classified as a PBP, which is defined as a protein bound to a chromophore known as a phycobilin. PBPs are water-soluble; in fact, they are not present within the membrane, but rather cluster in structures called "phycobilisomes" (PBS). The core of PBS is composed of allophycocyanin, while the rods are composed of PC and phycoerythrin (PE) [38], as shown in Fig 9. PBS forward the energy of the harvested light to chlorophyll for photosynthesis. Therefore, they serve as "secondary light harvesting pigments" [18].

The presence of PC has notably been observed in cyanobacteria and eukaryotic algae.

PC is classified into distinct categories, which are C-PC (obtained from cyanobacteria), R-PC (obtained from red algae) and R-PCII (obtained from *Synechococcus* species) [39].

Structurally, PC consists of two polypeptide chains. The former is the alpha chain and it is composed of 162 amino acids and one phycobilin, specifically phycocyanobilin. The other is the β one and is composed of 172 amino acids and two phycocyanobilins. These monomers aggregate to form trimers and hexamers. PC has a total molecular weight of approximately 220-230 kDa [40].

Scientific interest in PC is constantly growing thanks to their recognised biological activities with beneficial effects on human health [41]. As explained by Wu et al., PC shows great anti-inflammatory, anti-platelet, anti-cancer, nephroprotective and hepatoprotective properties that may be explained, at least in part, by its antioxidant activity [42].

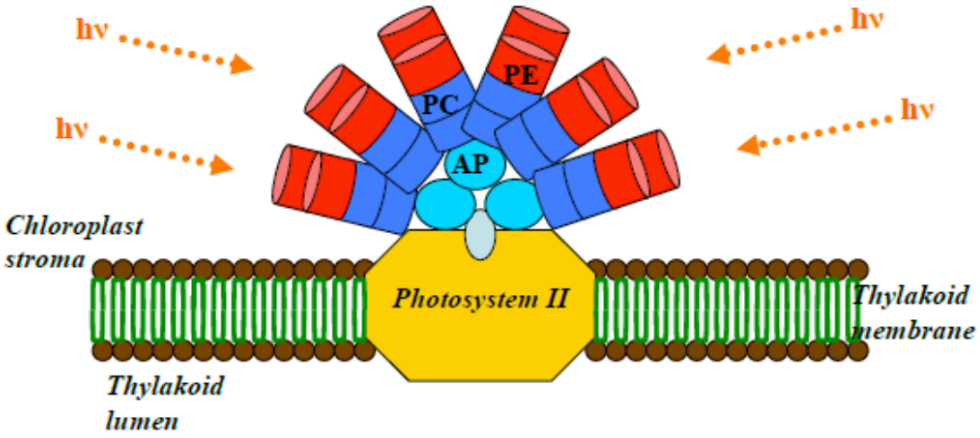

Fig. 9: PBS organization in Arthrospira platensis (Spirulina) [43]

CHAPTER 2

2.1 Green extraction techniques

The definition of green chemistry originated in the early 1990s [44] and is generally described as the "design of chemical products and processes aimed at reducing or eliminating the use and generation of hazardous substances" [45]. This definition has been adapted in relation to the green extraction of bioactive molecules as follows: "Green extraction is based on the discovery and design of extraction processes that reduce energy consumption, allow the use of alternative solvents and renewable natural products, and ensure a safe, high-quality extract/product" [46]. In recent decades, a growing number of researchers have adopted the term "green extraction" to describe an approach inspired by the principles of green chemistry. Green extraction of natural products aims to develop processes that minimize energy consumption and eliminate the use of toxic organic solvents, while still ensuring high extraction yields, safety, and extract quality [47].

2.1.1 The six Principles of green extraction

To describe more precisely the green extraction techniques, Chemat, et al. [46], proposed six main principles, that are reported below and represent innovative examples to follow.

- Principle 1: Innovation through the selection of varieties and the use of renewable plant resources.
- Principle 2: Use alternative solvents, primarily water or agro-solvents.
- Principle 3: Reduce energy consumption through energy recovery and innovative technologies.
- Principle 4: Produce co-products instead of waste through bio- and agro-refining industry.
- Principle 5: Reduce the number of unit operations and favour safe, robust and controlled processes.
- Principle 6: Aim for a non-denatured, biodegradable extract that is free from contaminants.

Adopting the principles of green extraction is a crucial step towards more sustainable and efficient processes. The aim is to ensure the responsible use of natural resources and the production of high-quality bioactive extracts.

2.1.2 Extraction techniques for recovery of bioactive molecules

The interest in natural bioactive molecules is steadily increasing, as they find applications in numerous fields, such as the food and nutraceutical industries, as well as the to the pharmaceutical and cosmetic sectors. To obtain these compounds, it is essential to adopt extraction methods that ensure their integrity and preserve their biological functionality [48].

Conventional techniques represent the most used methodologies for the recovery of bioactive compounds from various matrices, including plant, animal, and microbial sources. They are based on the principles of solubilization and mass transfer, generally using organic or aqueous solvents, but equire a large volume of solvents and a long extraction time [49]. The conventional extraction methods include [50]:

- Maceration: The powdered plant is then permitted to soak in a suitable solvent in a closed container at room temperature. In some cases, it may be necessary to periodically or continuously agitate the preparation to enhance the extraction process. When the concentration of metabolites in the extract and the plant material are equal, the extraction ultimately occurs. Usually the solid material is re-extracted [51].
- Percolation: it is a technique that works at room temperature. A percolator is made up of a narrow and cone-shaped vessel open at both ends. The matrix is placed inside a thimble holder and the solvent is allowed to percolate through it. The solvent extraction mixture is re-circulated in the system using a pump [52].
- Soxhlet extraction: the Soxhlet extractor consists of three parts, which are the percolator, the thimble, and the siphon (Fig.10). The plant powder is placed in cellulose thimble in an extraction chamber. A flask containing the chosen solvent is placed under the extractor and connected to a condenser placed above. The solvent is subjected to an increase in temperature, thereby initiating the process of evaporation. The vapors are then transported through a lateral tube, ultimately reaching the condenser. There, they cool and fall back as a liquid into the

chamber containing the sample. The chamber gradually fills with hot solvent, which solubilizes the matrix compounds. After numerous cycles, the solvent in the flask gradually becomes enriched with the extracted bioactive compounds. Finally, the solvent is removed by evaporation or rotavaporation, leaving the dry residue of the extract [53]. It's a relatively simple technique but consumes large amounts of solvents. It's a lengthy process that requires constant heating, with the risk of degradation for thermolabile molecules [54].

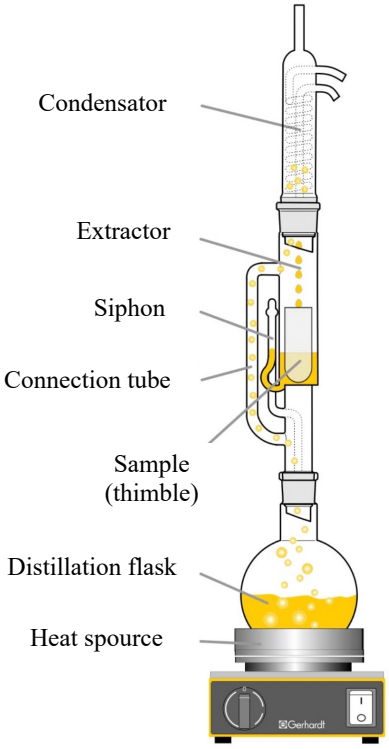

Fig. 10: Soxhlet extractor

- Reflux extraction: is an analytical apparatus that works based on the principle of condensation, as with the Soxhlet extraction. Unlike Soxhlet extraction, the matrix is directly immersed in the solvent inside the extraction flask. The solvent evaporates, condenses, and falls back into the same flask, without passing through a siphon system [55]. The sample is constantly in contact with the same volume of boiling solvent (Fig.11).

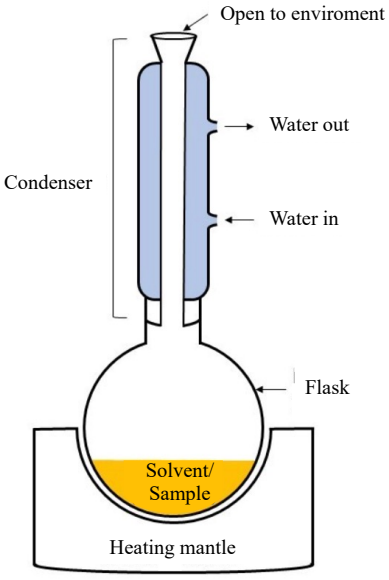

Fig. 11: Reflux

- Decoction: This procedure is suitable for extracting water-soluble, heatstable constituents. The plant material is generally broken into small pieces and boiled with water. Then, the extract is cooled, filtered and collected [56].

However, despite their effectiveness, conventional techniques have several limitations in terms of sustainability, efficiency, and selectivity. The critical issues associated with traditional methods include the use of large quantities of organic solvents, high energy consumption, and the risk of degradation of thermolabile compounds. Moreover, due to the growing interest in safer and more environmentally friendly processes, these drawbacks of conventional techniques have highlighted the need for more modern and eco-compatible approaches, capable of maximizing extraction yield while preserving natural resources [57].

For this reason, more innovative extraction techniques are now used, as described below:

- Microwave Assisted Extraction (MAE): the MAE technique involves exposing a sample to microwave energy, which causes alterations in the biomass cells. These alterations facilitate the release and extraction of the desired compounds from the sample matrix [58]. Microwave

devices consist of four main components, which are the magnetron, the waveguide, the applicator (containing the sample), and the circulator. Two categories can be distinguished: multimode or single-mode systems. The primary distinction between these two systems lies in their ability to support multiple electromagnetic modes. The electromagnetic field associated with the multimode system is distributed irregularly, exhibiting areas of various intensity. On the other hand, the single-mode system supports a single electromagnetic mode. In fact, the sample is exposed to a single magnetic field and it provides greater reproducibility and uniformity of extraction. The advantage of using a multi-system is that it allows to process more or larger samples, but with less uniform heating [59].

- Ultrasound Assisted Extraction (UAE): UAE is based on the use of high-frequency sound waves (typically ranging from 20 to 100 kHz) to facilitate the release of bioactive compounds from a solid matrix into a solvent. Ultrasonic waves generate cycles of compression and rarefaction in the liquid, forming cavitation microbubbles. The collapse of these bubbles produces shockwaves, and particle collisions cause the rupture of the cellular structure, facilitating mass transfer. This technique ensures an increase in both the extraction rate and yield [60].
- Supercritical Extraction (SC): SC is a green technique that exploits the unique properties of supercritical fluids. Fluids in supercritical conditions combine the characteristics of both gases and liquids. Like gases, they are characterized by low viscosity and high diffusivity, which facilitates their permeation through solid matrices. Nevertheless, due to their high density—comparable to that of liquids—they exhibit effective solubilization of target molecules. The most commonly used fluid is CO_2. [61]. Supercritical CO_2 is pumped through a tank containing the matrix and extracts the target compounds. The bioactive compounds dissolve in the fluid. The mixture is then depressurized and the CO_2 returns to the gaseous phase. The extracted compounds precipitate and are collected in solvent-free form [62]. Using CO_2 as a solvent offers several advantages: it is non-toxic, inexpensive, readily available, and has a

relatively low critical point (31.1°C, 73.8 bar). Under these conditions, CO_2 becomes an excellent solvent for non-polar or slightly polar compounds (lipids, terpenes, essential oils). To extract more polar compounds, modifying cosolvents, typically ethanol (EtOH) or water are used to increase the fluid's polarity [63].

- Pressurized Liquid Extraction (PLE): PLE is an extraction method that works at high temperatures and pressures using conventional solvents such as water and EtOH. The sample is placed inside an extraction cell. Then, the cell is filled with the extraction solvent and subjected to high pressure and temperatures. Under these conditions, the solvent exhibits reduced viscosity and penetrates the solid matrix more easily. The increased diffusivity accelerates mass transfer and an higher solubilization capacity for many bioactive compounds. Thus, the extraction rate is improved and extraction time is reduced [64].
- Pulsed Electric Fields (PEFs): It is a non-thermal technique that works at low temperatures and the release of bioactive compounds from biological matrices is caused by high-intensity electrical impulses. The system is composed of a high-voltage pulse generator, treatment chamber, fluid-handling system, and monitoring device. The sample, suspended in conductive medium, e.g. water or buffer, is placed between two electrodes that generate high-intensity pulses. These electric pulses cause membrane permeabilization (electroporation), allowing the release of metabolites [65]. The main advantage of this technique is that, by not using heat, it allows for better preservation of heat-sensitive components such as pigments. In fact, this technique is used for the extraction of thermolabile pigments [66].

Innovative methods are replacing traditional methods, because they have several limitations: they require large quantities of organic solvents (which are often toxic), very long extraction times, and in some cases high temperatures that can degrade sensitive molecules [57].

2.1.3 Green strategy for recovery of bioactive molecules

Innovative extraction techniques have become essential tools for implementing the principles of green extraction, providing more efficient processes with a reduced environmental impact [67]. To comply with the principles of green chemistry, it is crucial not only to replace toxic solvents and reagents but also to adopt miniaturization approaches, as both strategies can be applied universally without the need for costly or radical changes to laboratory infrastructure [68].

Miniaturization

The “green” approach to sample preparation also depends on the amount of matrix used and the volumes of reagents employed, as these factors directly influence waste production. Although the goal of “zero waste” is still far from being achieved in most analytical procedures, significant progress has been made in recent years thanks to the miniaturization of individual process steps, especially in sample preparation [69]. Miniaturized techniques also offer significant advantages in terms of portability and automation, making them more versatile than traditional methods. In addition, they are faster to use, which helps reduce operators' exposure to potentially hazardous substances [70]. However, the decision to use a miniaturized approach must be consistent with the purpose of the analysis. In the case of contaminant detection, these techniques have proven to offer efficient analytical results. On the contrary, when the objective is the quantitative extraction of compounds, such as the recovery of bioactive molecules from food waste, miniaturization is not an adequate solution. Miniaturized methods are divided into liquid-phase and solid-phase approaches. Among the most widely used are: Dispersive Liquid-Liquid Microextraction (DLLME), Hollow Fiber Liquid Phase Microextraction (HF-LPME), Solid Phase Microextraction (SPME), Stir Bar Sorptive Extraction (SBSE), Microextraction By Packed Sorbent (MEPS), and Single Drop Microextraction (SDME) [71]. Furthermore, although less widespread, the miniaturized Matrix Solid Phase Dispersion (MSPD) method [72]. has also been successfully applied to the analysis of numerous compounds, including secondary plant metabolites [73].

Green solvents

Another fundamental aspect of green chemistry is the use of green solvents. These are solvents that have one or more of the following properties: low or no volatility; non-flammability; no risk of inhalation (i.e. they are non-toxic and non-carcinogenic); recyclability; and biodegradability [74].

Water is considered the most natural solvent, and many researchers regard it as the most environmentally friendly in chemistry, from both experimental and industrial perspectives [67].

As an alternative, other solvents can be used, including micellar solvents, which are a convenient option thanks to their negligible toxicity, volatility and flammability. Micellar media are based on the formation of micelles through the addition of surfactants, amphiphilic molecules, to an aqueous solution. Micellar systems are mainly used in food analysis for the extraction and purification of proteins and enzymes, the simultaneous extraction of oils and proteins, and the development of effective antioxidants [75]. Another category of green solvents is bio-based solvents. These solvents can be classified according to the material they originate from: lignocellulose; sugars and starches; proteins and oils; or other forest or food residues. Alternatively, they can be classified according to the petrochemical solvent they are designed to replace. Bio-based solvents have proven particularly effective in extracting biomolecules from food matrices, such as fatty acids from oilseeds [76], caffeine from green tea leaves [77], and the peroxidase enzyme from bitter melon samples [78]. Ionic liquids (ILs) are generally defined as non-molecular solvents obtained by combining organic cations and organic or inorganic anions. They have melting points below 100 °C. Due to their extremely low vapour pressure, they are considered a greener alternative to traditional Volatile Organic Compounds (VOCs) [79]. Extraction systems based on ILs are widely used for extracting carbohydrates [80], organic acids (including lactic acid (LA) and polyketones) [81,82]. The disadvantages associated with ILs (the use of various reagents, volatile organic solvents, and the generation of by-products and waste) have stimulated the development and use of another class of solvents, Deep eutectic Solvents (DESs), which are considered greener and very promising alternatives in the field of extraction [83].

2.2 Green extraction solvents

2.2.1 Deep eutectic Solvents: DESs

The concept of "deep eutectic solvent" was first proposed by Abbott et al., in 2002 [84]. DESs are classified as a new class of mixtures, liquid at room temperature, that exhibit interesting solvent properties. DESs are a class of solvents formed by the interaction of two or more components, resulting in a stable liquid mixture with melting points markedly lower than those of the individual constituents [85].

They are composed of a Hydrogen Bond Acceptor (HBA) and a Hydrogen Bond Donor (HBD), which interact through hydrogen-bond formation in an appropriate molar ratio [86].

When both components are primary metabolites, such as amino acids, organic acids, sugars, or choline derivatives, they are referred to as Natural Deep Eutectic Solvents (NADESs) [87].

Fig. 12 shows the main constituents of DESs.

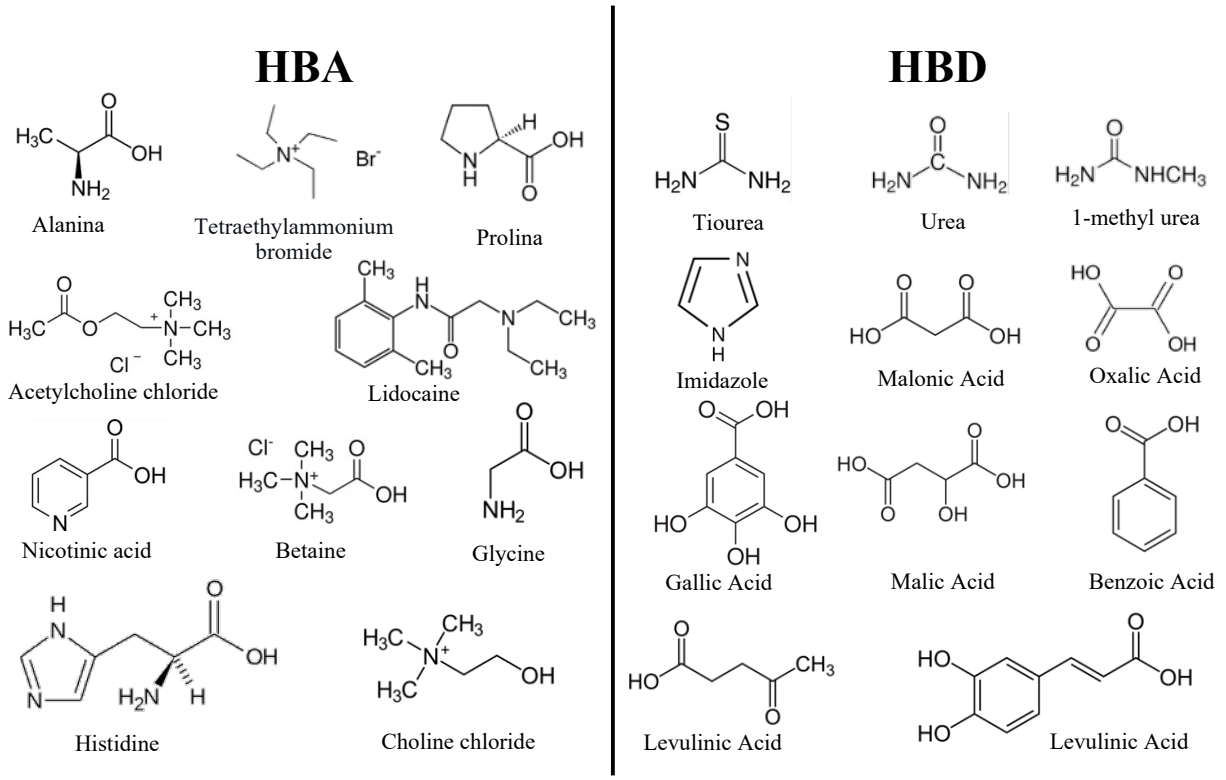

Fig. 12: This table shows the main HBAs and HBDs

In general, two main methods are employed for the synthesis of DESs: the heating method and the grinding method. The heating method, which is the most widely used, involves mixing the components and heating them to approximately 100 °C under continuous stirring until a homogeneous liquid is obtained. Alternatively, the grinding method consists of mixing the compounds at room temperature and grinding them in a mortar with a pestle until a clear liquid forms [88].

Gutierrez et al. [89], introduced an alternative synthesis method that involves the freeze-drying of aqueous solutions of the individual DES components. Separate aqueous solutions of the components are then prepared. These solutions are frozen, freeze-dried, and clear viscous liquids is obtained. However, water volumes detected in the freeze-dried mixture cannot be removed.

The formation of hydrogen bonds between the HBA and HBD components is central to the process, regardless of the method used for DES synthesis, resulting in the formation of a deep eutectic mixture [90]. The Fig.13 shows the formation of two different DESs from a combination of HBD and HBA. The dotted lines represent the hydrogen bonds that form between the chloride anion (Cl^-) of Choline Chloride (ChCl) and the hydroxyl groups (-OH) of the donor molecules, such as Ethylene glycol (EG) or Glycerol (Gly).

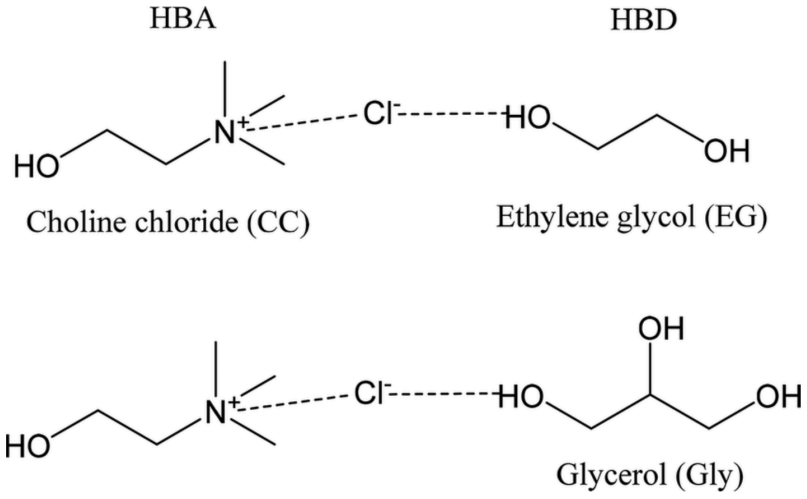

Fig. 13: Shows the interaction between HBD and HBA in two different DES.

The addition of water to this mixture is possible, but it requires some key considerations. Water can act both as a HBA an HBD due to its high polarity. Therefore, it can interact with the DES constituents. In fact, DES can be prepared using either the hydrated forms of their components, such as salts or hydrated acids, or the non-hydrated forms. When eutectic solvents are formed without hydrated components, water can be added and is assumed to become part of the solvent itself through stronger intermolecular hydrogen-bond interactions [91].

In addition to being green and environmentally friendly, DESs show a great ability to tune their composition and the nature of their components, resulting in mixtures with different physicochemical properties. These characteristics directly influence the applicability of DESs and the extraction efficiency across various extraction methods. These physicochemical properties depend on the interactions between the HBA and HBD [92]. Therefore, the nature and molar ratio of these components significantly influence the characteristics of the resulting mixtures.

As mentioned above, an eutectic mixture refers to a combination of two or more components that melts at a temperature lower than that of the individual pure components. The Fig.14 shows a general solid–liquid phase diagram of a binary mixture. Tm(A) and Tm(B) represent the melting temperatures of the two compounds A and B, respectively. The eutectic point represents the composition, it also represents the minimum melting temperature at which the melting curves of both compounds' melting curves meet at this point [93].

Therefore, the components of DESs interact through hydrogen bonds, and these interactions significantly lower the melting point compared to the pure components, resulting in a stable liquid at room temperature [93, 94].

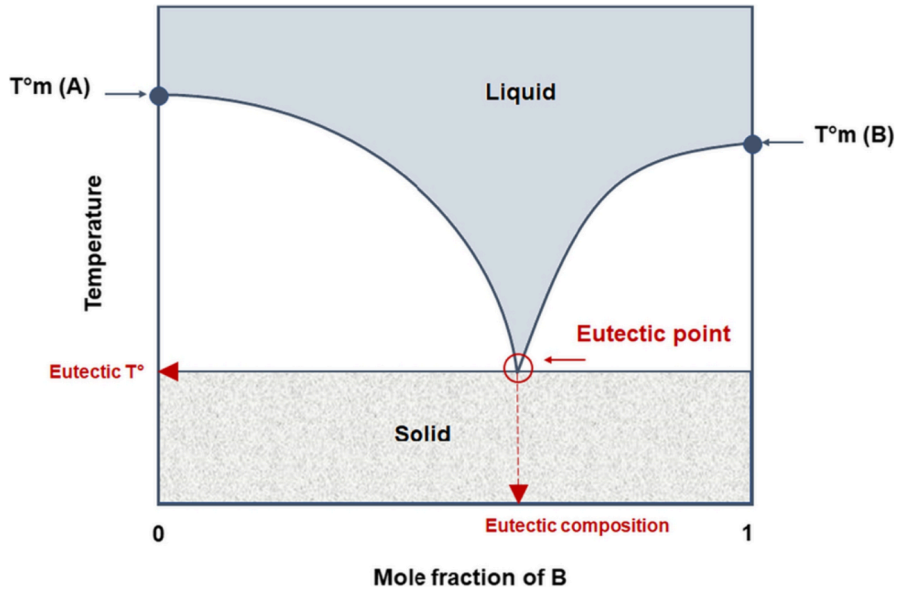

Fig. 14: Solid–liquid phase diagram of a binary mixture [93]

According to Martins et al. [95], the term "deep eutectic solvent" should be applied exclusively to mixtures with a melting point lower than the ideal eutectic temperature; otherwise, DESs could not be defined as 'deep' and would not be distinguishable from other mixtures. The melting points of most DESs generally range between –69 and 149 °C, but in all cases they are lower than 150 °C [92]. The nature and the molar ratio of the mixture components influence this characteristic. The preparation method can also affect the freezing point value, but not the eutectic composition, which must remain unchanged regardless of the method used [93].

In some articles, it has been observed that the lower the molecular weight of the HBD, the greater the freezing-point depression [96]. Some studies have shown that DESs composed of the same HBA exhibit variations in their melting points when different HBDs are employed [97].

Abbott et al. [94], synthesized several DESs using ChCl as the HBA and oxalic acid, malonic acid, and succinic acid as HBDs, which exhibited eutectic temperatures of 34 °C, 10 °C, and 71 °C, respectively. The authors attributed these differences to the distinct molecular structures of the HBDs.

Another important chemical-physical characteristic of DESs is represented by the viscosity. The resistance of a liquid to deformation at a given rate is described by its viscosity. This means that a

liquid flows easily when its viscosity is low. Higher viscosities, on the other hand, result in slower fluid flow [98]. Most DESs exhibit high viscosity due to the presence of numerous hydrogen bonds, as well as van der Waals and electrostatic interactions between their components. This characteristic represents a drawback for the use of DESs as extraction solvents, as it limits mass transfer and reduces extraction efficiency [99]. The viscosity of DESs is influenced by several factors, including the choice of HBD; DESs with small-sized HBDs exhibit lower viscosity [100]. The HBA/HBD ratio and the addition of water also significantly affect this parameter. Numerous studies have shown that the addition of water to the mixture reduces its viscosity. Dai et al. [101], demonstrated that the addition of water significantly reduced the viscosity of NADES; after adding 25% water (v/v) to glucose–ChCl–water and 1,2-propanediol–ChCl–water mixtures.

In general, viscosity decreases with increasing temperature, as a consequence of the increased molecular kinetic energy in the liquid and the reduction of the average intermolecular forces. Therefore, preheating is a simple and effective technique that can be employed to reduce viscosity prior to processing [102, 103].

In addition, the density of DESs depends also on the molar ratio and the combination of HBA and HBD. Shahbaz et al. [104], demonstrated that in DESs composed of ChCl and citric acid, density decreases with increasing amounts of ChCl.

Thanks to their easy preparation, low production costs, and low toxicity, DESs have attracted considerable interest across numerous application fields. One of the most prominent areas is their use as extraction solvents for bioactive compounds from natural matrices [105]. The extraction efficiency of DESs arises from the ability to modulate their physicochemical properties by varying the nature and molar ratio of the HBA and HBD. This flexibility allows the formation of mixtures with specific characteristics, capable of solubilizing a wide range of primary and secondary metabolites, including polyphenols, alkaloids, flavonoids, pigments, and proteins. Furthermore, DESs exhibit high compatibility with biological matrices and the potential to replace toxic organic solvents, reducing environmental impact and improving process safety [106].

However, the practical application of this technique is sometimes limited by the high viscosity of DES, which hinders the diffusion of solutes and reduces extraction efficiency [107]. As we mentioned earlier, one way to reduce viscosity is to increase the temperature. However, the applicability of this strategy involves higher energy consumption and depends on the thermal stability of the target analytes, which may undergo thermal degradation [108]. Alternatively, the addition of water to DESs represents an effective strategy to reduce viscosity, enhancing mass transfer and improving extraction efficiency. Water also affects other physicochemical properties of DESs, such as conductivity, polarity, and melting point. For these reasons, water content is a critical parameter to control during the extraction method development [109]. However, an excessive amount of water can compromise the DES structure by weakening the interactions between its components [110].

Another significant limitation of these mixtures as extraction solvents is the difficulty in recovering the compounds extracted from eutectic mixture. DESs, due to their low vapor pressure, cannot be removed by evaporation as is the case with organic solvents [111].

Della Posta et. Al. [112], reported the main strategies that can be employed for the recovery of bioactive compounds from DESs: the use of macroporous resins, the anti-solvent method, Solid Phase Extraction (SPE), the back exctracion method.

In 2013, Dai et al. [113], were the first group to attempt to evaluate a strategy for the recovery of hydroxyaflor yellow A and cartormin. carthamin, and tri-p-coumaroylspermidine from *C. tinctorius* (Asteraceae). L. (Asteraceae) from DES extracts using a **macroporous resin**.

The resins used for the recovery of analytes from DESs are porous solid materials capable of selectively adsorbing the analytes present in the matrix. These resins can be differentiated based on their polarity, chemical composition, specific surface area and pore diameter, characteristics that determine their adsorption efficiency and selectivity. In the separation process, the solution containing the DES and the analytes is passed through the column filled with the resin. The analytes are retained on the surface of the adsorbent material by chemical or physical interactions, while the DES passes through the resin and can be recovered later. Finally, the adsorbed analytes are eluted

from the resin using an appropriate solvent, which allows them to be released and collected for subsequent analytical steps [114].

The principle behind the **anti-solvent method** of recovering analytes from DESs is to add a large quantity of water, which breaks the bond between the HBA and HBD, thereby causing the DES to lose its properties and the desired compounds to precipitate. The anti-solvent used in the procedure must selectively solubilize the DES components or target analytes. The most common used anti-solvent is water. The first application of the anti-solvent method as a possible method to recover bioactive molecules from DESs dates back to 2015 [115].

Several studies employing this method, generally considered simple and economical, have been reported in the literature. [116, 117]. However, the efficiency of the process depends on several factors, including the type of DES used, the nature of the solvent chosen, the volume and ratio of the two phases, and the mixing time and intensity.

SPE involves the use of a cartridge containing a solid material. This material interacts with the analytes present in a liquid sample. The SPE method is a sample pretreatment technique that is widely used for the purification and isolation of analytes of interest.

Nam et al., e Jeong et al., apply the SPE method for the recovery of bioactive molecules from a DES extract [115,118]. In both cases, the method guaranteed excellent recovery yields in percentage terms: 90-100%.

The **back-extraction method** uses a biphasic system solvent that can extract analytes using DES. When the DES is diluted with a certain amount of water, the success of the back-extraction step depends on the selection of a solvent that is immiscible with the aqueous phase.The solvent selected as the back-extractant must be able to analyze the analytes of interest. For the first time, Xu et al. [119], attempted to recover analytes of interest from a DES extract using a back-extraction method.

2.2.2 Supramolecular Deep eutectic Solvents: SUPRADESs

Supramolecular Deep eutectic Solvents (SUPRADESs) represent an evolution of traditional DESs, characterized by the integration of supramolecular interactions, such as multiple hydrogen bonds,

π-π interactions, and electrostatic interactions, within the solvent structure. This is a particular class of DESs that incorporate cyclodextrins (CDs), host molecules capable of forming inclusion complexes with different analytes [120].

The integration of the properties of DESs and CDs gives these systems high application potential in industrial and laboratory settings, as confirmed by recent studies [121,122]. SUPRADESs are a relatively recently discovered class of solvents; the first publications reporting their use date back to 2020 [120]. Consequently, the information currently available on their physicochemical properties and potential applications is still limited. They certainly offer many of the advantages of traditional DES, such as low toxicity, biodegradability and ease of preparation. Furthermore, they offer the opportunity to develop "tailor-made" solvents thanks to the versatility of supramolecular interactions [123].

The key component of a SUPRADES is the CD, an oligosaccharide composed of six (α-CD), seven (β-CD), or eight (γ-CD) D-glucopyranose units linked by α-(1–4) bonds [124]. (Fig. 15)

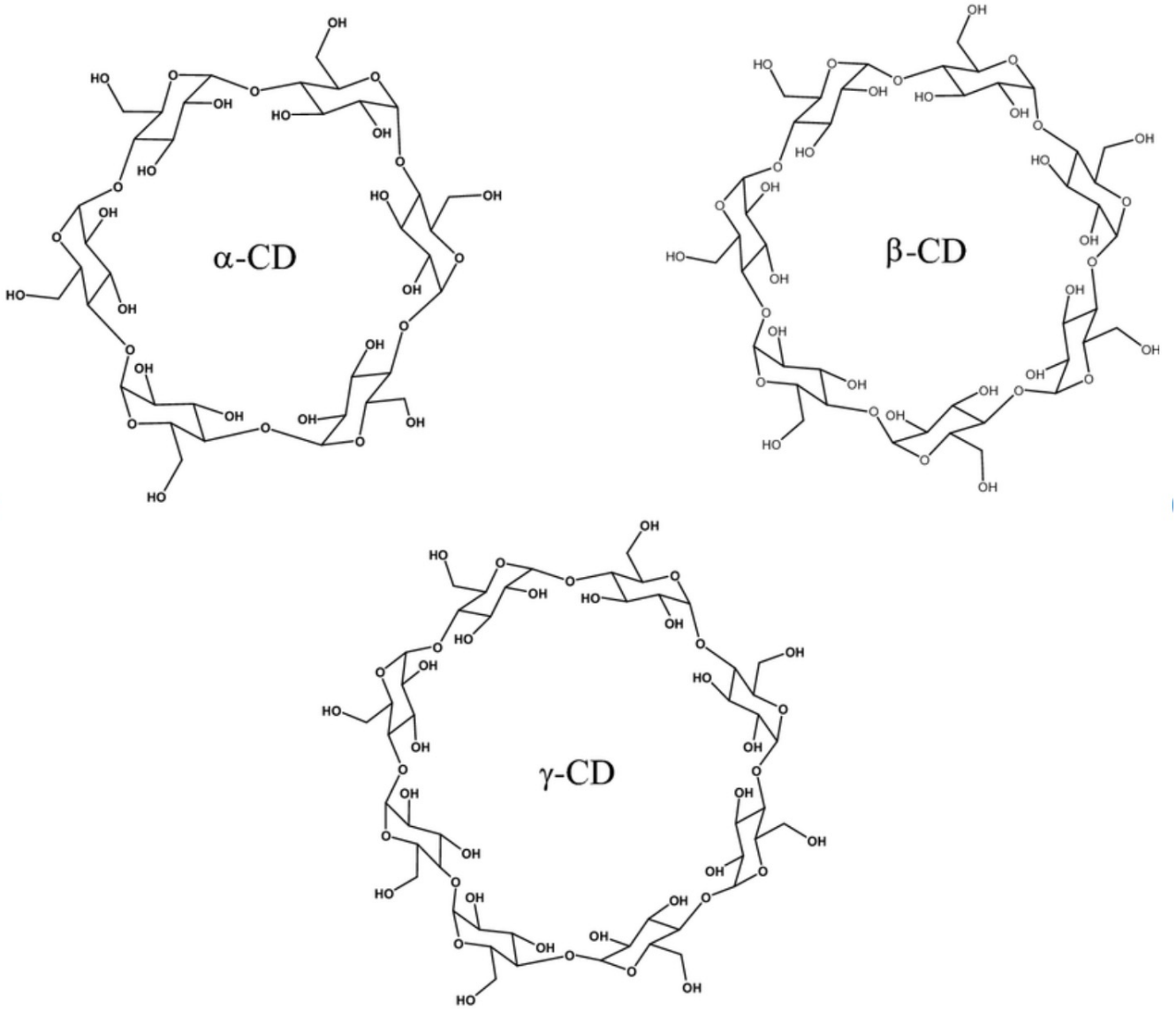

Fig. 15: Molecular structural representation of α, β, γ-CD [125]

CDs exhibit a typical truncated-cone structure with a hydrophilic outer surface and a hydrophobic internal cavity, which enables the formation of inclusion complexes through non-covalent host–guest interactions [126]. (Fig 16)

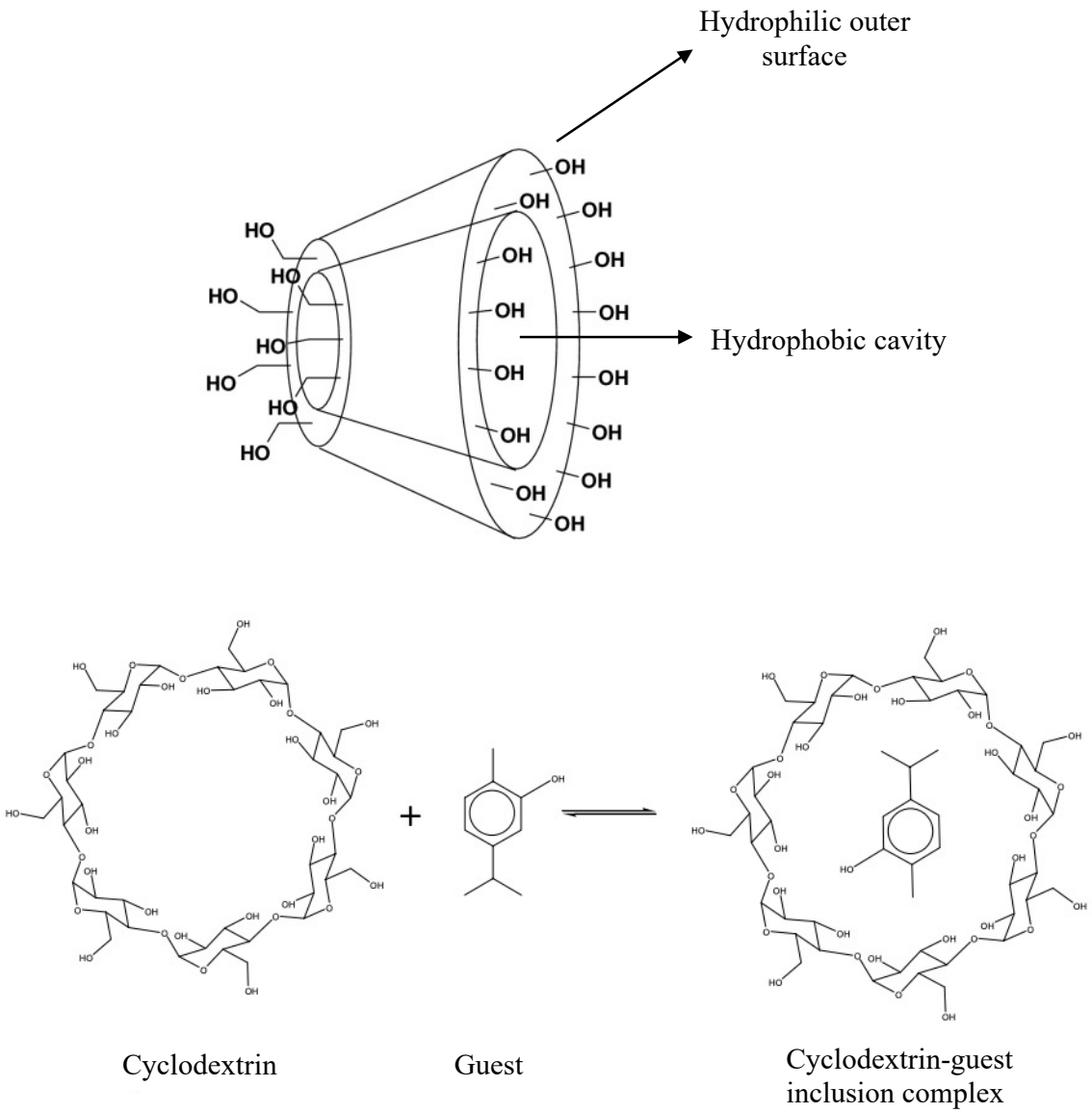

Fig. 16: Truncated cone shape with hydrophilic outer surfacace and CD-guest inclusion complex formation

Native CDs can be further modified by amination, esterification, or etherification reactions, introducing additional functional groups such as methyl, ethyl, hydroxyl, hydroxypropyl, carboxymethyl, or sulfobutylether. These modifications make it possible to obtain derivatives with greater solubility, chemical stability and ability to form inclusion complexes than native CDs [127,128].

The table 2 shows some characteristics of native CDs and their main derivatives: hydroxypropylated-β- CDs (HP-β-CD), randomly methylated β- CDs (RM-β-CD) sulfobutylether β- CDs (SBE-β-CD).

Charateristics and properties	Types of CDs						
	α-CD	β-CD	γ-CD	HP-β-CD	RM-β-CD	SBE-β-CD	
Number of glucopyranose	6	7	8	6	6	6	[129]
Molecular formula	$C_{36}H_{60}O_{30}$	$[C_6H_{10}O_5]_7$	$C_{48}H_{80}O_{40}$	$C_{63}H_{112}O_{42}$	$C_{54}H_{94}O_{35}$	$C_{42}H_{70}O_{35}$	[129]
Molecular weight (g/mol)	972	1135	1297	1540	1312	2163	[129]
Internal diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3				[125]
External diameter (Å)	14.6	15.4	17.5				[125]
Volume of the cavity (Å)	174	262	427				[125]
Solubility in water at 25°C (mg/mL)	130	18.4	249	>600	>500	>500	[125]
pK at 25°C	12,33	12,20	12,08				[125]
HBD Count	18	21	24	21	9	21	[129]
HBA Count	30	35	40	42	35	35	[129]

Table 2: Characteristics of native CDs and their main derivatives

SUPRADESs can be classified into binary and ternary systems [130]. (Fig. 17)

In binary SUPRADESs, the two precursor components (HBA and HBD) are mixed in precise molar ratios within a hermetically sealed vial. This mixture is then subjected to magnetic stirring at controlled temperature for a specific period of time [84]. The final product is a clear, stable, and homogeneous liquid at room temperature. In this system, CDs act as a HBA due to the presence of O-glycosidic bonds on its outer surface, while possible HBDs include carboxylic acids, alcohols, diols, and amides, similar to those used in conventional DESs [131].

On the other hand, CDs can be added to an already formed DES mixture to form ternary SUPRADESs. Their preparation is more complex than binary systems, since the simple mixing of DESs with CDs usually does not result in the formation of a stable liquid at room temperature. The addition of small amounts of water has been demonstrated to promote system organization by facilitating the formation of a DES–CD hydrogen-bonding network. This phenomenon, in turn, has been shown to lead to the stabilization of supramolecular structures [132].

The Fig. 17 shows some HBAs and HBDs used for the synthesis of binary and ternary SUPRADESs.

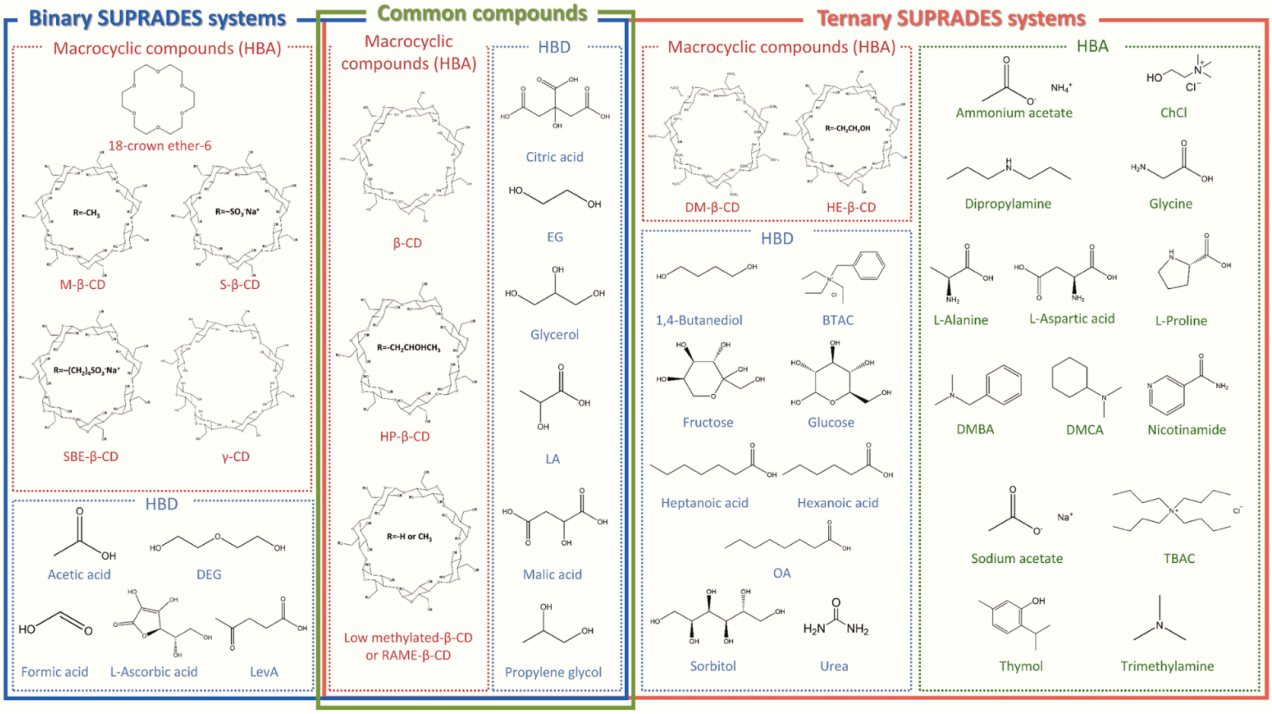

Fig. 17: HBAs and HBDs used for the synthesis of binary and ternary SUPRADES [133]

The use of SUPRADESs as green solvents for the extraction of bioactive molecules is gaining increasing attention, thanks to their ability to enhance yield, selectivity, and sustainability compared to conventional solvents [134]. The extraction efficiency of SUPRADESs is strongly dependent on their physicochemical properties [135].

An essential parameter to consider is the melting point: to be employed as liquid extraction solvents at room temperature, their melting point must be below 25 °C. However, most SUPRADESs do not meet this requirement due to the high melting points of CDs. To obtain binary systems with melting points below 25 °C, it is therefore necessary to significantly reduce the CD content in the mixture [136].

The decomposition temperature of SUPRADESs is a crucial parameter for assessing their suitability for high-temperature processes, such as solvent regeneration. At elevated temperatures, the structure of SUPRADESs may undergo phase changes and the hydrogen bonds and electrostatic interactions between HBA, HBD, and CD may be disrupted. For binary SUPRADES, available data indicate degradation temperatures ranging from 195 to 270 °C. Moreover, an increase in CD content tends to raise the decomposition temperature due to the high thermal stability of CD and the strength of its bonds with the HBD [131].

To achieve effective phase separation in an extraction process, it is essential that the SUPRADESs density differs from that of the sample matrix, being either higher or lower. Generally, DESs exhibit higher densities than water, ranging from 1.0 to 1.35 g/cm³ at 20 °C, and most binary and ternary SUPRADESs fall within a similar range [93]. Increasing the CD content in SUPRADESs tends to raise their density, whereas the addition of water can reduce it, but only in hydrophilic SUPRADESs [137]. Moreover, a decrease in temperature lowers the kinetic energy of the molecules, leading to an increase in density due to the reduction of the molar volume of the eutectic complexes [131].

SUPRADESs generally exhibit higher viscosities compared to DESs [85], primarily due to strong hydrogen bonding between CDs and HBDs, the larger size of CD molecules, and reduced free volumes (steric effect) [138]. Viscosity is influenced by the type and proportion of CD: in binary

systems, it increases in the order α-CD < β-CD < γ-CD and rises significantly with higher CD content [135]. The use of modified CDs or the addition of water in ternary SUPRADESs can markedly reduce viscosity. Overall, viscosity is a critical parameter for SUPRADESs applications: low viscosity facilitates solvent handling and reduces energy costs, whereas high viscosity can limit mass transfer efficiency [133].

When selecting an extraction solvent, it is essential to consider its compatibility with the sample matrix. SUPRADESs are generally hydrophilic due to the pronounced hygroscopic nature of CDs [128]. Despite their hydrophobic internal cavity, non-covalent bonds are more easily formed with other hydrophilic substances because of the hydrophilic properties of the CDs outer surface. However, this characteristic imposes limitations on the application of SUPRADES for the extraction of compounds from water or wastewater. The literature reports only a single study on hydrophobic SUPRADESs, based on thymol:heptanoic acid (1:1) and its derivatives, showing that their hydrophobicity can be tuned by adjusting the pH [139].

SUPRADESs offer remarkable versatility and find applications across various fields; they have proven effective in fuel desulfurization, combining advanced extraction and catalytic capabilities [140].

Numerous studies have highlighted that SUPRADESs exhibit significantly higher performance than conventional solvents in the extraction of polyphenols. Polyphenols can act as HBDs and interact with the HBA present in SUPRADESs, potentially generating antagonistic effects [141]. This phenomenon may arise from the fact that β-CD reduces the polyphenol–HBA interaction due to the instauration of stronger bonds within the β-CD/polyphenol inclusion complexes. At the same time, β-CD enhances the solubility of less polar polyphenols by entrapping them, influencing the overall extraction outcome [136]. Recent studies highlighted that SUPRADESs exhibit higher extraction efficiency compared to conventional DESs [142].

CHAPTER 3

3. Techniques for bioactive molecules determination

The separation of bioactive compounds present in plant extracts is a crucial and complex step in the identification, characterization and exploitation of analytes. This step is essential for understanding the chemical composition of an extract and for correlating the presence of specific molecules with their biological, antioxidant or pharmacological activities [143].

A wide range of chromatographic techniques are available for this purpose, differing in terms of separation principle, efficiency, sensitivity and applicability. The most commonly used methods include Thin Layer Chromatography (TLC) and its advanced, high-performance counterpart High Performance Thin Layer Chromatography (HPTLC), as well as paper chromatography, column chromatography, gas chromatography (GC), High Performance Liquid Chromatography (HPLC), and the more recent Ultra High Performance Liquid Chromatography (UHPLC) [144].

Separation techniques enable individual analytes to be isolated and characterized, both qualitatively and quantitatively. These techniques are essential for studying complex mixtures derived from plant matrices. The most suitable technique is chosen based on the chemical nature of the compounds to be analyzed (e.g. polarity, molecular weight, volatility and thermal stability), the complexity of the matrix, and the analytical objectives set [143].

Among instrumental techniques, HPLC is the most widely used and well-established method for separating and quantifying **phenolic compounds**. HPLC is generally performed using a reverse-phase C18 column coupled with a Diode Array Detector (DAD), and sometimes a mass spectrometer (MS) is also used to provide detailed structural information on the analytes [144].

The introduction of UHPLC, which uses columns packed with particles smaller than 3 μm in diameter and pumps capable of sustaining higher pressures, has further improved the method's resolution, reduced analysis times, and increased its sensitivity [145].

UV-Vis spectrophotometry is an analytical techniques, rapid, inexpensive and widely used tool for determining the presence of classes of bioactive compounds, such as polyphenols and flavonoids. It

is often used alongside specific colorimetric assays, including the Folin–Ciocalteu method to determine total polyphenol content, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay to evaluate antioxidant activity and the Trolox Equivalent Antioxidant Capacity (TEAC) assay to quantify antioxidant capacity equivalent to Trolox [146]. This spectrophotometric method can be used to determine compounds that contain chromophore groups such as **PC**.

Further analytical insights can be obtained using advanced spectroscopic techniques, such as Fourier Transform Infrared Spectroscopy (FTIR), which is useful for the functional identification of chemical groups and for the qualitative evaluation of the overall molecular composition. This technique has recently been used, for example, to identify tannic compounds in complex plant matrices, such as the cobs of mutant sorghum lines [147].

Today, new analytical technologies combined with green chemistry allow us to obtain a detailed and comprehensive analytical picture. This enables not only the precise identification and quantification of bioactive compounds, but also the study of their biological properties, stability, and potential applications in the food, nutraceutical, cosmetic, and pharmaceutical sectors [144].

3.1 High Pressure Liquid Chromatography (HPLC)

Liquid chromatography (LC) is a separation methodology based on molecular properties such as size, shape, and charge. In particular, the discrimination of analytes is determined by their different solubility between the stationary and the mobile phase. For this reason, it is important to know the physical-chemical properties of the analytes, such as solubility, polarity, pKa and pH, before setting up a chromatographic method [143].

In HPLC, the stationary phase is enclosed within a column, while a pump ensures the flow of the mobile phase through it. The chemical compounds are detected by means of a detector that generates a signal proportional to their respective concentrations, which is subsequently processed by a data acquisition and analysis system through a dedicated software interface [144].

Depending on the nature of the mobile phases, LC techniques can be classified as Normal Phase (NP) or Reversed Phase (RP) chromatography:

- In NP-LC, the mobile phase is nonpolar, while the stationary phase exhibits polar characteristics. Consequently, polar analytes are more strongly retained by the stationary phase. An increase in the polarity of the solute molecules leads to a higher adsorption capacity, resulting in longer elution times. Chemically modified silica, functionalized with groups such as cyanopropyl, aminopropyl, or diol, is generally employed as the stationary phase [148].

- RP- LC is based on the principle of hydrophobic interaction and features a non-polar stationary phase, typically composed of silica modified with alkyl groups (such as C18, C8, or C4). The mobile phase is polar. Non-polar compounds interact more strongly with the stationary phase and therefore exhibit longer retention times. Polar compounds interact less with the stationary phase and elute more rapidly [148].

In most cases, these separations are performed in reverse phase using a C18 column with UV detection. You can also choose between an isocratic or gradient method. Isocratic HPLC: the mobile phase maintains a constant composition during the analysis. Gradient HPLC: the mobile phase changes its composition over time (for example, by gradually increasing the percentage of organic solvent in reverse phase) [148].

As shown in the schematic diagram (Fig.18), HPLC instrumentation includes a solvent reservoir, pump, injector, column and detector.

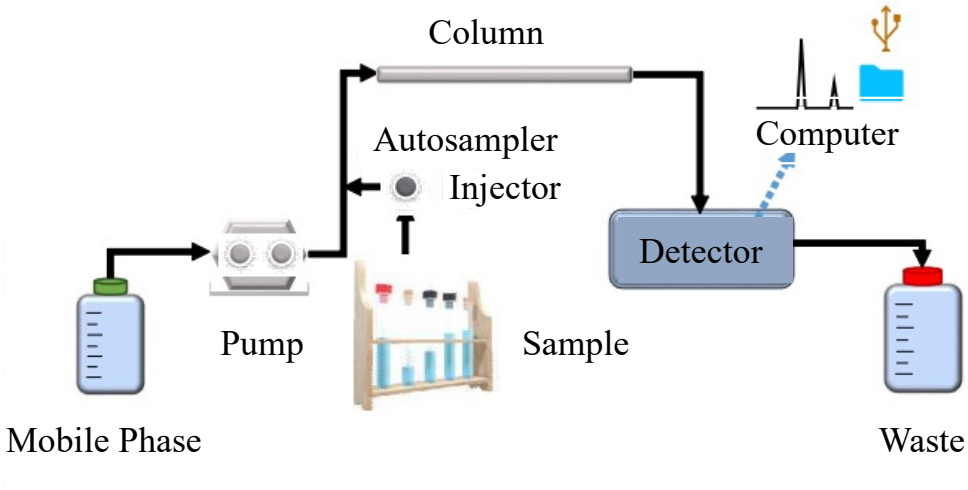

Fig. 18: Component of HPLC [149]

Solvent reservoir: Mobile phase contents are contained in a glass reservoir. The nature of the mobile phase varies depending on the chromatographic mode used. In NP-LC the mobile phase is non-polar, while the stationary phase is polar. Conversely, RP-LC, the mobile phase is polar, generally consisting of mixtures of water and organic solvents such as MeOH or acetonitrile, and the stationary phase is non-polar. Systems are often provided to remove dissolved gases in powder form from solvents [148].

Pump: The role of the pump is to push the mobile phase through the LC aphy at a specific flow rate, expressed in millilitres per minute (mL/min). Normal flow rates in HPLC are between 1 and 2 mL/min. Typical pumps can reach pressures between 6000 and 9000 psi (400 to 600 bar) [149]. Common used pumps are:

- Syringe pumps: these operate by pushing the mobile phase through the controlled movement of a piston inside a precision syringe. The main advantage of this system is the absence of pulses in the flow, which ensures a regular and precise delivery of the solvent. However, they have a limited tank capacity.

- Reciprocating pumps: these operate on the basis of the reciprocating movement of a piston that cyclically fills and empties a pumping chamber. During this operation, small flow pulsations are generated, which are dampened by dampers or pressure accumulators. The advantages of reciprocating pumps are their reduced internal volume, high external pressures, easy adaptability to gradient elutions and constant flow rates, which are largely independent of column back pressure and solvent viscosity. Most commercially available chromatographs use reciprocating pumps.
- Pneumatic pump: these use a compressible solvent reservoir, placed in a container pressurised by compressed gas (such as nitrogen or air). The gas pressure pushes the mobile phase through the system, ensuring a pulse-free flow and excellent stability. These pumps are simple, economical and do not cause pulses; their disadvantages are limited capacity and outlet pressure and the dependence of the pumping speed on the viscosity of the solvent, so they are not suitable for gradient elution.

Injector: The most widely used method of introducing a sample in LC is based on the use of a sampling loop. Interchangeable samplers are often available, allowing samples of various sizes, between 5 and 500 µL. Many HPLC instruments contain an autosampler with an automatic injection system that can continuously inject variable volumes [149].

Column: LC columns are generally made of stainless steel tubing, but glass or Tygon tubing can also be used for pressures below 600 psi. Standard columns are between 10 and 30 cm long and have an internal diameter of 2–5 mm. The packing materials consist of particles with a diameter between 3 and 10 µm. The most common packing material is silica, prepared by agglomerating submicrometric particles to obtain particles of uniform diameter. In addition to silica, the following are also used: Alumina (Al_2O_3), Porous polymers, Ion exchange resins. It is important to note that the column is divided into N sections, each of which has a stationary and mobile phase in equilibrium. Each of these sections is defined as a theoretical plate. If the total length of the column is L, then the height

equivalent to a theoretical plate (HETP) is: HETP = L/N. The efficiency of a column is expressed in terms of the number of theoretical plates (N). The greater the number of theoretical plates, the greater the column efficiency. A narrow peak corresponds to good chromatographic separation. According to the above formula, the greater the number of theoretical plates, the smaller the theoretical plate height and the greater the efficiency of the column [149].

Detector: After chromatographic separation, the analyte of interest is detected using appropriate detectors. Among the commercially available detectors for LC are UV detectors, fluorescence detectors, electrochemical detectors, refractive index (RI) detectors, and MS detectors. The choice of detector depends on both the type of sample and the objectives of the analysis. In the case of multicomponent analysis, the absorption spectra may be shifted to longer or shorter wavelengths compared to the main compound [148].

3.2 Mass spectrometry

Mass spectrometry (MS) is an advanced analytical technique used to quantify target analytes and identify unknown compounds within a sample. Typically, the MS detector is coupled with GC or LC systems, resulting in GC/MS or LC/MS configurations, respectively [150].

It is a destructive method, as the molecule does not remain intact after analysis but is ionized and fragmented into ions with different mass-to-charge ratios (m/z). The generated ions are separated based on their m/z, and the detector measures their abundance, producing a mass spectrum in which each peak corresponds to a specific component with a defined m/z ratio.

This technique facilitates the determination of molecular masses, both nominal and exact, and the acquisition of fragmentation profiles specific to each compound [146].

A typical mass spectrometer is composed of three fundamental components: the ion source, the mass analyzer, and the detector. (Fig. 19)

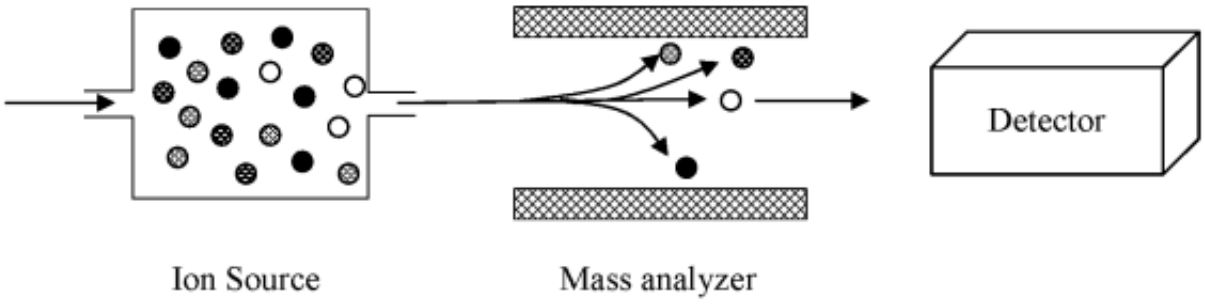

Fig. 19: Shows th key components of the MS [150]

Ion Source: This is the first part, which provides the analyte ionization [150]. The development of many different ionization techniques has made it possible to convert almost any large, non-volatile, and thermally labile compound into a gas-phase ion [151].

Ion sources can be categorized into two main types and can be classified as hard or soft. Hard ion sources transfer such a high amount of energy to the molecule that it undergoes fragmentation directly within the source. In contrast, soft sources provide only the amount of energy necessary to induce ionization. The most commonly employed gas-phase sources are Electron Impact (EI) and Chemical Ionization (CI). Among the desorption sources, Electrospray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI), and Matrix Assisted Laser Desorption/Ionization (MALDI) are included. Among all of them, the only source classified as hard is EI. ESI represents the preferred option for the analysis of polar compounds separated by LC, due to its very high sensitivity and its ability to be easily integrated with HPLC systems [150].

Mass analyzer: The most common used analyzers are [150]:

- Quadrupole: as show in Fig.20, consists of four parallel metal rods arranged in square configuration. Opposite rods are subjected to combined electric potentials: a Direct Current (DC) voltage and a high-frequency Alternating Current (AC) voltage. Ions entering the quadrupole are influenced by the oscillating electric field generated by the rods, which affects

their trajectories. Only ions with a specific m/z follow a stable path and reach the detector, while ions with different m/z values become unstable, collide with the rods, and are removed. By appropriately varying the DC and AC voltages, the quadrupole can scan a range of m/z values, either selecting individual ions one at a time or allowing multiple ions to pass simultaneously, depending on the operational mode, such as Selected Ion Monitoring (SIM) or full scan.

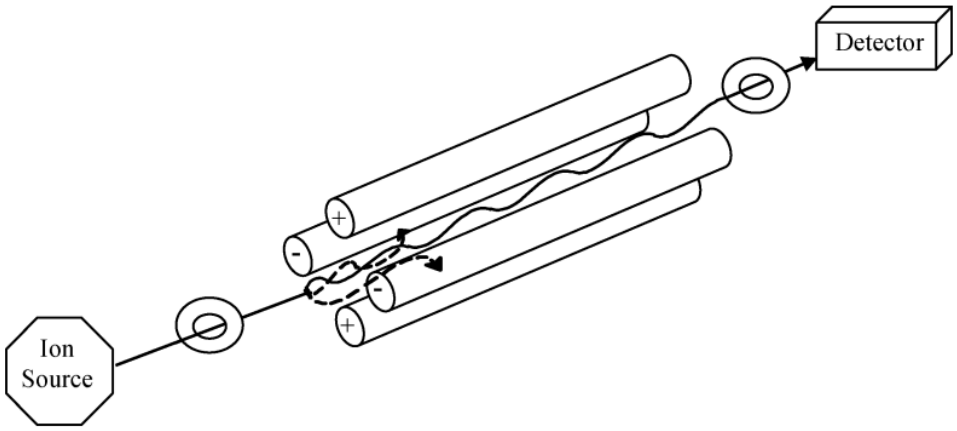

Fig. 20: Rapresentation of a quadrupole mass analyzer

- Ion trap: is a mass analyzer that traps and accumulates ions within a confined cavity. The ion trap consists of three main electrodes: a central ring electrode, which surrounds the cavity where ions are trapped, and two hemispherical electrodes (inlet and outlet) positioned on either side of the ring electrode. Each of the lateral electrodes has a small central aperture that allows controlled ion passage (Fig.21). The cavity defined by these three electrodes is referred to as the ion trap. To generate a mass spectrum, the electric potential is varied so that ions are sequentially ejected from the trap toward the detector according to increasing m/z.

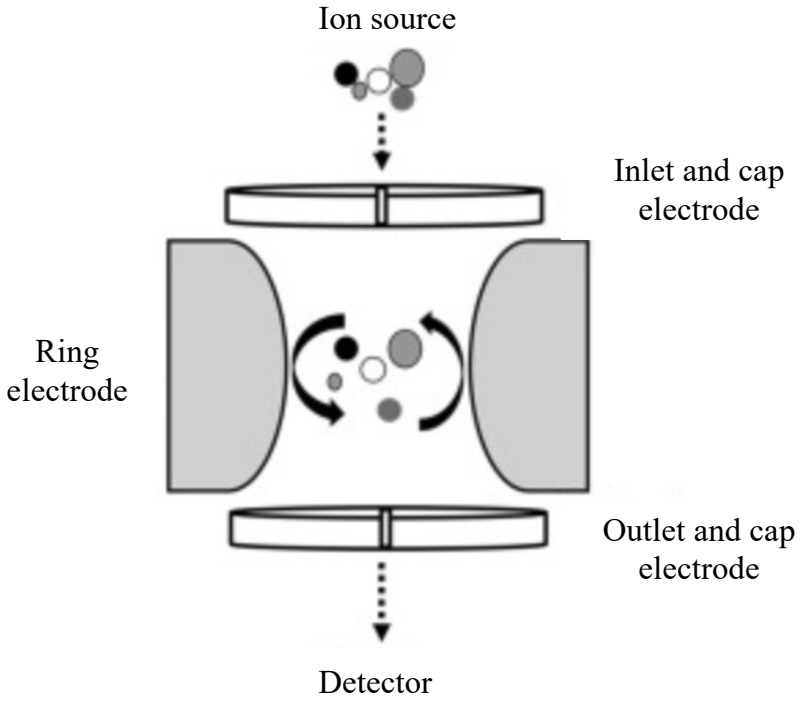

Fig. 21: Rappresentation of ion trap mass analyzer

- Time Of Flight (TOF): It separates ions based on the time they take to travel a fixed distance. This time depends on the ions' m/z. Lighter ions travel faster than heavier ions when accelerated with the same energy.

Detector: is the component of the MS responsible for recording ions converting their passage into a measurable signal. It transforms the ions into electrical signals proportional to their abundance, allowing the generation of the mass spectrum, in which each peak corresponds to an ion with a specific m/z value [150].

3.3 Spectrophotometric assays

Spectrophotometric methods are based on the interaction between light and matter, relying on the principle that molecules absorb electromagnetic radiation at specific wavelengths. The observed

absorption is proportional to the concentration of the analyzed compound, thus allowing its quantification.

The Folin-Ciocalteu assay is a widely used method for determining the Total Phenolic Content (TPC) or antioxidant compounds in a sample [146]. The underlying principle is relatively straightforward: phenolic compounds present in the sample reduce the Folin-Ciocalteu reagent, which consists of a mixture of phosphomolybdate and phosphotungstate. The reaction occurs in a basic environment, typically through the addition of sodium carbonate, leading to the formation of an intense blue complex with a maximum absorption around 750 nm. Measuring the absorbance of the blue solution using spectrophotometry allows estimation of the TPC. For quantitative analysis, the sample absorbance is compared to a calibration curve constructed using a standard phenol, such as gallic acid, and the results are expressed as Gallic Acid Equivalents (GAE) per gram of sample [146].

The literature contains numerous analytical assays that are based on spectrophotometric measurement. TEAC is one of the most widely used methods for determining the antioxidant activity of an extract. The method uses a UV-Vis spectrophotometer to measure the absorbance of a solution containing the ABTS• radical+, generated by the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)), a colourless substance that takes on a visible colour when in its radical form as it absorbs characteristic wavelengths in the visible range. Adding antioxidant molecules to the ABTS•+ radical solution can cause reduction of the radical to its colourless form via hydrogen or electron transfer, resulting in discolouration of the mixture. This change is proportional to the decrease in absorbance over time, measured at a specific wavelength (734 nm). The antioxidant power is then expressed by comparing the obtained absorbance values with those measured for known quantities of a reference antioxidant molecule, generally ascorbic acid or Trolox [152]. Other assays reported in the literature for determining the antioxidant capacity of an extract include: Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC) and the DPPH [152].

CHAPTER 4

4. Aims of the Ph.D. research project

The general aim of this doctoral project was to develop analytical methods based on green extraction techniques for the determination of bioactive molecules in plant matrices. The study was prompted by the increasing demand for environmentally friendly, efficient methods with a low environmental impact that can replace traditional extraction processes, which often use toxic solvents and require high energy consumption. These green methods must also be capable of preserving the integrity and biological activity of the compounds of interest. Specifically, two research activities were carried out.

The first line of research focused on the development and optimization of a green extraction technique for the extraction of phenolic compounds and PC from Spirulina, described in Chapter 5. In particular, the most effective DES was identified, with the aim of obtaining an extract enriched in both phenolic compounds and PC, characterized by high stability, greater purity, and prolonged preservation of bioactivity. The optimization of the extraction procedure involved the systematic evaluation of various parameters, including the choice of DES composition, the optimal water content necessary to modulate viscosity and diffusivity, the biomass/solvent ratio, and the temperature and duration of extraction. The extract obtained was characterized qualitatively and quantitatively using analytical techniques such as HPLC-MS and spectrophotometric assays, for the determination of phenolic compounds and PC, respectively.

The second line of research focused on the study of SUPRADES as extraction solvents for bioactive molecules, described in Chapter 6. In particular, the interaction of SUPRADES with quercetin was examined. SUPRADES are the evolution of DES thanks to the presence of CDs. Quercetin is a flavonoid widely found in the plant kingdom, but with poor bioavailability due to its low water solubility and rapid metabolic degradation. The use of SUPRADES therefore represents a promising approach to overcoming these critical issues, thanks to the interaction between quercetin and β-CD, allowing for more efficient and selective extraction of bioactive compounds and, at the same time,

better preservation and biological availability. Initially, preliminary tests were conducted to evaluate the main extraction parameters and verify the ability of SUPRADES to form stable complexes with quercetin. The interaction between quercetin and SUPRADES was evaluated using HPLC-DAD.

Both lines of research adhere to the principles of green chemistry, sharing the aim of developing safer, more sustainable and highly efficient extraction processes. DES and SUPRADES are excellent extraction solvents, providing a safe and sustainable alternative to traditional toxic organic solvents. They therefore represent sustainable and highly versatile solutions, suitable for the development of new processes in the nutraceutical, pharmaceutical, and cosmetic sectors, where the demand for safe, efficient, and environmentally friendly methods is increasing.

CHAPTER 5

5. Deep Eutectic Solvents based green approach for bioactive molecules recovery from Spirulina

5.1 Introduction

Spirulina is a unicellular, filamentous microalga that naturally thrives in highly alkaline environments, especially in the salt lakes of Africa and Mexico, as well as in some freshwater ecosystems [153]. To date, fifteen distinct species of Spirulina have been identified, with *Arthrospira maxima* and *Arthrospira platensis* being the most extensively cultivated and studied due to their remarkable nutritional and biochemical properties [154]. This cyanobacterium has attracted growing scientific and commercial interest as a sustainable source of nutrients and bioactive molecules. Its nutritional profile is exceptionally rich, featuring a protein content that can reach up to 70% of its dry weight—considerably higher than that of many traditional plant and animal sources. In addition to proteins, Spirulina contains substantial amounts of carbohydrates, lipids, vitamins, minerals, pigments, enzymes, and a wide range of phenolic compounds [155].

Phenolic compounds are secondary metabolites characterized structurally by one or more hydroxyl groups attached to aromatic benzene rings. They play a crucial role in the defense systems of algae, acting as chemical protectants against biotic and abiotic stressors such as pathogen attacks and UV radiation. Because of their ability to neutralize reactive oxygen species, phenolic compounds are recognized as one of the most important classes of natural antioxidants [156]. Extraction of these bioactive molecules from Spirulina is typically achieved using solvent-based techniques, including UAE, MAE, and High Pressure Temperature Extraction (HPTE) [157]. Reported TPC values for Spirulina vary widely, generally ranging between 0.2 and 15 mg of GAE per gram of sample [158]. This variability is influenced by factors such as the cultivation conditions, extraction method, and solvent type used.

Beyond phenolics, Spirulina owes many of its biological and health-promoting effects to a class of water-soluble pigment–protein complexes known as PBPs. Among them, PC is the predominant form, accounting for approximately 20% of the organism’s dry weight [159]. PBPs are composed of

apoproteins covalently bound to linear tetrapyrrole chromophores called phycobilins. Based on their spectral properties, PBPs are divided into four major types: PE, PC, phycoerythrocyanin (PEC), and allophycocyanin (APC). In cyanobacteria such as *Spirulina platensis*, PBPs are organized into large protein complexes known as PBS, which serve as accessory light-harvesting antennae during photosynthesis [160].

A typical PBS consists of a central core, mainly composed of APC and linker proteins, surrounded by rod-like structures formed by PE, PEC, and PC. However, in *Spirulina platensis*, the rods contain only PC, reflecting a unique adaptation of its photosynthetic system [160]. PBPs are stabilized by covalent linkages between cysteine residues of the apoprotein and phycobilin molecules. Four principal types of phycobilins have been identified, each exhibiting distinct colors based on the number of conjugated double bonds: phycocyanobilin (PCB, blue), phycoviolobilin (PVB, violet), phycoerythrobilin (PEB, red), and phycourobilin (PUB, yellow) [161], as illustrated in Fig. 22.

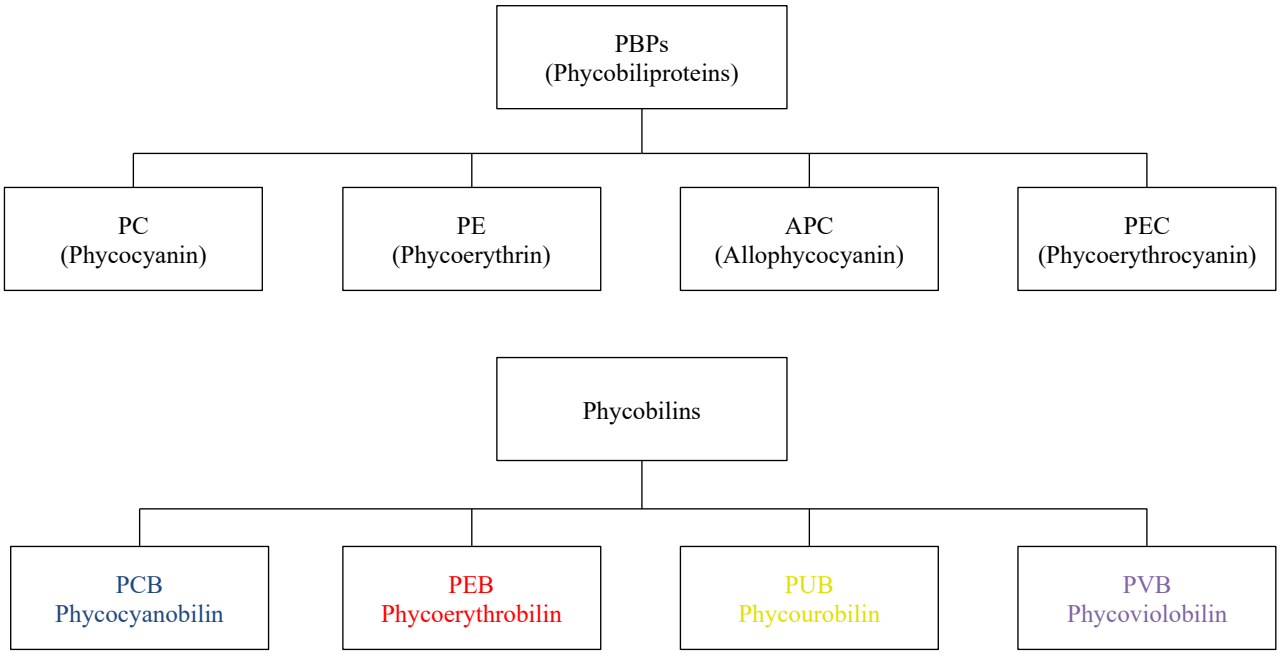

Fig. 22 Classification of phycobiliproteins (PBPs) and their associated phycobilin chromophores.

PC is a blue-colored biliprotein composed of two types of polypeptide subunits: an α chain consisting of 162 amino acids with a molecular weight ranging from 10 to 19 kDa, and a β chain composed of

172 amino acids with a molecular weight between 14 and 21 kDa. The α subunit carries a single PCB chromophore covalently attached to the cysteine residue at position 84, while the β subunit contains two PCB molecules bound at cysteine residues 84 and 155. The α and β subunits associate to form an αβ heterodimer, which further assembles symmetrically into trimers and subsequently into hexamers, denoted as $(\alpha\beta)_6$. In aqueous solutions, PC exists as a heterogeneous mixture of monomers, trimers, hexamers, and higher-order oligomers, exhibiting molecular weights ranging approximately from 44 to 260 kDa [40]. This structural complexity contributes to its remarkable optical, antioxidant, and anti-inflammatory properties, making PC one of the most valuable natural compounds derived from Spirulina for applications in in food, pharmaceutical, and cosmetic industries.

Phycobilins are the chromophoric groups responsible for the absorption of light energy in the visible spectrum, specifically between 500 and 650 nm. Among these pigments, PC exhibits a strong absorption maximum at approximately 620 nm and a fluorescence emission peak at around 640 nm, which make it highly effective in photosynthetic light harvesting and useful for a variety of biotechnological applications [162].

Despite its numerous potential uses, the practical application of PC is often limited by challenges associated with its extraction and stability. Conventional extraction techniques tend to yield extracts with relatively low purity and limited pigment stability. Typically, PC is isolated through a Solid Liquid Extraction (SLE) process using either water or phosphate buffer solutions as the solvent medium. As it is known that Spirulina possesses a robust peptidoglycan-rich cell wall that impedes intracellular release, cell disruption is an essential preliminary step. Common mechanical and physical strategies include freezing–thawing cycles, bead milling, vigorous mixing, and high-shear homogenization [163].

Among these, the freezing–thawing technique using water as the solvent is simple and widely employed, but it usually produces crude extracts with purity ratios ranging from 0.07 to 0.4 [163]. Therefore, subsequent purification is required to achieve higher purity levels. Common downstream methods include ion-exchange chromatography, purification through pH-gradient elution, or

ultrafiltration systems capable of separating components based on molecular weight [164]. Depending on the final purity, PC can serve different applications: food-grade PC requires a purity greater than 0.7, cosmetic-grade PC exceeds 1.5, and analytical-grade PC must reach a purity above 4.0 [165].

During extraction and handling, it is essential to consider the physicochemical sensitivity of PC. The pigment is thermolabile, showing degradation at temperatures above 50 °C, and exhibits optimal stability within a pH range of 5 to 8. Quantitative analyzes reported in the literature indicate that the PC content in Spirulina can vary between 20 and 200 mg per g of dry biomass, depending on strain type, cultivation conditions, and extraction parameters. These values are typically determined using spectrophotometric methods based on the Bennett–Bogorad equation [163].

However, PC dissolved in water tends to be unstable over time, undergoing degradation and loss of color intensity. Faieta et al. [166], demonstrated that the inclusion of saccharides in the extraction medium enhances the stability of aqueous PC solutions. This effect is attributed to the ability of sugars to form a hydrogen-bonding network around protein molecules, thus reducing conformational changes and oxidative degradation. Consequently, the use of sugar-based or polyol-containing solvents represents a promising strategy for improving PC stability.

In recent years, DESs has emerged as a sustainable alternative to traditional organic solvents. DESs are composed of two or more components mixed at a specific molar ratio to form a eutectic mixture through hydrogen bonding interactions. One component typically acts as a HBD—such as sugars, organic acids, polyols, or amines—while the other functions as a HBA, most commonly a quaternary ammonium salt [167]. When both components are of natural origin, the resulting mixture is termed a NADES. The physicochemical properties of DESs, including their polarity, viscosity, and solvating power, can be finely tuned by adjusting the molar ratio of their constituents and by controlling the amount of water added to reduce viscosity.

DESs have been successfully applied for the extraction of a wide range of bioactive compounds, including phenolic molecules, flavonoids, and alkaloids, due to their ability to dissolve both

hydrophilic and moderately hydrophobic compounds [106]. Furthermore, several studies have reported that DESs can act as biomass pre-treatment agents, weakening or partially disrupting the cell wall structure of microalgae, which enhances mass transfer and facilitates the release of intracellular compounds without the need for energy-intensive methods such as freeze–thaw cycles [168].

An additional and particularly attractive feature of NADES is their protein-stabilizing capability. They are composed of osmolytes—such as betaine, trehalose, glycerol, and urea—naturally produced by living organisms to counteract stress conditions like dehydration, freezing, and oxygen deprivation, so NADES can provide a protective environment for sensitive biomolecules. Tian et al. [169], demonstrated that a DES composed of trehalose and betaine effectively stabilized trypsin, preventing its structural denaturation. In this system, trehalose acts as a glass-forming agent that immobilizes proteins and limits their exposure to degradation, while betaine functions as an osmotic stabilizer that maintains protein integrity.

The present work aimed to develop and optimize a rapid, efficient, and environmentally sustainable extraction method for Spirulina, utilizing a NADES-based system. The aim was to produce an extract enriched in both phenolic compounds and PC, exhibiting high stability, enhanced purity, and long-term preservation of bioactivity. The adoption of NADES offers several advantages: it simplifies the extraction process by eliminating the need for repetitive freeze–thaw cycles, minimizes solvent toxicity, and provides intrinsic stabilization for sensitive proteins such as PC. Optimization of the extraction procedure involved systematic evaluation of several parameters, including the choice of NADES composition, the optimal water content needed to modulate viscosity and diffusivity, the biomass-to-solvent ratio, and extraction temperature and duration. Through this approach, it becomes possible to design a green, high-efficiency method capable of simultaneously recovering phenolic compounds and PC from Spirulina powder—yielding a multifunctional extract suitable for applications in the nutraceutical, cosmetic, and pharmaceutical industries.

5.2 Materials and methods

5.2.1 Chemicals

ChCl, glucose, glycerol, betaine, and LA were obtained from Sigma-Aldrich (Milan, Italy). The Folin–Ciocalteu reagent was also purchased from Sigma-Aldrich (Milan, Italy). HPLC–MS grade water and formic acid (95–97%) were supplied by the same manufacturer. Acetonitrile (99.9%, hypergrade for LC–MS) and gallic acid were procured from Merck KGaA (Darmstadt, Germany).

5.2.2 Samples

Spirulina powder was supplied by a nutraceutical company. Prior to the experimental procedures, the powder was dried in an oven (Heraeus Scientific Instruments, Hanau, Germany) at 50 °C for one hour to eliminate residual moisture.

5.2.3 NADESs preparation

HBAs and HBDs were weighted according to the predetermined molar ratios and heated at 80 °C until a homogeneous liquid mixture was obtained. Subsequently, 30% (v/v) of water was added to each mixture to reduce viscosity, followed by vortex mixing to ensure complete homogenization. The prepared NADES formulations were then stored at room temperature until use. The NADES compositions tested as potential solvents for the extraction of phenolic compounds and PC from Spirulina are listed in Table 3.

HBAs	HBDs	Molar ratio w/w
Glucose	Glycerol	1:2
Betaine	Glucose	1:2
Betaine	Lactic acid	1:2
ChCl	Glucose	1:2
ChCl	Glycerol	1:2
ChCl	Lactic acid	1:2

Table 3: NADES components and molar ratios

5.2.4 Extraction method

According to previous studies, the most effective method for disrupting the Spirulina cell wall is through repeated freezing–thawing cycles [163]. However, in the present study, this approach was not feasible due to the physicochemical properties of the NADESs used. When placed at -20 °C, the NADES mixtures solidified and did not revert to their liquid state upon thawing. Instead, cell disruption was achieved through the combined effect of ultrasonic treatment and the intrinsic properties of the NADESs, which facilitate cell wall weakening and improve the extraction efficiency of phenolic compounds and PC even without the use of freeze–thaw cycles [168]. For the extraction process, 0.5 g of Spirulina powder was mixed with 7.35 mL of a NADES composed of betaine and glucose in a 1:2 (w/w) molar ratio. The mixture was vortexed to ensure uniform dispersion and then subjected to ultrasonic treatment in an ultrasonic bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) at 50 °C, operating at a frequency of 37 kHz and a thermal power of 200 W, for one hour. Following sonication, the sample was centrifuged at 5000 rpm for 15 minutes, and the resulting supernatant was collected as the extract.

5.2.5 Optimization of the extraction procedure

Optimization of the extraction process was conducted using a One Factor At A Time (OFAT) experimental design, with the concentration of PC and the TPC serving as the main response variables. Both parameters were quantified using spectrophotometric methods. Initially, a screening phase was performed to evaluate six different NADESs formulations (as reported in Table 3) in order to identify those providing the highest simultaneous extraction yields of PC and phenolic compounds. Once the most promising NADES compositions were identified, their viscosity was further optimized by testing two different water contents 30% and 40% (v/v), added to the solvent mixture. Subsequently, the influence of the matrix-to-solvent ratio on extraction efficiency was investigated by comparing two ratios: 1:20 and 1:30 (w/w). After determining the most effective NADES formulation, optimal water content, and matrix-to-solvent ratio, the effects of extraction time and temperature were explored. Extraction durations of 30 minutes, 1 hour, and 2 hours were tested, along

with two operating temperatures, 25 °C and 50 °C, to identify the conditions that maximized the recovery of both PC and phenolic compounds.

5.2.6 Determination of total phenolic content (TPC)

TPC of the extracts was quantified using the Folin–Ciocalteu colorimetric method. In an alkaline environment, the Folin–Ciocalteu reagent undergoes reduction, resulting in the formation of blue-colored anions whose intensity is proportional to the phenolic concentration. For the assay, 20 µL of sample were mixed with 100 µL of Folin–Ciocalteu reagent and 1580 µL of distilled water. The mixture was kept in the dark for 8 minutes to allow the initial reaction to occur. Subsequently, 300 µL of a sodium carbonate (Na_2CO_3) aqueous solution at a concentration of 0.2 g/mL were added, and the mixture was gently agitated for 2 hours under dark conditions to prevent photodegradation. The absorbance of the resulting solution was measured at 765 nm using an Infinite M200 PRO Tecan microplate spectrophotometer (Tecan Trading AG, Männedorf, Switzerland). Quantification was performed through a calibration curve prepared with gallic acid as the reference standard, in a concentration range of 0–2000 µg/mL. The TPC results were expressed as grams of g GAE per 100 grams of Spirulina powder.

5.2.7 Determination of PC

The PC content in the extracts was quantified spectrophotometrically following the Bennett–Bogorad method. Measurements were performed using a UV-1800 UV–Vis spectrophotometer (Shimadzu, Milan, Italy). The absorbance of each sample was recorded at three wavelengths: 620 nm, 650 nm, and 280 nm, corresponding respectively to the highest absorption of PC, allophycocyanin, and TPC. The concentration of PC was then calculated by applying the Bennett–Bogorad equation, which ensures the correction of overlapping absorption among PBP, providing an accurate estimation of PC content in the extract.

$$[PC] = \frac{[A_{620} - 0.474(A_{650})]}{5.34}$$

5.2.8 Purity determination of PC extract

Traditionally, the purification of PC extracts relies on labor-intensive and costly techniques such as Ion Exchange Chromatography (IEC) and purification by pH-gradient elution [164]. In the present study, an alternative and more straightforward approach was explored, involving the use of Amicon® ultrafiltration cartridges to enhance the purity of the extract.

The purity of the obtained PC extract was evaluated spectrophotometrically. Specifically, the purity index was calculated as the ratio of the absorbance measured at 620 nm (corresponding to the characteristic absorption peak of PC) to the one measured at 280 nm (associated with total protein content). This ratio provides a reliable indicator of extract purity, with higher values reflecting lower contamination from other proteins or impurities.

$$Purity = \frac{A_{620}}{A_{280}}$$

5.2.9 Evaluation of extract stability

Previous studies have indicated that sugars can enhance the stability of PC and that NADES are capable of stabilizing protein structures [169]. To evaluate and corroborate these findings, the stability of PC was assessed in both aqueous and NADES-based extracts. Both types of extracts were stored at 4 °C in the dark, and the PC content was monitored daily over a period of two months using a spectrophotometric assay. This approach allowed for a comparative analysis of the stabilizing effects of NADES versus conventional aqueous solutions on PC over time.

5.2.10 HPLC-MS qualitative analysis

The phenolic compound profile of the extracts was analyzed using a Shimadzu Prominence LC-20A liquid chromatograph (Shimadzu, Milan, Italy) equipped with dual LC-20 AD XR pumps, an autoinjector (SIL-10ADvp), a column oven (CTO-20 AC), a degasser (DGU-20 A3), and coupled to a DAD (SPD-M10Avp) and a single quadrupole mass spectrometer (LCMS-2010 EV, Shimadzu, Tokyo, Japan) with an ESI interface. Data acquisition and analysis were performed using Shimadzu LC Solution software, version 3.7 (Shimadzu, Tokyo, Japan). Separation was carried out on an

Ascentis® Express 90 Å F5 column (15 cm × 2.1 mm, 2.7 μm particle size; Merck KGaA, Darmstadt, Germany) maintained at 35 °C. The mobile phase consisted of 0.1% (v/v) formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), delivered at a constant flow rate of 0.2 mL/min. The injection volume was 2 μL, and the gradient program for phenolic separation was as follows: 0 min, 5% B; 30 min, 35% B; 35 min, 100% B; 42 min, 100% B. Chromatograms were monitored at 323 nm using the PDA detector with a spectral range of 200–400 nm.

MS data were acquired in negative ionization mode with the following parameters: nitrogen nebulizing gas flow, 1.5 mL/min; dwell time, 1 s; m/z acquisition range, 100–800 m/z; scan rate, 1000 amu/s; detector voltage, 1.5 kV; interface temperature, 250 °C; CDL temperature, 300 °C; heated block temperature, 300 °C; interface voltage, 3.50 kV; Q-array voltage, 0.0 V; and Q-array RF, 150.0 V. This setup allowed for precise qualitative and quantitative analysis of phenolic compounds in the Spirulina extracts.

5.3 Results

5.3.1 Selection of the extraction solvent for phenolic compounds and PC from Spirulina

Six NADESs were selected as potential extraction media for phenolic compounds and PC from Spirulina powder: glucose–glycerol (1:2), betaine–glucose (1:2), betaine– LA (1:2), ChCl–glycerol (1:2), ChCl–glucose (1:2), and ChCl– LA (1:2). Among these, glucose–glycerol and betaine– LA NADESs had been previously tested for PC recovery from Spirulina, whereas the remaining formulations have been widely reported in the literature as effective solvents for the extraction of bioactive molecules from plant matrices [146]. The use of NADESs composed of naturally occurring molecules found in plant biomass offers several advantages. In particular, NADES can stabilize protein structures [169] and, as previously described, ensure the extraction to be performed without freeze–thaw cycles, reducing the environmental impact of the process [168]. To select the most suitable extraction solvent, all six NADESs were tested under standardized conditions: a matrix-to-solvent ratio of 1:20 (w/w) and sonication in an ultrasonic bath at 50 °C for 1 hour. Following sonication, the mixtures were centrifuged at 5000 rpm for 15 minutes, and the supernatants were

collected as extracts. The extraction efficiency of the NADESs was compared to that of water, which is traditionally used for phenolic and PC recovery from Spirulina. As illustrated in Fig.23, NADESs generally followed a similar trend in their ability to extract both phenolic compounds and PC. The lowest extraction yields for TPC and PC were observed with NADESs containing LA as the HBD. The high acidity of these NADESs (pH ≈ 2) caused partial degradation of PC and structural alterations in phenolic compounds [170].

Betaine–glucose, betaine–glycerol, and ChCl–glucose NADESs demonstrated comparable efficiency in extracting phenolic compounds, yielding TPC values in line with those reported in the literature for organic solvent-based extractions [171]. Water showed reasonable phenolic compound extraction but was less effective for PC, particularly in the absence of freeze–thaw cycles necessary to disrupt the cell wall. Among the tested solvents, sugar-based NADESs provided the highest PC extraction efficiency, likely due to the stabilizing effect of sugars on PC structure. However, ChCl–glucose NADES exhibited poor reproducibility for phenolic compound extraction. Considering both extraction efficiency and reproducibility, betaine–glucose NADES was identified as the most suitable solvent, combining high yields for PC with reliable phenolic compound recovery. Consequently, it was selected for further optimization in subsequent experiments.

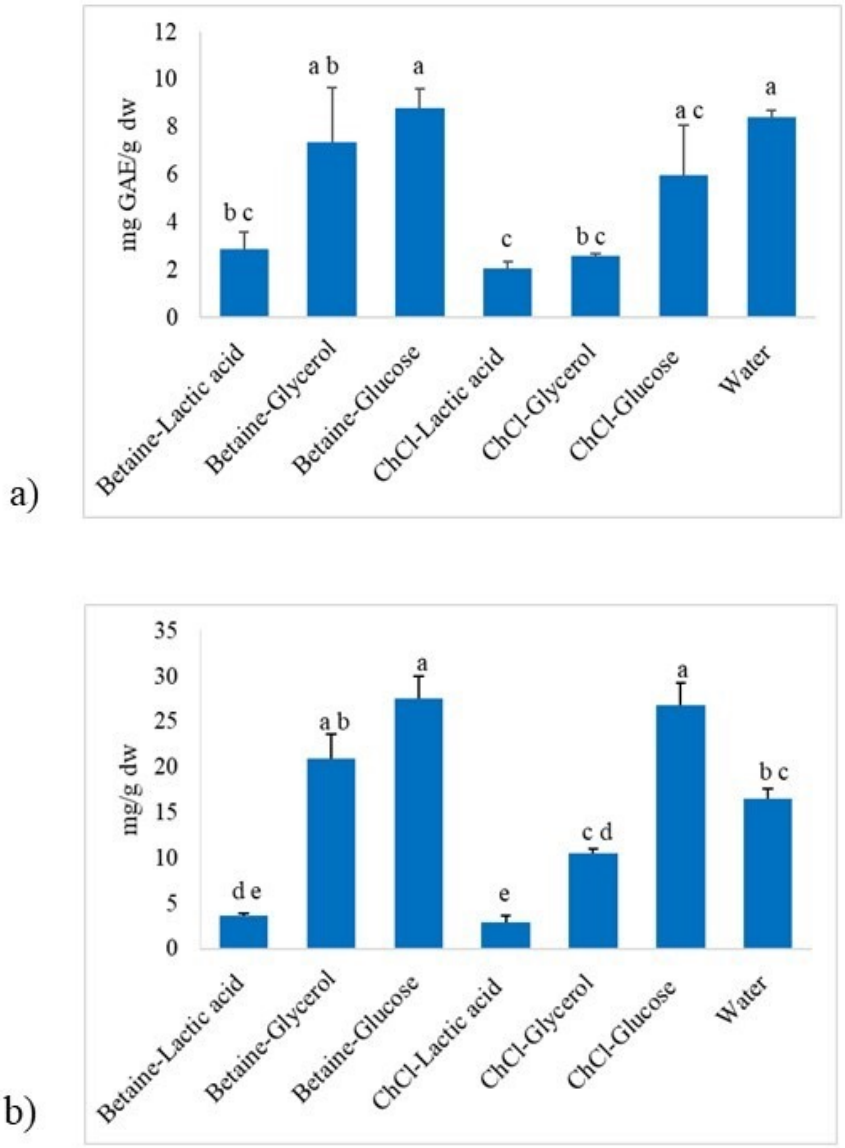

Fig. 23: TPC (a) and PC concentration (b) obtained using tested NADESs. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results

5.3.2 Percentage of water to be added to the DES

DESs are generally characterized by high viscosity, which can negatively impact extraction efficiency and reproducibility. One common strategy to mitigate this issue is the addition of water to the DES. The high viscosity of betaine–glucose NADES has been previously reported in the literature [172], making water addition a critical parameter for optimization. To determine the optimal water content, two SLEs were conducted at 50 °C for 1 hour using betaine–glucose NADES (1:2 molar ratio) supplemented with either 30% or 40% (v/v) water. Lower water percentages (<30%) were not tested,

as the NADES remained excessively viscous and prevented effective separation between the Spirulina matrix and the solvent. Water concentrations above 40% were avoided because at approximately 50% water content, the DES structure begins to collapse, leading to a marked reduction in extraction efficiency. As shown in Fig. 24, the highest recovery of both TPC and PC was achieved with betaine–glucose NADES containing 30% (v/v) water. These findings are consistent with previous reports in which betaine–glucose NADES was employed as an effective solvent for the extraction of bioactive compounds from plant materials [146]. Accordingly, all subsequent extraction optimization experiments were carried out using betaine–glucose DES supplemented with 30% water as the extraction solvent.

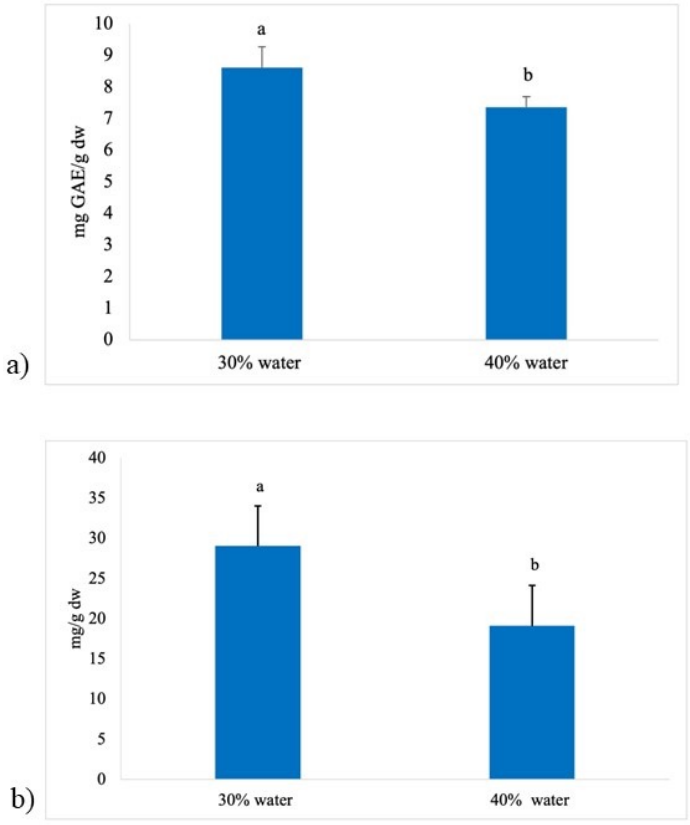

Fig. 24: TPC (a) and PC concentration (b) obtained considering different percentages of water to be added to the NADES. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results

5.3.3 Selection of matrix-to-solvent ratio

Extraction of PC and phenolic compounds from Spirulina typically requires relatively high matrix-to-solvent ratios to ensure effective solubilization of the target compounds. Lower ratios often result in poor separation between the Spirulina biomass and the solvent. In this study, two matrix-to-solvent ratios, 1:20 and 1:30 (w/w), were evaluated. For each extraction, 0.5 g of Spirulina powder was mixed with the corresponding amount of betaine–glucose NADES containing 30% water, according to the selected ratios. The mixtures were sonicated in an ultrasonic bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) for 1 hour at 50 °C. As illustrated in Fig. 25, no significant differences in TPC or PC content were observed between the two tested ratios. Considering that the 1:20 (w/w) ratio requires a smaller volume of extraction solvent while achieving comparable yields, it was selected for all subsequent optimization experiments.

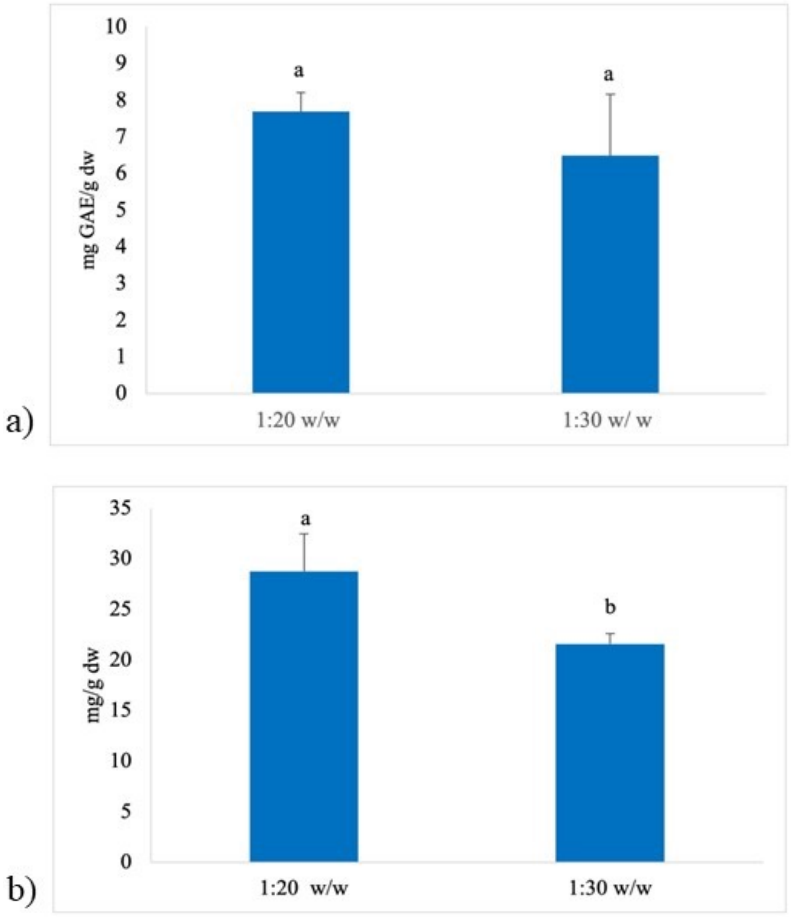

Fig. 25: TPC (a) and PC concentration (b) obtained by considering different quantities of matrix-to-solvent ratio. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results.

5.3.4 Extraction temperature and time optimization

The efficiency of analyte extraction from Spirulina is strongly influenced by both the temperature and the duration of the extraction process. In general, longer extraction times are expected to increase yield, as the solvent has more opportunity to penetrate the biomass and solubilize target compounds [173]. Similarly, higher temperatures can enhance extraction by improving solvent diffusivity and mass transfer; however, excessive heat may lead to degradation of sensitive molecules such as PC and phenolic compounds [163]. To determine the optimal extraction temperature, two experiments were conducted using betaine–glucose NADES supplemented with 30% water as the solvent, maintaining a matrix-to-solvent ratio of 1:20 (w/w). In both cases, 0.5 g of Spirulina powder was mixed with the appropriate amount of NADES and sonicated in an ultrasonic bath for 1 hour at either 25 °C or 50 °C. As shown in Fig. 26, extraction yields for both TPC and PC were significantly higher at 50 °C. This improvement can be attributed to enhanced mass transfer of the solvent into the biomass at elevated temperatures, which facilitates the release of bioactive compounds. Once the optimal temperature was established at 50 °C, the effect of extraction time was investigated. Using the same solvent system and conditions, three different sonication durations—30 minutes, 1 hour, and 2 hours—were tested. As presented in Fig. 27, no statistically significant differences were observed in TPC across the different extraction times. In contrast, PC content increased when extending the extraction time from 30 minutes to 1 hour, after which a plateau was reached, showing no further improvement with a 2 hour extraction. Based on these results, an extraction temperature of 50 °C combined with a duration of 1 hour was selected as the optimal condition. This balance ensures efficient recovery of both PC and phenolic compounds while minimizing the risk of thermal degradation and avoiding unnecessarily prolonged processing times, improving the overall efficiency and reproducibility of the extraction method.

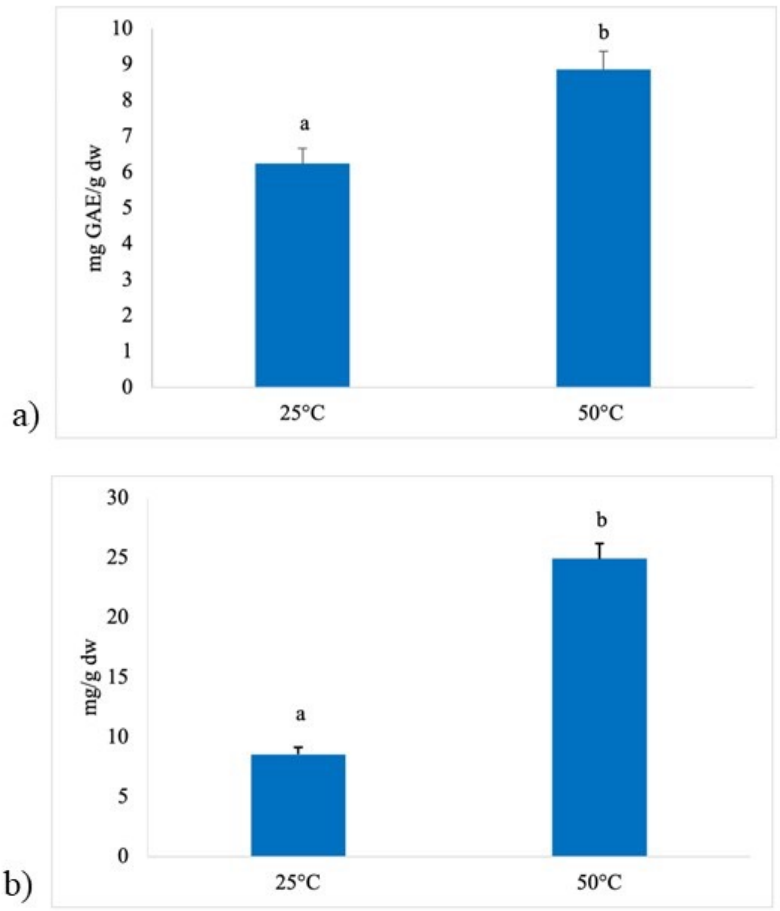

Fig. 26: TPC (a) and PC (b) concentration obtained by considering extraction temperatures at 25°C and 50°C. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistical statistically significant differences among results.

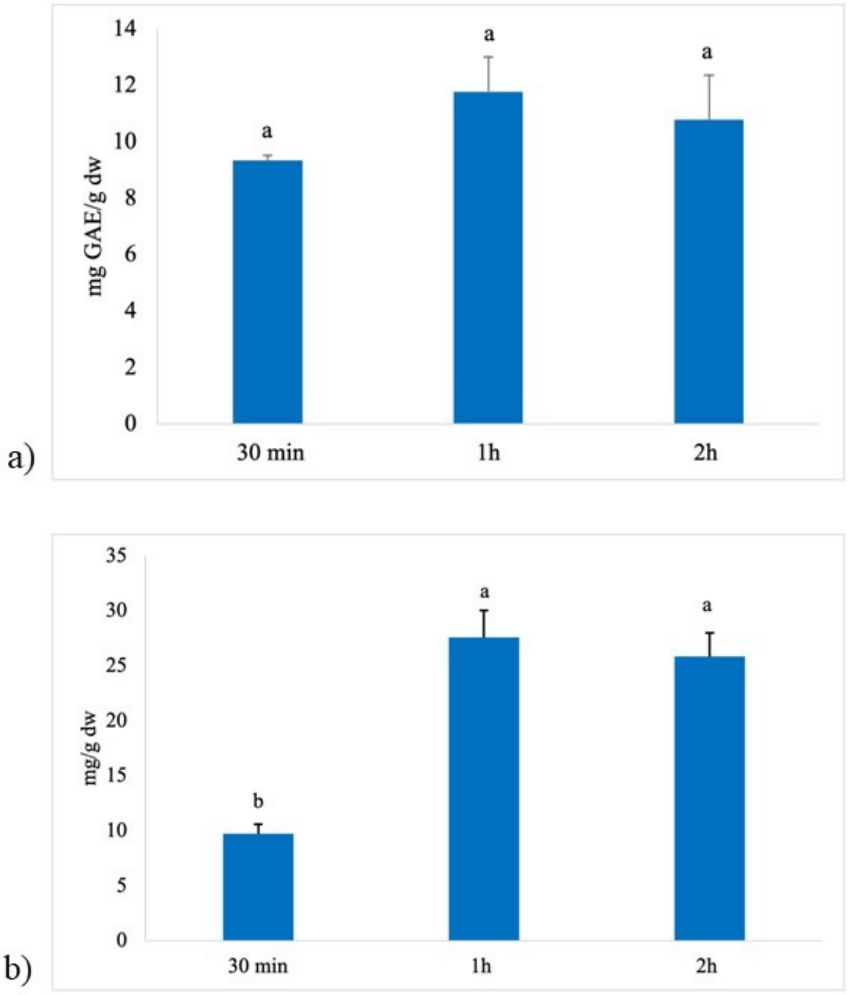

Fig. 27: TPC and PC concentration obtained by testing the extraction at different times. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results

5.3.5 Purity of the extract

An extract enriched in both phenolic compounds and PC was prepared under the previously optimized conditions. Specifically, 0.5 g of *Spirulina* powder was mixed with 7.35 mL of betaine–glucose NADES at a 1:2 molar ratio. The mixture was vortexed to ensure homogeneity and then placed in an ultrasonic bath at 50 °C, operating at a frequency of 37 kHz and a thermal power of 200 W, for 1 hour. After sonication, the samples were centrifuged at 5000 rpm for 15 minutes, and the supernatant was collected as the extract. The initial purity of the extract was 0.2. To enhance the purity of PC, the extract was subjected to ultrafiltration using Amicon® Ultra-15 centrifugal filters with a 100 kDa molecular weight cut-off. Subsequent analysis demonstrated that this filtration step effectively

increased the PC purity from approximately 0.2 to 0.5, as illustrated in Fig. 28 The use of Amicon® Ultra-15 centrifugal filters represents an innovative and successful approach for improving the purity of PC in NADES-based extracts, offering a simple and efficient alternative to more complex and time-consuming purification techniques.

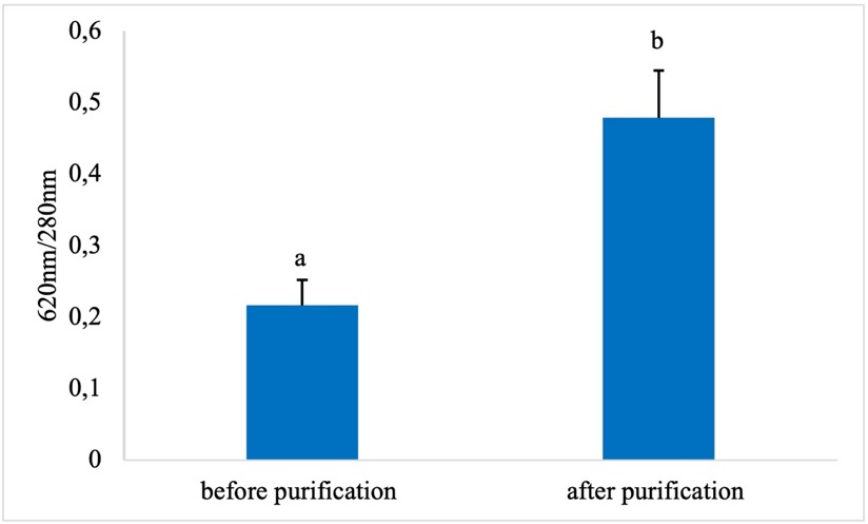

Fig. 28: Extract purity before and after centrifugation with Amicon Ultra-15. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results

5.3.6 Stability of extract

The PC stability in the extract obtained using betaine–glucose NADES under optimized conditions was evaluated and compared with that of a PC extract prepared in water. Both extracts were analyzed daily over a 60 days period using the spectrophotometric method described in Section 2.7 [166], and PC concentrations were determined accordingly.

The absorbance data collected during the 60 days storage period demonstrated that PC exhibited significantly higher stability in the NADES-based extract compared to the aqueous extract. As illustrated in Fig. 29, a gradual decrease in PC concentration was observed in both extracts over time. However, after 60 days, PC content in the water extract had decreased by approximately 53%, whereas the NADES extract showed a much smaller reduction of only 20%. These results indicate that betaine–glucose NADES is an effective solvent for obtaining a PC-enriched extract with

improved long-term stability. The stabilizing effect is likely associated with the osmoprotective role of betaine, which helps prevent protein degradation, while glucose may function similarly to trehalose, providing additional protection against structural denaturation. Although the precise mechanisms by which NADES stabilize proteins remain unclear, these findings support the hypothesis that NADES components—naturally occurring in biomass—can enhance protein stability through their inherent roles as osmolytes [169].

The optimized extraction procedure enabled the efficient recovery of both phenolic compounds and PC from Spirulina powder. Under the selected conditions—using betaine–glucose NADES supplemented with 30% water as the extraction solvent, a matrix-to-solvent ratio of 1:20 (w/w), an extraction temperature of 50 °C, and a sonication time of 1 hour—the yield of phenolic compounds was 11.77 ± 1.23 mg GAE per gram of Spirulina powder, while the PC content reached 27.56 ± 2.46 mg per gram of biomass. The resulting extract demonstrated enhanced stability of PC over time and a purity index of 0.5, indicating a significant reduction in contamination from other proteins or matrix components. By employing betaine–glucose NADES, the proposed method avoids the need for energy-intensive and environmentally demanding freeze–thaw cycles typically required to disrupt the microalgal cell wall. Furthermore, the sugar and NADES osmolyte composition contributes to the stabilization of PC, helping maintain its structure and functionality during storage. Overall, this approach represents an effective and sustainable strategy for the simultaneous extraction of phenolic compounds and PC from Spirulina. It produces an enriched extract with high bioactive content and improved stability, while reducing environmental impact and simplifying the extraction process compared to traditional methods that rely on organic solvents or repeated freeze–thaw treatment.

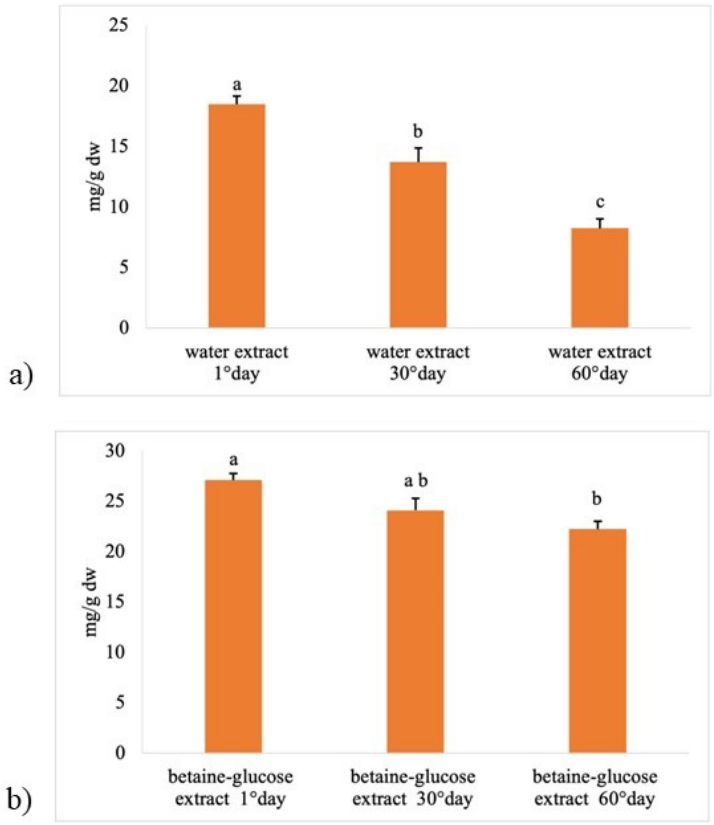

Fig. 29: Figures show the concentrations of PC in the water extract (a) and in DES extract (b) at 30- and 60-days intervals. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to define statistically significant differences. Different letters mean statistically significant differences among results.

5.3.7 HPLC/ESI-MS qualitative analysis

Spirulina is recognized as a rich source of phenolic compounds, including caffeic acid, chlorogenic acid, salicylic acid, quinic acid, gallic acid, and catechin [174]. To tentatively identify the phenolic compounds present in Spirulina powder, the extract obtained under the optimized extraction conditions was analyzed using the HPLC-PDA/ESI-MS method described in Section 5.2.10. The analysis allowed for the tentative identification of four phenolic compounds—caffeic acid, quercetin, rosmarinic acid, and chlorogenic acid—as well as two organic acids, citric acid and succinic acid, which are known for their potential therapeutic activities. The identification was based on the detection of the deprotonated molecular ions $[M-H]^-$ in the mass spectra. The chromatogram, shown in Fig. 30, was extracted at a wavelength of 323 nm to highlight the phenolic signals. Each peak in the chromatogram corresponds to an analyte listed in Table 4, which provides the identification

number, compound name, retention time (t_R), and m/z $[M-H]^-$ values. It is important to note that the identified compounds mainly represent the most abundant or detectable species under the adopted experimental conditions. In particular, both the NADES-based extraction procedure and the analytical technique employed may have influenced the observed profile. The extraction system may selectively favor specific classes of compounds, while the use of HPLC coupled with a single quadrupole mass spectrometer may not provide sufficient sensitivity and resolution for the identification of compounds present at low concentrations. Therefore, the limited number of identified compounds does not necessarily reflect the full chemical composition of the sample. The application of high-resolution mass spectrometry techniques could enable the identification of additional metabolites, including those present in trace amounts. In agreement with the literature, Spirulina exhibits a broader phenolic profile, including compounds such as gallic acid, salicylic acid, catechins, and other phenolic derivatives, which were not detected in the present study, likely due to methodological limitations or matrix effects. These results nevertheless confirm that the optimized NADES-based extraction method is effective in recovering a variety of bioactive phenolic and organic compounds from Spirulina, further supporting its potential as a source of nutraceutical ingredients.

Peak number	**Analyte**	**t_R**	**m/z $[M-H]^-$**
1	Citric acid	2.6	191
2	Caffeic acid	3.9	179
3	Succinic acid	9.5	117
4	Quercitrin	11.1	447
5	Rosmarinic acid	21.1	359
6	Chlorogenic acid	34.7	353

Table 4: Identification number (given in accordance with the elution order), name, tR and m/z ion of phenolic compounds and organic acids identified in the Spirulina extract.

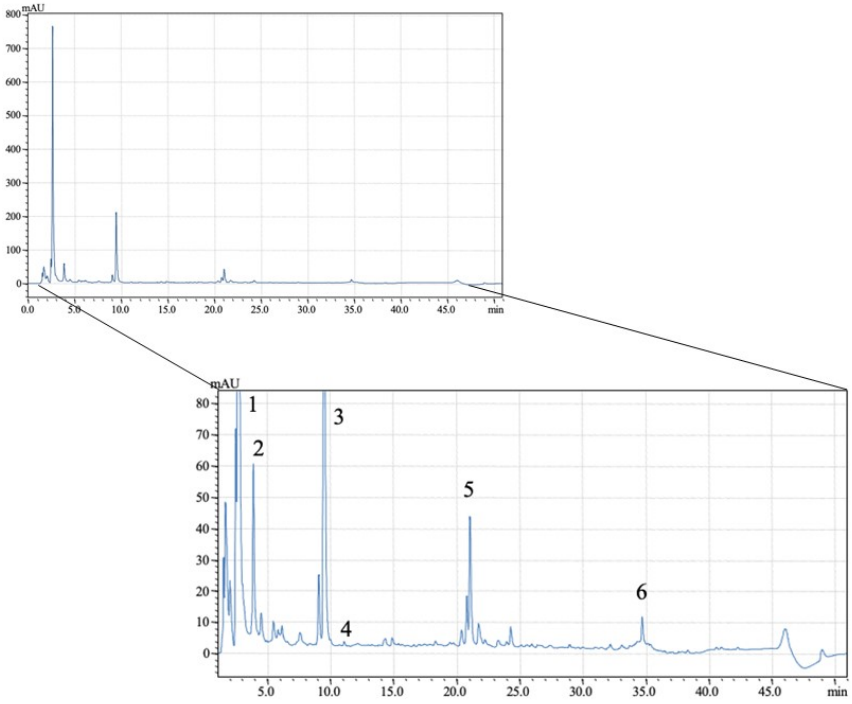

Fig. 30: HPLC-PDA phenolic compounds chromatogram (wavelength at 323 nm) of Spirulina extract. Peak number refer to Table 4

5.4 Conclusion

The developed and optimized extraction procedure represents an innovative, rapid, and environmentally friendly alternative to conventional methods for recovering phenolic compounds and PC from Spirulina. This approach is the first to successfully target the simultaneous extraction of both phenolic compounds and PC, providing an extract enriched in bioactive molecules suitable for applications in nutraceutical and cosmeceutical industries. The study confirmed the remarkable efficiency of NADESs in extracting phenolic compounds, achieving yields that surpass those obtained with traditional organic solvents. Beyond enhancing extraction efficiency, NADESs improve the permeability of the microalgal cell membrane, eliminating the need for labor-intensive freeze–thaw cycles typically required to release PC. This feature makes the method not only faster but also more sustainable and environmentally responsible. For applications in nutraceuticals, it is crucial to obtain a PC extract free from contaminating proteins. In this context, the use of Amicon® Ultra-15

centrifugal filters proved to be a highly effective and practical strategy for increasing the purity of PC, offering a simple and reproducible alternative to more complex purification techniques. Furthermore, the combination of betaine and glucose in the NADES contributes to the long-term stabilization of PC, thanks to the osmoprotective properties of betaine and the potential protein-stabilizing effect of glucose. As a result, the optimized method produces a bioactive extract with high purity, enhanced stability, and a rich content of both phenolic compounds and PC. This makes it a powerful and versatile approach for the development of functional ingredients and products in the fields of nutraceuticals, cosmeceuticals, and beyond, where the preservation of bioactivity and structural integrity of compounds is essential.

CHAPTER 6

6. Supramolecular deep eutectic solvents (SUPRADESs) as extraction media for bioactive molecules from plant matrices

6.1 Introduction

Phenolic compounds represent one of the largest and most important classes of secondary metabolites produced by plants. These molecules, characterized by the presence of one or more aromatic rings with hydroxyl groups, are widely distributed throughout the plant kingdom and play a crucial role in both plant physiology and interactions with the surrounding environment [12].

From a chemical standpoint, phenolic compounds are distinguished by their benzene ring-based structure, which confers specific physicochemical properties, such as the ability to form hydrogen bonds and participate in redox reactions. This structure underpins the fundamental role of phenolic compounds in protecting plants from various forms of oxidative and environmental stress [175].

The diversity of phenolic compounds is extensive, encompassing numerous subclasses, each with distinct characteristics. These subclasses include phenolic acids, flavonoids, tannins, and lignins. Phenolic acids, including caffeic and ferulic acid, have been identified as key components in the reinforcement of cell walls, thereby contributing to mechanical strength and defense against pathogens [22]. Flavonoids, one of the most extensively studied families, contribute to the pigmentation of flowers, fruits, and leaves, facilitating ecological processes such as pollination and seed dispersal. Tannins are high-molecular weight polyphenols that act as chemical deterrents against herbivores due to their ability to bind proteins and reduce digestibility. Lastly, lignins are phenolic polymers that confer rigidity and impermeability to cell walls, supporting the development of woody tissues [176].

The functions of phenolic compounds in plants are essential for survival. They participate in defense mechanisms against biotic stress, such as infections by pathogens and herbivore attacks, as well as abiotic stress, including exposure to UV radiation, drought, and salinity. They exhibit antioxidant properties, as they can neutralize free radicals generated under stress conditions, protecting plant cells

from oxidative damage. Moreover, phenolic compounds contribute in regulating plant growth and development through interactions with hormones. They also influence ecological interactions by promoting symbiotic relationships with beneficial microorganisms, such as mycorrhizal fungi and rhizosphere bacteria [175].

Beyond their role in plant physiology, phenolic compounds are of great interest for their impact on human health. Several studies have highlighted their antioxidant, anti-inflammatory, antimicrobial, and potentially anticancer properties. For this reason, these compounds are gaining increasing importance in the fields of nutraceuticals and pharmacology [177]. Their ability to modulate oxidative and inflammatory processes in the human body positions them as key components in the prevention of chronic and degenerative diseases [178].

Quercetin is one of the most abundant and extensively studied flavonoids in plants, belonging to the flavonol subclass. Chemically, it is a polyphenol with a three-ring structure and multiple hydroxyl groups that determine its chemical and biological properties. (Fig. 31) Its molecular formula is $C_{15}H_{10}O_7$, and its structure allows quercetin to act as an effective antioxidant [179].

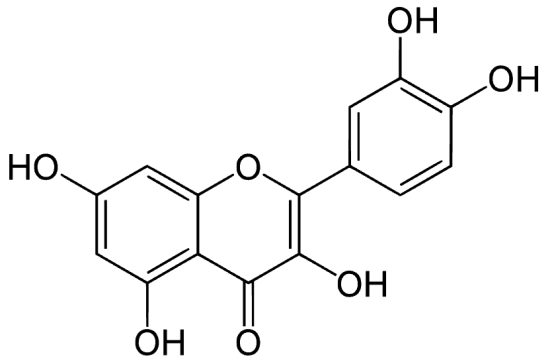

Fig. 31: Quercetin structure

Quercetin is present in many plants, particularly in fruits, vegetables, leaves, and seeds. Rich dietary sources include apples, onions, green tea, citrus fruits, berries, and grapes. It is well known for its ability to neutralize free radicals, protecting cells from oxidative damage [180].

Biologically, quercetin performs multiple functions. In addition to its potent antioxidant activity, it exhibits anti-inflammatory, antiviral, antibacterial, and anticarcer properties. Numerous *in vitro* and

in vivo studies have demonstrated its potential to inhibit inflammatory pathways, modulate immune responses, and interfere with abnormal cell proliferation [181].

In the nutraceutical and pharmacological fields, quercetin is being studied for its beneficial effects in the prevention and treatment of chronic diseases such as cardiovascular disorders, diabetes, neurodegenerative diseases, and certain types of cancer. Its ability to improve endothelial functions, reduce hypertension, and regulate glucose levels in blood highlight its value for human health [182].

Despite its numerous potential benefits, quercetin's bioavailability in the human body is relatively low, due to its poor water solubility (logP:1.5) and rapid metabolism. As a result, research is also focused on the development of innovative formulations to enhance its absorption and therapeutic efficacy. One effective strategy involves encapsulation in CDs, a family of cyclic oligosaccharides derived from starch [183].

CDs are cone-shaped molecules composed of glucose units linked together to form a ring with a hydrophobic internal cavity and a hydrophilic external surface. This structure enables CDs to host hydrophobic or poorly water-soluble molecules, such as quercetin, by forming inclusion complexes. The encapsulation of quercetin within CDs improves its aqueous solubility while simultaneously protecting it from chemical and metabolic degradation [184]. Additionally, this technique enhances the molecule's thermal and photochemical stability, making it more resistant during storage and administration [185].

From a pharmacokinetic perspective, quercetin–CD complexes facilitate intestinal absorption and controlled release, improving bioavailability and therapeutic efficacy. Multiple studies have shown that the oral administration of CD-quercetin complexes increases the phenol's concentration in plasma, ensuring more pronounced biological effects with lower doses [186].

Beyond improving bioavailability, CD encapsulation may also reduce gastrointestinal side effects and enhance tolerability, making this technology particularly attractive for the development of quercetin-based dietary supplements and pharmaceutical products [187,188].

SUPRADESs, as previously discussed in Paragraph 2.2.2, are innovative solvents characterized by the presence of CDs, which confer unique supramolecular properties. These properties enable SUPRADESs to form inclusion complexes with hydrophobic or poorly soluble molecules. This capability makes SUPRADESs highly effective as extraction solvents, improving both selectivity and efficiency in the recovery of bioactive compounds [189].

They are particularly suited for extracting natural substances such as flavonoids and alkaloids, often found in complex matrices such as plants or agro-industrial residues. In addition to enhancing the solubilization of these compounds, the inclusion of CDs also protects them from chemical and oxidative degradation during the extraction process, ensuring greater stability and preserving their functional and therapeutic properties [189].

Numerous studies in the literature have highlighted the SUPRADESs potential as effective extraction solvents. For instance, Huang et al. [190], tested various SUPRADES-based formulations for extracting phenolic compounds from tea, achieving promising results.

The high extraction efficiency of SUPRADESs is largely attributed to their extensive hydrogen bonding network, which facilitates interaction with polyphenols and enhances their solubility. In this way, SUPRADESs have proven to be versatile and powerful tools for the extraction of bioactive molecules from natural matrices [190].

Zhang et al. [191], developed and optimized an innovative method for extracting flavonoids from Scutellariae Radix using SUPRADESs. Specifically, they tested various DES-based formulations enriched with both native and modified CDs. Among the tested formulations, the one composed of DMBA and OA with the addition of 5.5% β-CD proved to be the most effective. The presence of CDs facilitated host–guest interactions with flavonoids, significantly improving extraction yield compared to conventional DESs.

SUPRADESs have shown promising developments in the field of analytical applications, serving versatile roles not only as extraction solvents but also as desorption agents, sorbent materials, phase modifiers, and chiral selectors. Table 5 lists several types of SUPRADESs along with their respective

applications, highlighting the diversity and effectiveness of these systems in various analytical contexts [133].

Method	SUPRADESs	Sample	Analyte	Analytical instrument	Results Extraction yield	Refs.
UA-SLE	1.5 % w/v β-CD in L-LA/ammonium acetate (7:1)	Medicinal plants (thyme, oregano, Greek sage, and sage	Polyphenols	UV-Vis	62.0-117.5 mg CAE/g	[141]
SA-SLE	1.0 % w/v HP-β-CD in glycerol/ sodium acetate (6:1)	Moringa oleifera leaves	Polyphenols	Uv-Vis and LC-DAD-MS	66.3 0.5 mg GAE/g	[192]
SA-SLE	0.7 % w/v β-CD in L-LA/ammonium acetate (7:1)	Olive leaf	Polyphenols		113.7 mg CAE/g	[193]
UA-SLE	3 % w/w HP-β-CD in ChCl/malic acid (1:1)	Chokeberry fruits	Anthocyanins	HPLC-DAD	N.R.	[194]
UA-SLE	β-CD/LA (1:1)	Bayberry	Polyphenols	Uv-Vis	28.9 1.3 mg GAE/g	[195]
UA-SLE	HP-β-CD/L-LA (1:5)	White tea	Polyphenols	Uv-Vis	111.4 2.3 mg GAE/g	[190]
VA-SLE	5.5 % w/w β-CD in DMBA/OA (1:2)	Scutellarie Radix	Flavonoids	HPLC-UV	158 mg/g	[191]

Table 5: Applications of SUPRADESs in analytical studies

The project therefore aimed to extract quercetin using SUPRADESs. The working hypothesis underlying the study was that the formation of an inclusion complex between quercetin and CDs could improve the solubility and stability of the molecule, as well as reduce its metabolic rate,

resulting in an increase in overall bioavailability. Quercetin is a flavonoid widely found in the plant kingdom, but its application in nutraceuticals and pharmaceuticals is limited by its low water solubility and rapid metabolic degradation. The use of SUPRADES therefore represents a promising approach to overcoming these critical issues, allowing for more efficient and selective extraction of bioactive compounds and, at the same time, their better preservation and biological availability. Before applying the methodology to actual plant matrices, preliminary tests were conducted under controlled laboratory conditions to evaluate the main extraction parameters and to verify the ability of SUPRADES to form stable complexes with quercetin. These initial tests made it possible to optimize the operating conditions and confirm the validity of the proposed approach, laying the foundations for the application of the method to real samples of plant origin.

6.2 Materials and methods

6.2.1 Chemicals

ChCl, Urea, β-CD, levulinc acid, quercetin standard, methanol (MeOH) and EtOH (HPLC grade), Phosphate buffer prepared from sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4), hexan, Isoamyl acetate (reagent grade 98%) were supplied by Sigma-Aldrich (Milan, Italy). Water (HPLC-MS grade) and formic acid (95–97 %) were purchased by Sigma-Aldrich (Milan, Italy). Acetonitrile (99.9 %) hypergrade for LC-MS and gallic acid were supplied by Merck KGaA (Darmstadt, Germany).

6.2.2 Samples

Preliminary tests were carried out on standard quercetin solutions to evaluate the extraction efficiency of SUPRADESs in controlled conditions prior to proceeding with complex plant matrices.

Solutions with a known concentration (100 μg/mL) of quercetin in MeOH and isoamyl acetate were prepared from the standard solution of quercetin in dimethyl sulfoxide (DMSO) (10.000 μg/mL).

6.2.3 SUPRADESs preparation

Two types of SUPRADESs were prepared: β-CD–levulinic acid (1:27 molar) and ChCl–urea (1:2) + 2% β-CD.

The β-CD and levulinic acid were weighed in the indicated molar proportions and mixed at 60 °C under magnetic stirring for one hour, until a homogeneous and transparent liquid was obtained.

To prepare the ternary SUPRADES, DES was first produced consisting of ChCl and urea in a molar ratio of 1:2. The two components, weighed appropriately, were heated to 80°C until a homogeneous liquid was obtained. Subsequently, 2% (w/w) β-CD was added and the mixture was kept at 60°C for about 1 hour to obtain the final SUPRADES.

The systems obtained were characterized by FTIR spectroscopy to verify the formation of hydrogen bonds typical of DESs and the stability of the supramolecular system.

6.2.4 Absorption experiments

Two different SUPRADESs were tested; one consisting of β-CD–levulinic acid (1:27) and the other of ChCl–Urea + 2% β-CD—to evaluate their effectiveness in recovering quercetin from a standard solution (100 µg/mL).

For the SUPRADES formed by β-CD–levulinic acid, 1000 µL of this SUPRADES were added to 500 µL of a quercetin standard solution (100 µg/mL) prepared in MeOH.

The absorption capacity of SUPRADES formed by ChCl–Urea + 2% β-CD was evaluated by mixing 1000 µL of SUPRADES with 500 µL of a standard solution of quercetin (100 µg/mL) prepared in isopentyl acetate.

It was necessary to change the solvent of the standard in order to ensure its immiscibility with SUPRADES, thus allowing the formation of two separate phases. In fact, for both experiments, after shaking for 5 minutes, the system separated into two phases: a lower phase represented by SUPRADES, which should retain quercetin, and an upper phase represented by the solvent of the standard solution. The upper phase was collected and analyzed by HPLC.

Once the most effective SUPRADES was identified, three versions of this SUPRADES were tested to evaluate their quercetin absorption capacity: one containing 1% CD, one with 2% CD, and one consisting of pure DES without CDs.

After selecting the SUPRADES with the best absorption ability, different volume of quercetin standard solution- volume of SUPRADES ratios were tested:

- 500 µL of quercetin standard (100 µg/mL) + 1000 µL of SUPRADES (1:2)
- 500 µL of quercetin standard (100 µg/mL) + 500 µL of SUPRADES (1:1)
- 1000 µL of quercetin standard (100 µg/mL) + 500 µL of SUPRADES (2:1)

6.2.5 Optimization of quercetin release from SUPRADES

To estimate the actual recovery of quercetin from the standard solution using SUPRADES, an HPLC analysis of the extract was performed. However, when the SUPRADES containing quercetin was injected, no signal was detected. Assuming that quercetin was indeed contained within the CDs, it was necessary to break the quercetin–β-CD complex to release the quercetin. Since the complex is stabilized by hydrogen bonds, van der Waals forces, and hydrophobic interactions, these forces had to be overcome to free the molecule. Hexane and a phosphate buffer at pH 7 were tested as potential solvents to break the quercetin–β-CD complex. A volume of 500 µL of the two solvents was added to the recovered SUPRADES. The mixtures were stirred for 30 minutes, then the solvents that should have contained quercetin were collected and analyzed by HPLC.

Since the results were not satisfactory, it was decided to first test a mixture of phosphate buffer and MeOH, and then MeOH alone.

After identifying the most efficient solvent (MeOH) for quercetin release, both the SUPRADES volume-solvent volume ratio the contact time were optimized. Specifically, the SUPRADES volume was kept constant at 500 µL, while solvent volumes of 1 mL and 2 mL were tested. Once the most effective solvent volume was selected, the contact time between the SUPRADES and the chosen

solvent was optimized. Three conditions were tested: 30, 60, and 90 minutes, in order to identify the optimal conditions for maximizing quercetin release.

To increase the environmental sustainability of the process EtOH was tested as green solvent to replace the organic one, while maintaining the optimized conditions.

6.2.6 Evaluation of CDs efficiency

After optimizing the absorption and release procedure, another test was performed to evaluate the efficiency of the CDs. Three versions of the optimized SUPRADES were tested to evaluate their ability to absorb quercetin: one containing 1% CD, one containing 2% CD, and one composed of pure DES without CD.

6.2.7 HPLC analysis

Quercetin determination was performed through; Shimadzu Prominence LC-20A chromatograph (Shimadzu, Milan, Italy) was used, equipped with two pumps (LC-20 AD XR), an autoinjector (SIL-10ADvp), a column furnace (CTO-20 AC), a degasser (DGU-20 A3) and coupled with a DAD (SPD-M10Avp). Shimadzu LC solution Ver. 3.7 (Shimadzu, Tokyo, Japan) program was selected. The column selected for analysis was an Ascentis® C18 (15cm x 4.6 mm x 3 μm) (Merck KGaA, Darmstadt, Germany). The analytes were eluted with a constant flow of 1 mL min− 1 with a column temperature of 40°C. The mobile phase was composed by an acidified aqueous solution with 0.1 % (v/v) formic acid (A) and acetonitrile acidified with 0.1 % formic acid (B). The injected volume was 2 μL and the gradient used for the separation of the phenolic compounds was: t0' =10 % B; t20'=100 %B; t25'=100 % B

6.3 Results

6.3.1 Absorption experiments

SUPRADESs β-CD–levulinic acid (1:27) and ChCl–Urea + 2% β-CD were tested as extraction solvents to evaluate their ability to recover quercetin from a standard quercetin solution (100 μg/mL). Extraction with 500 μL of quercetin standard (100 μg/mL) and 1000 μL of SUPRADES (β-CD–

levulinic acid) showed a theoretical recovery of 50% quercetin. Meanwhile, the extraction performed with SUPRADES composed of ChCl–Urea + 2% β-CD showed a theoretical recovery of 100% for quercetin. Fig. 32 clearly shows that the most efficient SUPRADES is ChCl–Urea + 2% β-CD.

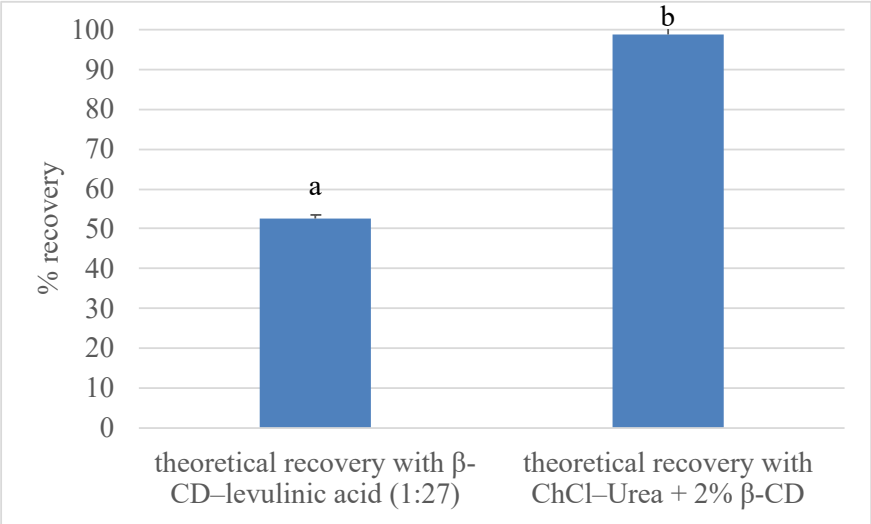

Fig. 32: Theoretical recovery of quercetin using two different types of SUPRADES: β-CD–levulinic acid (1:27) and ChCl–Urea + 2% β-CD

The most efficient SUPRADES were selected for subsequent testing. In particular, the following ratios between the volume of the quercetin standard solution and the volume of SUPRADES were tested:

- 500 µL of quercetin standard (100 µg/mL) + 1000 µL of SUPRADES (1:2)
- 500 µL of quercetin standard (100 µg/mL) + 500 µL of SUPRADES (1:1)
- 1000 µL of quercetin standard (100 µg/mL) + 500 µL of SUPRADES (2:1).

As can be seen in Fig. 33, the extraction performed with SUPRADES consisting of ChCl–Urea + 2% β-CD showed a theoretical quercetin recovery of 100% with both a 1:1 and a 1:2 ratio, while with a 2:1 ratio (1000 µL of quercetin and 500 µL of SUPRADES) the recovery dropped to 31%. Based on these results, the 1:1 ratio was selected as the most effective.

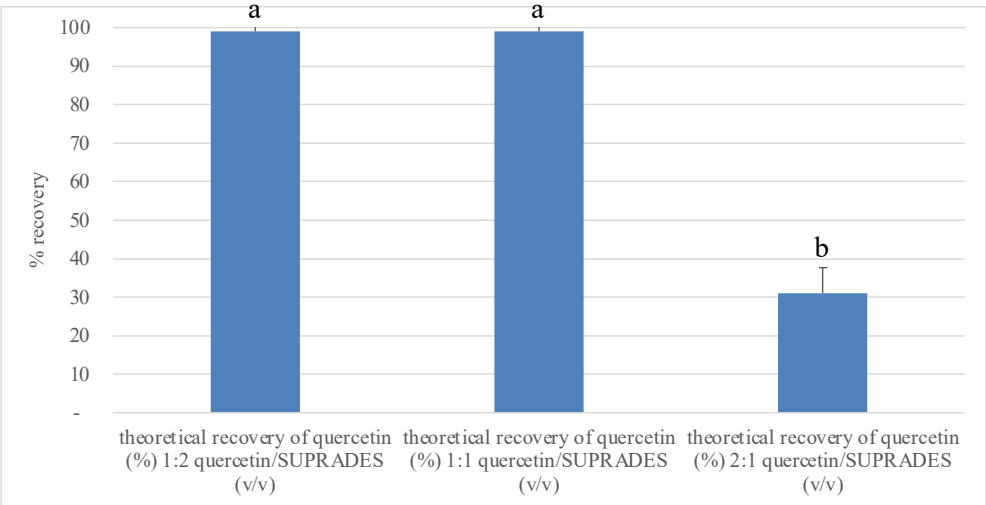

Fig. 33: The figure shows the theoretical recovery percentages of the experiments conducted at different quercetin: ChCl–Urea + 2% β-CD ratios.

6.3.2 Optimization of quercetin release from SUPRADESs

To optimize the release of quercetin from SUPRADES, different solvents were tested: hexane, phosphate buffer at pH 7, phosphate buffer at pH 7 combined with MeOH, and only MeOH. Considering that this complex is stabilized by hydrogen bonds, van der Waals interactions, and hydrophobic interactions, several strategies were developed to overcome these intermolecular forces. First, hexane was used, selected for its ability to disrupt hydrophobic interactions. In parallel, a phosphate buffer at pH 7 was employed, chosen for its potential to interfere with hydrogen bonds. However, neither the addition of 500 µL of hexane nor the use of 500 µL of phosphate buffer at pH 7 allowed a detectable release of quercetin.

Based on these results, a new strategy was therefore developed, based on the combination of phosphate buffer and MeOH, with the aim of combining the effect of a polar solvent with that of an organic co-solvent capable of reducing hydrophobic interactions. This treatment allowed the release of approximately 30% of the quercetin. To evaluate the role of the buffer, pure MeOH was then tested (1 mL of MeOH added to 500 µL of SUPRADES containing quercetin), resulting in an 80% release. (Fig. 34) This indicated that the buffer was not necessary; therefore, MeOH was selected as the solvent for quercetin release.

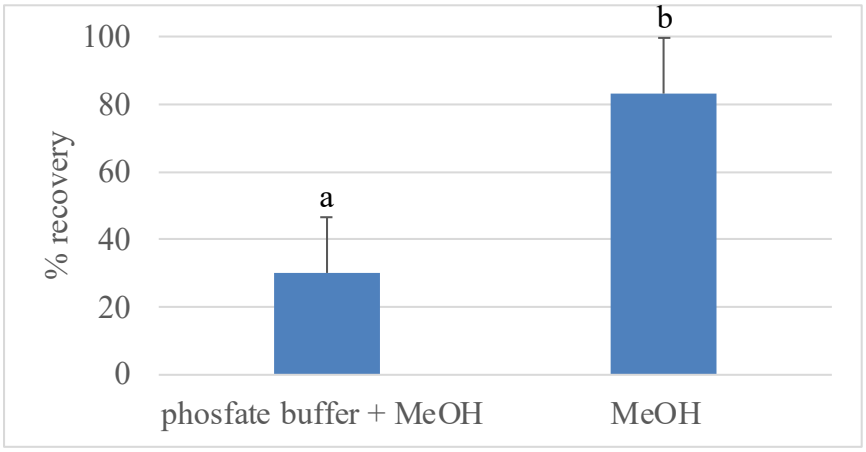

Fig. 34: The Figure shows the percentage of quercetin released from SUPRADES using phosphate buffer + MeOH and only MeOH.

Subsequently, both the SUPRADES volume and the solvent volume ratio, as was well as the contact time, were optimized. Specifically, the SUPRADES volume was kept constant at 500 µL, while solvent volumes of 1 mL and 2 mL were tested. As shown in the Fig. 35, increasing the MeOH volume up to 2 mL did not improve quercetin recovery; therefore, 1 mL was chosen as the optimal volume. While extending the contact time (30, 60 or 90 minutes) led to a decrease in release, probably due to the re-encapsulation of quercetin in the β-CD cavity (Fig. 36), for this reason, the best time is 30 minutes.

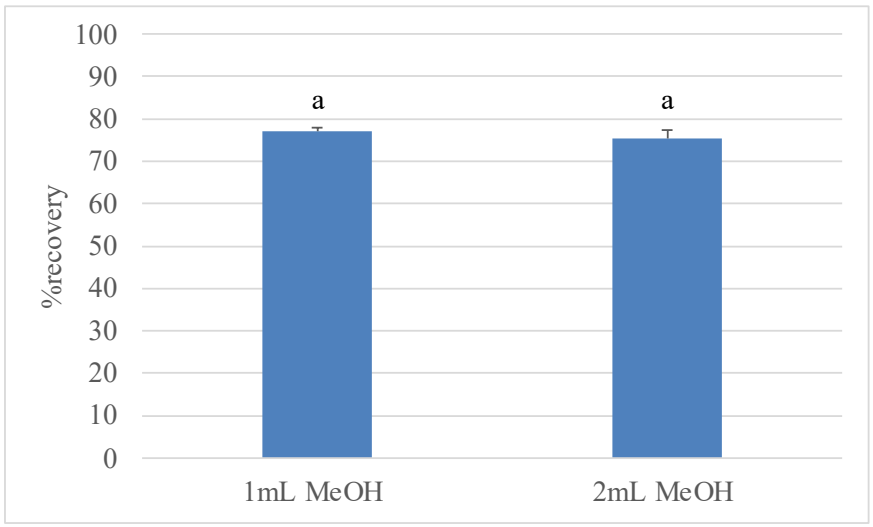

Fig. 35: The figure shows the percentage release of quercetin from SUPRADES using 1 mL of MeOH and 2 mL of MeOH.

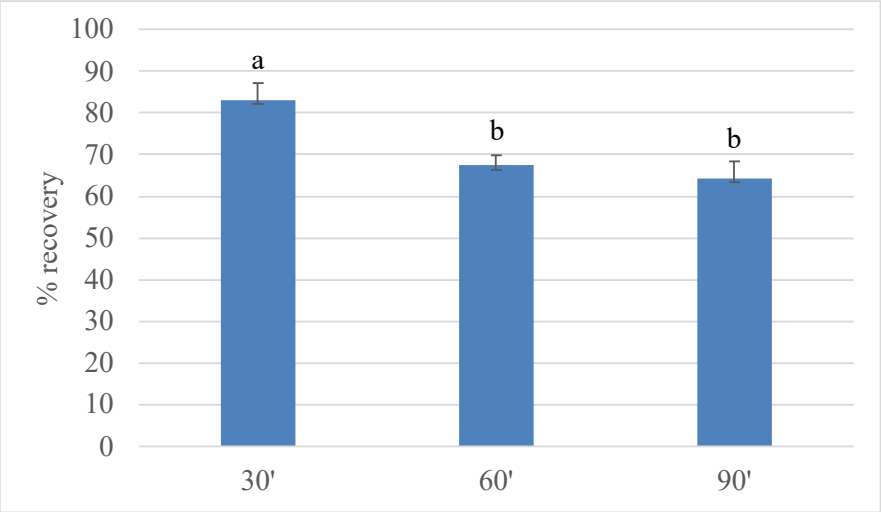

Fig. 36: The graph shows the percentage of quercetin released from SUPRADES by increasing the contact time between SUPRADES and MeOH.

Since MeOH is toxic and not considered a "green" solvent, EtOH was tested as a safer alternative. Keeping the previously optimized conditions, EtOH was used as the release solvent. In practice, adding 1 mL of EtOH to 500 µL of the SUPRADES–quercetin complex, followed by shaking for 30 minutes and HPLC-PDA analysis, allowed 70% of the quercetin to be released (Fig. 37). Although the release efficiency with EtOH is slightly lower than with MeOH, EtOH was still preferred because it is non-toxic and therefore safer.

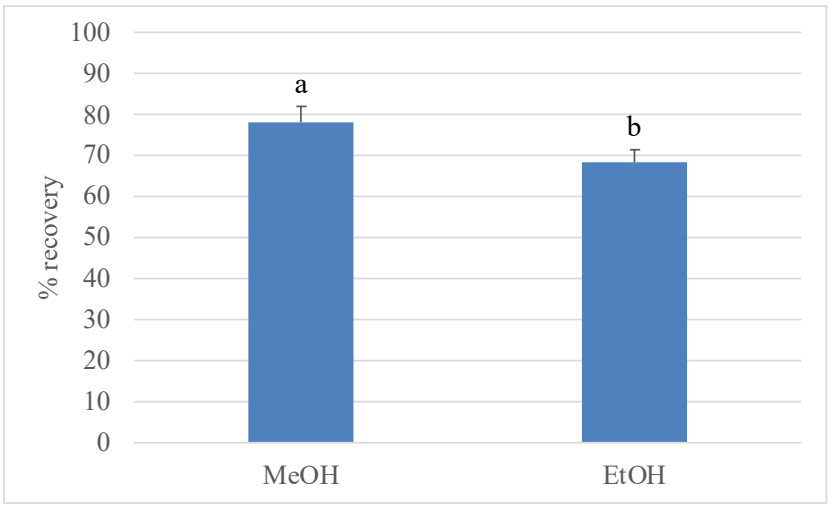

Fig. 37: The figure shows the percentage of quercetin released from SUPRADES using MeOH and EtOH.

6.3.3 Evaluation of CDs efficiency

The optimized procedure can be described as follows: SUPRADES composed of ChCl–Urea + 2% β-CD was used a 1:1 v/v ratio with the quercetin standard solution. Subsequently, the addition of 1 mL of ethanol to SUPRADES, followed by 30 minutes of stirring, resulted a 70% release of quercetin. To evaluate the actual efficiency of CDs, and maintaining the optimized conditions, three tests were then performed using: SUPRADES containing 1% CD, SUPRADES containing 2% CD, and DES without CDs. The results confirmed the importance of CDs. In fact, as can be seen from the Fig. 38, we obtain a greater release using SUPRADES with 2% CD.

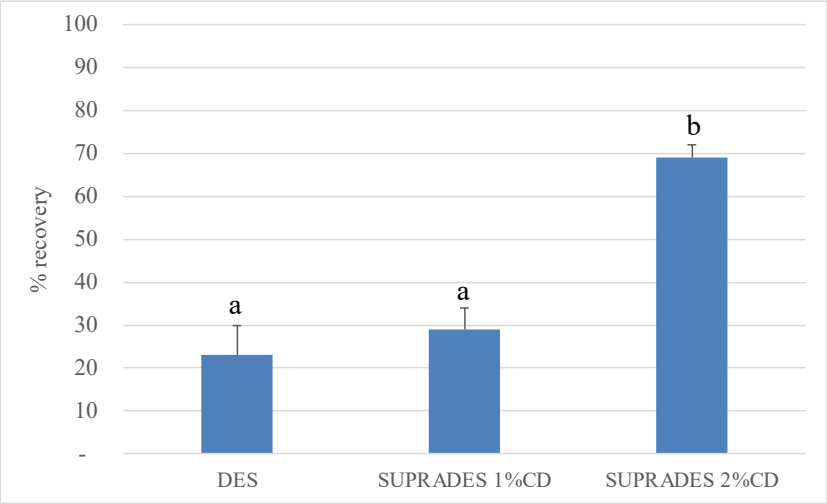

Fig. 38: Percentage of quercetin released using the SUPRADES made of ChCl–Urea with 1% β-CD, 2% β-CD, and without β-CD (pure DES).

6.4 Conclusion

The study demonstrated that SUPRADES containing β-CD are highly effective systems for the extraction of poorly soluble bioactive molecules, such as quercetin. In particular, using the SUPRADES composed of ChCl–Urea + 2% β-CD appears that almost all quercetin can be recovered from the standard solution. However, the complex formed is not detectable by HPLC analysis. For this reason, it was important to optimize a method for releasing quercetin from the complex so that it

could be quantified. The use of EtOH as the release solvent showed a good compromise between efficiency (70%) and environmental sustainability. The next stages of the research involve applying the optimized method to natural matrices rich in quercetin, such as onion skins, capers, and Ginkgo biloba leaves, to verify its effectiveness under real operating conditions. Further objectives include evaluating the stability of the quercetin–β-CD complex over time and investigating the potential applications of SUPRADES in the nutraceutical and pharmaceutical sectors, where the use of sustainable technologies and environmentally friendly processes is an emerging priority.

CHAPTER 7

7. Conclusion

Research has shown that both DES and SUPRADES are excellent alternatives to traditional solvents for extracting bioactive molecules from plant matrices. Both projects were developed in line with the principles of green chemistry, with the aim of reducing the environmental impact of extraction processes while improving their efficiency, selectivity, and compatibility with nutraceutical, cosmetic, and pharmaceutical applications.

In particular, the first project, “Deep Eutectic Solvents based green approach for bioactive molecules recovery from Spirulina,” demonstrated that DES are excellent extraction solvents for both phenolic compounds and PC, with yields superior to those obtained with traditional organic solvents.
The result was an extract of high PC purity. Furthermore, the combination of betaine and glucose in NADES helps to stabilise PC in the long term, thanks to betaine's osmoprotective properties and glucose's potential to stabilize proteins.
Consequently, the optimized method produces a highly pure, stable extract with a rich content of both phenolic compounds and PC. A subsequent study could focus on the use of such an optimized extract for the development of products in the nutraceutical and cosmeceutical fields, where preserving the bioactivity and structural integrity of the compounds is essential.

The second line of research highlighted how SUPRADES, obtained by integrating β-CD into DES matrices, have a remarkable ability to extract poorly soluble molecules such as quercetin. The formation of inclusion complexes between CDs and target compounds demonstrates the potential of these supramolecular systems as new tools for the purification and valorization of secondary metabolites of natural origin. Using the SUPRADES composed of ChCl–Urea + 2% β-CD, it appears that almost all of the quercetin in the standard solution can be recovered. However, the formed complex is not detectable by HPLC analysis. Therefore, it was important to optimize a method of releasing quercetin from the complex for quantification. Using EtOH as the release solvent achieved

a good balance between efficiency (70%) and environmental sustainability. This result confirms the feasibility of incorporating SUPRADES into 'green' extraction and purification processes, thereby reducing the ecological impact.

Overall, the results obtained suggest that DES and SUPRADES are a genuinely environmentally friendly alternative to conventional solvents, offering advantages in terms of efficiency, safety, sustainability and reusability. This work therefore contributes to progress in green analytical chemistry, providing a solid foundation for future developments in the sustainable extraction of bioactive compounds from natural sources.

Looking ahead, a particularly promising goal is to apply SUPRADES to the extraction of quercetin directly from plant matrices. This approach would not only yield an extract rich in quercetin, but also promote its encapsulation in situ within the CD already present in the SUPRADES system. Such a strategy could significantly increase the bioavailability of the molecule, overcoming the known limitations associated with its poor solubility and rapid metabolic degradation.

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List of papers and congress

Della Posta, S., Cutè, E., De Gara, L., & Fanali, C. (2025). Deep Eutectic Solvents based green approach for bioactive molecules recovery from Spirulina. Journal of Chromatography A, 1743, 465695.

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XXVIII Congresso Nazionale della Società Chimica Italiana (SCI 2024) "Development and optimization of analytical method for the extraction of polyphenols and phycocyanin from Spirulina followed by HPLC-MS analysis" (poster presentation)