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A GREEN APPROACH FOR THE RECOVERY OF CHLOROPHYLL FROM UNSOLD SPINACH: STABILIZATION AND ENCAPSULATION

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Abstract

Food waste represents a major problem from an economic and environmental point of view. In the perspective of the Circular Economy, food waste may be an excellent source for the recovery of high added value molecules, such as colorants. Riding the wave of ever-increasing demand of natural colorants recovered by *green* methods, the aim of this PhD thesis project is to develop a custom-made protocol for the extraction, stabilization and encapsulation of chlorophyll (Chl) from vegetable waste, to be used as a natural food colorant. A tailored Enzyme-Assisted Extraction (EAE) approach has been developed for the conservative recovery of Chl-containing chloroplast from unsold spinach, ensuring the stability and protection of pigment. Furthermore, since Chl can undergo a series of reactions leading to the loss of central magnesium, the use of zinc as substituent (able of restoring stability) has been studied. The plant matrix was characterized in terms of cell wall polysaccharide composition, and a tailored enzymatic mix based on cellulase (40%) xylanase (41%) and polygalacturonase (19%) was formulated. The process variables [temperature (°C), time (h), enzyme mix dose (U/g), $ZnCl_2$ concentration (ppm), and buffer/substrate ratio (B/S)] and their interactions were studied by Response Surface Methodology (RSM). The overlay plot made it possible to identify the process conditions (T: 25 °C, Zn: 150 ppm e B/S: 17.5, t: <2 h and enzyme mix dose between 12 and 45 U/g) to maximize the amount of Chl, and concurrently, the quality of the green color of the extract. The novel colorant was, then, applied in the production of a real food (baghrir). Moreover, the Chl extraction kinetic and recovery yield was further investigated using an organic solvent-free method, based on limited dosing options (biocatalyst and $ZnCl_2$) for the quick and mild recovery of Chl. The kinetic of pigment extraction turned out to be a very useful tool to identify the proper conditions, in terms of biocatalyst dose (0.10–50 U/g), extraction time (1–48h), and $ZnCl_2$ amount (50–300 ppm), both for enhancing the recovery yield and preserving the greenness. Considering the extraction kinetic, the recovery yield, and the colorimetric data, the suitable conditions to produce a stable green and food-grade colorant are: 0.10 U/g of enzyme, 3h, and 150 ppm of $ZnCl_2$ at 25 °C. The extraction yield of Chl (4863μg/U) was about 51% greater than control, with a higher extraction rate constant (5.43×10^{-4} g/(μg min)).

Considering the impact of $ZnCl_2$ amount on Chl, its protective action resulted to be more noticeable toward Chl *a*: at 150 ppm, an increased amount of about 2.5 and 1.5 times was found for Chl *a* and Chl *b*, respectively, in comparison to the reference (0 ppm $ZnCl_2$). The EAE and the simultaneous use of two metal ions (zinc and copper) combined at a 10:1 ratio, was also tested in order to improve the pigment's stability keeping it inside intact chloroplasts. Optimized via RSM, this combined approach allowed us to increase the amount of recovered intact chloroplasts, improving Chl stability and preserving its green color. Applied to meringues, the pigment maintained the green color without affecting sensory qualities at lower concentrations (1-3% w/w). Finally, since Chl application is limited by its poor solubility and stability in aqueous solutions, especially under acidic conditions, a novel approach to enhance the solubility and stability of zinc modulated Chl (Chl-Zn^{2+}) by complexation with whey protein isolate (WPI) and alginate (ALG) was investigated. Encapsulation of Chl-$Zn^{2+}$with WPI prevented its precipitation between pH 3 and pH 7, except at the isoelectric point (pI) of WPI (around pH 4.5). The visual appearance, colorimetric measurements, and absorbance spectra indicated that Chl-Zn^{2+} formed stable complexes with WPI at various pH. Additionally, the inclusion of ALG prevented isoelectric precipitation at pH 4.5, likely due to electrostatic interactions between WPI and ALG, leading to the formation of stable Chl-WPI-ALG complexes. These complexes significantly improved the color stability and solubility of Chl-Zn^{2+} during storage under both neutral and acidic conditions. Fluorescence spectroscopy and particle size measurements confirmed the formation of these complexes and their enhanced stability. Finally, this study suggests that the use of WPI and ALG to encapsulate Chl-Zn^{2+}could be an effective method to expand its application in acidic food products, providing a stable and vibrant natural colorant.

Keywords: Circular economy; Unsold vegetables; Food colorant; Enzyme-assisted extraction; Chlorophyll, Color stability; Encapsulation.

List of abbreviations

ADI = Acceptable Daily Intake

ALG = Alginate

B/S = Buffer/Substrate ratio

BLG = β-Lactoglobulin

C* = Croma

Chl = Chlorophyll

CL = Cellulase

DF = Diluition Factor

DM = Dry Matter

DOE = Design Of Experiment

d_w = Dry weight

EAE = Enzyme-Assisted Extraction

EFSA = European Food Safety Authority

F = Fluorescence in presence of quencher

F_0 = Fluorescence in presence of quencher

FAO = Food and Agriculture Organisation

FDA = Food and Drug Administration

FW = Food Waste

f_w = Fresh weight

GRAS = Generally Recognized As Safe

h° = Hue angle

JECFA = Joint Expert Committee on Food Additives

K_q = Stern-Volmer quenching constant

K_{sv} = Bimolecular quenching constant

MAE = Microwave Assisted Extraction

PEF = Pulsed-Electric Field

PG = Polygalacturonase

pI = Isoelectric point

PLE = Pressurized liquid extraction

Q = Concentration of quencher

RSM = Response Surface Methodology

SA = Solubility yield

SE = Solvent Extraction

SFE = Supercritical fluid extraction

SPI = Soy Protein Isolate

T = Temperature

t = Time

UAE = Ultrasound-assisted extraction

VMAE = vacuum microwave assisted extraction

WHO = World Health Organisation

WPI = Whey Protein Isolate

XL = Xylanase

t_q = Average lifetime of unquenched fluorophore

1.Introduction and State of the Art

1.1 Food waste

Food Waste (FW) is a global issue with far-reaching consequences, that continues to grow, with significant economic, environmental, and social impacts (Teigiserova *et al*., 2020). This problem has been overlooked for a long time, leading to increasing amounts of waste throughout the entire food supply chain (Peira *et al.*, 2018). FW may be defined as "*all the foodstuffs discarded from the food supply chain for economic or aesthetic reasons or owing to the nearness of the 'use by' date, but which are still perfectly edible and fit for human consumption and, in the absence of any alternative use, are ultimately eliminated and disposed of, generating negative externalities from an environmental point of view, economic costs and a loss of revenue for businesses*" (European Parliament resolution, 2012). FW occurs at every stage of the production chain: on farms, during processing, in shops, restaurants, and at home.

It is important to distinguish between food loss and FW. Food loss occurs early in the supply chain, during primary production and processing. It is unintentional and result from a variety of factors, including inadequate infrastructure, lack of proper handling, transportation methods, and insufficient storage facilities. Whereas FW happens at the final stages, including distribution and consumption, such as in wholesale, logistics, retail, food services, and households (Parfitt *et al.*, 2010). FW is a combination of both factors, leading to a significant reduction in the quantity and quality of edible food available for human consumption. FW is a subset of food loss and consists of material intended for human consumption that is not consumed. Indeed, this waste represents a loss of edible food discarded from the supply chain due to choice or negligence by its actors (Okawa, 2015; Peira *et al.*, 2018).

FW may begin even before primary agricultural production, as the amount of food produced is often determined by pre-established trade agreements that do not always match actual demand (Peira *et al.*, 2018). Defining FW and loss can be challenging, but it is clear that this phenomenon involves all stakeholders in the food chain, including those involved in processing, handling,

distribution, and consumption of food products (Cicatiello *et al.*, 2016). The Waste Resources Action Programme (WRAP) (WRAP, 2009) divides the concept of waste within the food chain into four different phases: production and processing, retail, restaurant services and home consumption, whilst Peira *et al*., (2018) reported five stages, namely, agricultural production, post-harvest production and storage management, processing and packaging, distribution (wholesale and retail) and consumption.

Recent research indicates that the global production of FW amounts to 1.6 billion tons annually, with 1.3–1.6 billion tons being edible, representing a market value of $750 million. The majority of FW is generated by typical households (approximately 47 million tons per year), followed by the processing stage (17 million tons), catering services (10.5 million tons), primary production (9.1 million tons), and distribution (4.6 million tons). FW is primarily composed of meat and fish (30%), vegetables (19%), and dairy products (17%), totaling $27 billion in value (Peira *et al.*, 2018). According to data from the European Statistical Institute (Eurostat), the wholesale and retail food sector is responsible for 5% of FW in the EU. Analysis shows that large grocery stores (supermarkets and hypermarkets) have 46 tonnes of unsold food per year, medium-sized stores have 11 tonnes, and small stores have 1.7 tonnes (Piirsalu *et al.*, 2022). Approximately 12% of this unsold food is donated to food banks or other charitable organizations. In Italy, the General Wholesale Market of Milan generates about 1500 tonnes of unsold vegetables annually, which are collected for redistribution by charitable organizations (Tedesco *et al*., 2021). However, due to the high perishability of these products, national legislation limits the donation of unsold food, resulting in most of it becoming food waste destined for landfill (Bonadonna *et al*., 2018). To harmonize unsold food legislation across member states, the European Parliament adopted resolution no. 2018/C 307/03 (Katsarova, 2019).

Moreover, FW causes huge increases of greenhouse gases in the atmosphere, both from the production as well as the consumption level, which in turn generates effects of climate change. In fact, FW also has a strong environmental impact, according to WRAP every ton of wasted food is

responsible for the release of 4.5 tons of CO_2 into the environ E140ment and a water consumption equal to 170 billion cubic meters.

Furthermore, the social and health costs related to waste must also be considered. These include the adverse effects due to exposure to pesticides, soil erosion, loss of biodiversity and social conflicts over natural resources. At the same time, the demand for food continues to grow and by 2050 the FAO estimates a 50% increase in global demand for agricultural products compared to current levels. The fight against FW therefore becomes fundamental to actively contribute to safeguarding the planet and food security.

The issue of FW also has an ethical dimension. This ethical concern arises from the fact that approximately 1.3 billion tons of food are wasted globally each year, while around 1 billion people suffer from hunger. Wasting unnecessary food is ethically problematic because it depletes the earth's resources. The overexploitation of these resources is expected to worsen in the future due to increasing consumption demands and population growth. It is estimated that the global population will reach about 9 billion by 2050, further straining available resources (Godfray *et al*., 2010). According to the latest report on global food security "The State of Food Security and Nutrition in the World", published in 2019, almost 690 million inhabitants of planet have suffered from hunger while every year the value of food waste is around 1.2 trillion dollars.

It is estimated that without initiatives to reduce FW, it could increase by about 40%, reaching 126 million tons per year (Peira *et al.,* 2018). Effective waste management may only be achieved through comprehensive food chain control and better utilization of limited resources (Battilani, 2015). In line with Circular Economy principles, recent advancements have focused on using plant-based waste as a source of natural pigments.

1.2 Circular economy

One of the pillars of sustainable development is the Circular Economy model. Indeed, this model views waste as a secondary resource (Teigiserova *et al.*, 2020). According to the definition of the

European Parliament: "*The Circular Economy is a model of production and consumption that involves sharing, lending, reusing, repairing, reconditioning and recycling existing materials and products for as long as possible*." (European Parliament, 2016). The Circular Economy approach is based on three key factors: reduce, reuse, and recycle. This involves minimizing waste and refuse, reusing items where possible, and recycling what cannot be reused to create new secondary resources that may be reintroduced into the production cycle. The aim of this model is to retain resources and their value within the production cycle for as long as possible, thereby reducing waste and the pressure on the planet's natural resources. In contrast, the principles of the Circular Economy differ from the traditional linear economic model (Fig. 1.2.1), which follows the 'extract, produce, use, and dispose' scheme, relying on the availability of large quantities of easily accessible and low-cost materials and energy.

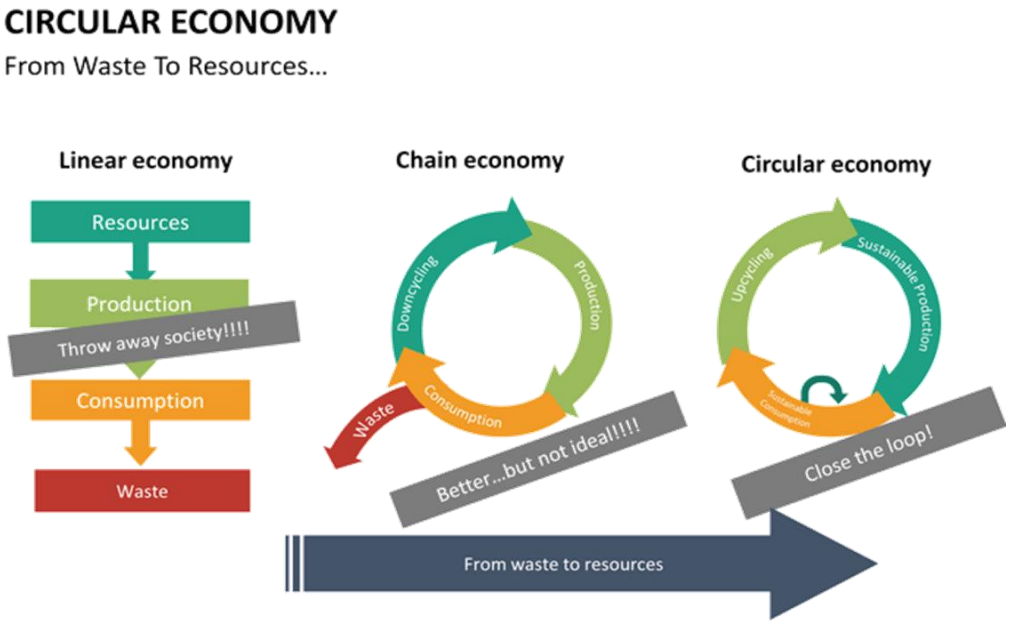

Fig. 1.2.1 Graphic representation of the linear economy and the virtuous circle of the Circular Economy

The Circular Economy may define the background requirements for the management of FW and other kinds of waste, generating new business opportunities. FW may be applied to produce several

biomaterials (Mirabella *et al.,* 2014), bioenergy (Dahiya *et al*., 2018), and high-value products (Galanakis, 2012; Sagar *et al.,* 2018).

Taking into account the definition of waste by Circular Economy (waste = secondary resource), EU has approved an action plan to incorporate it into Member States legislation (Directive EU, 2018/851), which includes a commitment to achieve the global Sustainable Development Goal (SDG) 12.3 "*halve food waste per capita at the retail level and of consumers and reduce food losses along production and supply chains (including post-harvest losses) by 2030*" (Flanagan *et al.*, 2018). FW legislation in Europe has always been ambiguous. The key EU directive, Waste Framework Directive (European Parliament, 2008, Directive EU, 2008/98/EC), does not differentiate FW from other types of bio-waste, such as garden waste (Teigiserova *et al.,* 2020). In this context, a Circular Economy program was developed to manage FW and minimize it as much as possible. This model is illustrated using a pyramid chart (Sanchez *et al.,* 2020), which outlines the actions to be implemented (Fig. 1.2.2).

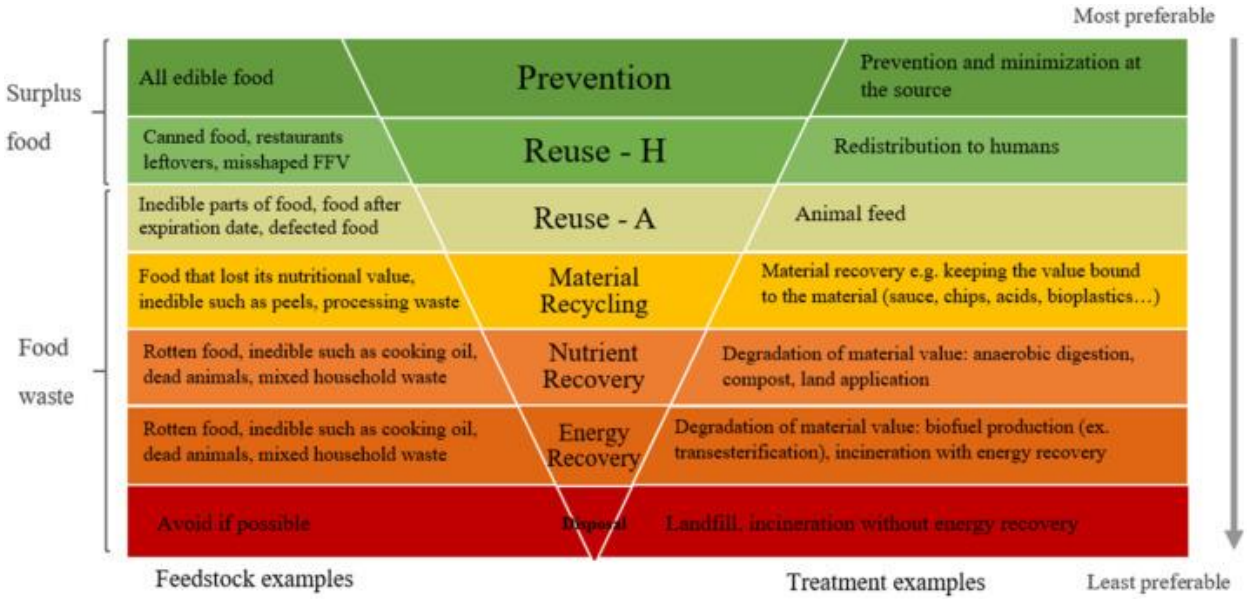

Fig. 1.2.2 Updated hierarchy for food surplus and waste proposed herein building on terminology from major European and national projects (UNEP, 2014; WRAP, 2013; FUSIONS: Östergrenet al., 2014). *FFV fresh fruits and vegetables. Source: Teigiserova et al., (2020)

This pyramid describes the optimal strategies, focusing first on prevention actions, which are at the top of the pyramid (Fig. 1.2.2). Prevention may be achieved through various strategies that act directly at the source, improve logistics and management tools (production, processing, and retail), or aim at educating consumer behavior and habits (Papargyropoulou *et al.,* 2014; Garrone *et al.,* 2014). In particular, these efforts should primarily focus on the most resource-intensive and polluting foods, such as red meat and dairy products (Teigiserova *et al.,* 2020). The second level involves the reuse of surplus food for human consumption (Fig. 1.2.2). The food surplus is the only fraction that can be directly reused for human consumption (Mourad, 2016; Garrone *et al*., 2014). Due to the highly perishable nature of food, the reuse of excess food is subject to strict safety and hygiene regulations, which may limit the amount of food that can be reused and consequently increase food waste. The animal feed industry has its own food safety regulations, thus forming a separate reuse category (Fig. 1.2.2). In this context, the vision is to reuse excess food exclusively for humans, while food waste can be repurposed for animal feed. Safety precautions for the reuse of food waste for animal feed must prevent the development and spread of diseases that could be serious for both animals and humans, such as bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) (Teigiserova *et al.,* 2020). Moving down the hierarchy, the categories of "recovery" and "recycling" often overlap, although they are distinct categories (Fig. 1.2.2). In terms of food, redistribution is sometimes referred to as "recovery" (Garrone *et al.,* 2014), while food waste used as animal feed can be considered "separate collection." The recovery of nutrients and energy must also be distinguished into two different categories (Fig. 1.2.2) because they provide distinct services, although the differences can sometimes be blurred. A prime example is the anaerobic digestion of food waste, which produces both fertilizer (as digestate) and energy (such as methane or storable hydrocarbons), and thus could fall into both categories (Fig. 1.2.2). At the bottom of the pyramid, as a last option, we find food waste disposal.

Circular Economy can significantly reduce the negative impacts of resource extraction and use on the environment and help restore biodiversity. Therefore, in line with the Green Deal's climate neutrality objective (by 2050), in March 2020 the European Commission proposed the first package of measures to accelerate the transition to a Circular Economy, as announced in the Circular Action Plan. The proposals include the enhancement of sustainable products, consumer empowerment towards the green transition, the revision of the Construction Materials Regulation and a strategy on sustainable textiles.

Through greater use of recycling and reuse, the proposed actions are aimed at generating maximum value and maximum use of raw materials, products and waste, promoting energy savings and reducing greenhouse gas emissions.

Circularity and sustainability need to be integrated into all stages of the supply chain to achieve a fully circular economy: from design to production to the consumer. Therefore, in line with the Sustainable Development Goals and in the context of Directive 2008/98/EC 38 referred to in section 4.1, the Commission will propose a target on food waste reduction as a key action in the upcoming EU Farm to Fork Strategy, which will cover the food supply chain.

The Commission will also consider specific measures to increase the sustainability of food distribution and consumption. With the Circular Economy Action Plan, the EU is committed to halving food waste per capita at retail and consumer levels by 2030, giving further impetus to the sector and complementing the Farm to Fork Strategy and the Biodiversity Strategy.

At National level, the main measures in force are the National Recovery and Resilience Plan (PNRR), which represents the Italian version of the Next Generation EU, and the Waste Decree (Legislative Decree 116/2020), which constitutes the reference legislation for waste management and disposal. This decree has made it possible to align Italian legislation with European guidelines, which are incorporated into it. With regard to the PNRR, within Mission 2 "*Green Revolution and Ecological Transition*", there is a Component (C1) dedicated to the Circular Economy and sustainable agriculture.

The advantages of reducing food waste from a circular perspective encompass environmental, economic, and social impacts. Environmentally, food waste significantly contributes to climate change and greenhouse gas emissions, and it consumes substantial amounts of energy and materials (Krishnan *et al*., 2020; Närvänen *et al*., 2020). Economically, circular waste management lowers costs and leads to reduced food prices (Despoudi *et al.,* 2021). Socially, minimizing food waste by utilizing unwanted food helps alleviate hunger (Chauhan *et al.,* 2018). Circular supply chain management integrates all supply chain functions, enhancing control over all stages or processes, which increases efficiency, reduces costs, and improves food quality and safety (Corrado & Sala, 2018; Krishnan *et al.,* 2020; Närvänen *et al.*, 2020). A closed-loop food supply chain adopts a circular economy approach through reverse logistics systems, involving food waste recycling and reuse (Jabbour *et al.,* 2021). Recent studies emphasize the need for further research on implementing circularity, focusing on improved processes such as collection, storage, new technologies, and the development of new infrastructure and transport (Ciccullo *et al*., 2021; Santeramo, 2021). Additionally, more knowledge on new behaviors and cooperative arrangements with other stakeholders is required. New routines and habits among consumers and retailers are essential for reducing food waste from a circular and green economy perspective (Aschemann-Witzel et al., 2017; Welch *et al*., 2018; Santeramo, 2021). From the perspective of Circular Economy, various biotechnological methods and green processing technologies (such as microwave, pressurized liquid, ultrasound, supercritical fluid, pulsed electric field, and enzyme-assisted extraction) may be utilized to extract high-value molecules from unsold fruits and vegetables, meeting consumer expectations and reducing the environmental footprint (Augustin *et al*., 2020; Benucci *et al.,* 2022).

1.3 Food waste as substrates for the extraction of high value-molecules

FW contain several molecules and phyto-complexes with a significant biological and economic value, in particular, they contain a significant amount of biologically active compounds such as polyphenols, dietary fiber, essential oils, pigments, organic acids, etc. (Kumar *et al.,* 2017). These compounds may be extracted, purified and characterized using appropriate technologies, allowing the development of new commercial applications in the food and non-food sectors (pharmaceutical, biomedical, cosmetic, etc.) (Galanakis *et al.,* 2012). Research is mainly focused on the recovery of functional compounds derived from agricultural and food processing by-products. These sources are plentiful, concentrated in a few locations, and less prone to deterioration compared to waste generated at the end of the food chain. The latter is typically spread across many households and accumulates in various regions due to meal preparation (Galanakis *et al.,* 2012).

In particular, regarding the recovery of high-added value molecules, five distinct recovery phases may be observed. However, these phases may sometimes be adjusted depending on the matrix and/or molecule being studied. The methods typically progress from the macroscopic to the macromolecular level, followed by the extraction (or removal) of specific micro-molecules, and finally, the purification of the target compounds (Fig. 1.3.1).

Macroscopic pretreatment (Fig. 1.3.1) aims to tailor the food waste matrix based on water content, enzymatic activity, and permeability of bio-source tissues. This step involves processes that vary depending on the nature and structure of the substrate (e.g., solid, sludge, or wastewater). For instance, if the substrate is a fruit or vegetable by-product, a wet grinding step is necessary to facilitate and enhance the yield of subsequent separation and extraction steps (Oreopoulou & Tzia, 2007). Often, thermal pretreatment is required, which reduces water content and activates or deactivates key enzymes (e.g., pectin methylesterase or polyphenol oxidase), thereby influencing the yield. Alternatively, dehydration may be performed through mechanical pressing to avoid

thermal yield reduction and loss of functionality (Fig. 1.3.1). However, non-thermal processes (e.g., freeze-drying) have disadvantages such as increased costs due to vacuum conditions and the low shelf-life of the treated matrix, as thermal treatment also prevents microbial growth. Other technologies like centrifugation or microfiltration are also suggested during the pretreatment stage (Fig. 1.3.1) because they can remove solids, oils, and fats. These components are prone to auto-oxidation, causing substrate deterioration and limiting mechanical processes such as substrate flow, mixing, and homogenization (Díaz et al., 2004; Galanakis *et al*., 2010).

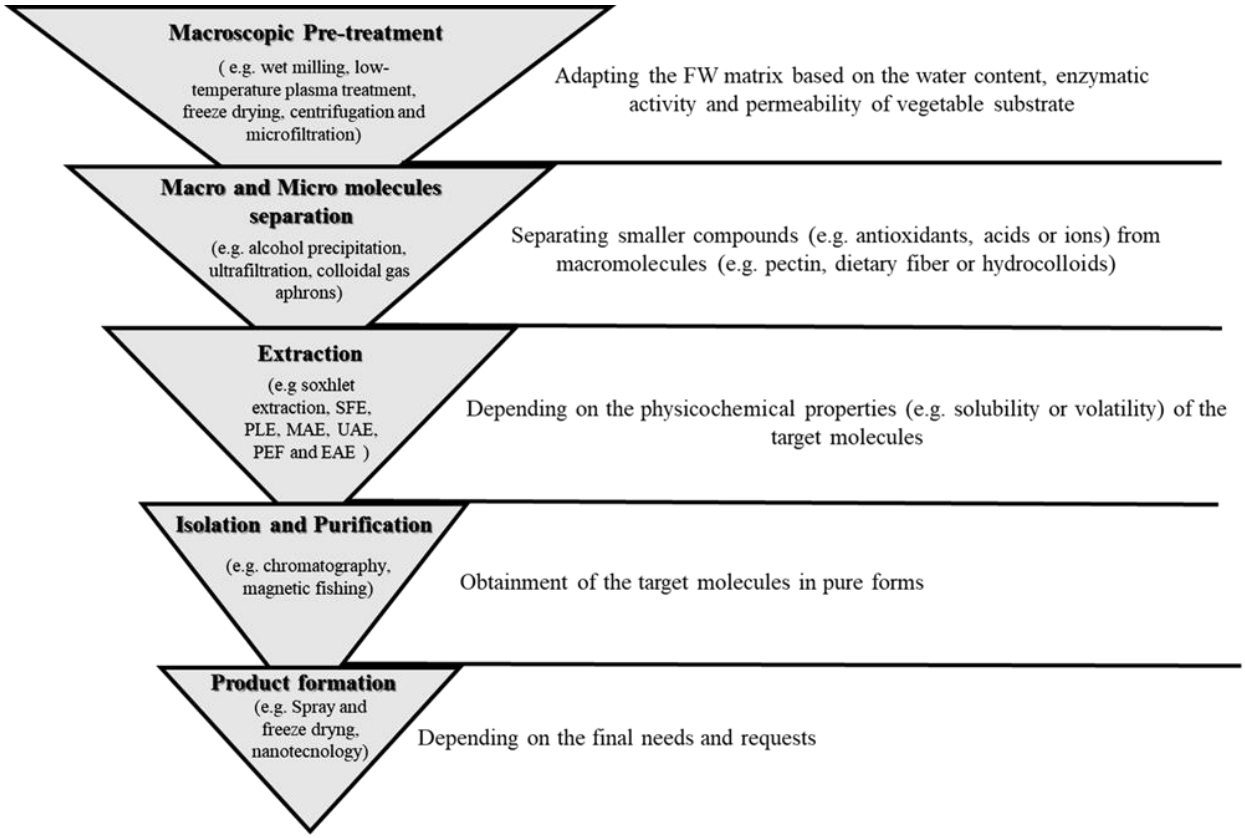

Fig. 1.3.1 Recovery phase of high-added value compounds from food wastes (FW). Abbreviations: EAE, enzyme-assisted extraction; MAE, microwave-assisted extraction; PEF, pulsed electric field; PLE, pressurized liquid extraction; SFE, supercritical fluid extraction; UAE, ultrasound-assisted extraction, Source: Benucci et al., (2022)

In the second recovery stage (Fig. 1.3.1), alcohol precipitation is the most popular method for separating smaller compounds (e.g., antioxidants, acids, or ions) from macromolecules (e.g.,

pectin, dietary fibers, or hydrocolloids), which are collected in the so-called alcohol-insoluble residue. This method is chosen because it is inexpensive, non-toxic, and easy to use. However, it is not selective and cannot separate complexes between smaller molecules (e.g., phenols) and larger molecules (e.g., proteins, pectin). Extraction, the next and most crucial step in downstream processing (Fig. 1.3.1), is well-documented with various methods used for targeted molecules based on their physicochemical characteristics (e.g., solubility or volatility). Solvent extraction is considered the conventional method due to its cost-effectiveness, as the solvent acts as a physical carrier to transfer targeted molecules between different phases (e.g., solid, liquid, and vapor). The fourth recovery step focuses on isolating and purifying target compounds from co-extracted impurities (Fig. 1.3.1). The simplest and most common method is adsorption, supported by resins, activated carbon, or polysaccharide-based materials. Chromatographic methods, although laborious, solvent-intensive, and time-consuming, can ensure the acquisition of pure target molecules for pharmaceutical and functional applications. The final step in downstream processing is product formation (encapsulation or drying) (Fig. 1.3.1), which is not a recovery operation but should be considered based on final needs and requirements (Galanakis *et al.,* 2012).

The recovery is implemented with the ultimate goals of maximizing the yield of target compounds, meeting the requirements of industrial processing, separating high-value ingredients from impurities and toxic compounds, preventing deterioration and loss of functionality during extraction and ensuring the final product maintains its ‘food-grade’ status.

These high-value molecules, including proteins, peptides, polysaccharides, dietary fibers, and functional bioactive substances (such as polyphenols, antioxidants, antimicrobial compounds, and natural pigments), may be utilized as natural food additives (Ayala-Zavala *et al*., 2010). Among these, vegetable pigments (e.g. carotenoids, betalains, anthocyanins, and chlorophylls) are considered safe and healthy as they possess, in addition to coloring power, antioxidant, anti-inflammatory and antimutagenic activities (Sharma *et al*., 2021).

1.4 Food coloring agents

Coloring agents fall into the category of food additives, that are defined as "any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities" (Codex Alimentarius Commission, 2017). Worldwide, the primary regulatory authorities for food additives are the European Food Safety Authority (EFSA) and the United States Food and Drug Administration (FDA). Currently, over 2,500 additives are deliberately added into foods to preserve specific qualities or prolong shelf life. Over the years, numerous additives have been prohibited, with some bans being worldwide and others specific to certain countries (Branen *et al.,* 2001). In the European Union, food additives are classified into 26 functional groups based on their role in food (Council Regulation, 1333/2008). In contrast, the United States categorizes them into six classes (Carocho *et al*., 2014). Beyond these classifications, additives, including food colorants, may also be grouped by their origin and production method into four categories: natural additives (derived from animal or plant sources), similar to natural additives (those that mimic natural ones), modified from natural (natural additives that have been chemically altered), and artificial additives (synthetic compounds) (Carocho *et al*., 2014).

In details, food coloring agents or dyes are used to alter or give color to food to increase its attractiveness to consumers (Sigurdson *et al.,* 2017) and they are defined as "any dye, pigment or substance which when added or applied to a food, drug or cosmetic, or to the human body, is

capable (alone or through reactions with other substances) of imparting color" (Martins *et al*., 2016).

Food colorants may be classified into five categories based on their chemical structure: azo compounds, quinophthalone derivatives of quinoline yellow, triarylmethane group, xanthenes, and indigo dyes (Sarikaya *et al.,* 2012).

Azo compounds, characterized by the functional group R-N=N-R′ where R and R′ can be aryl or alkyl, are widely used in food products. Some of the most commonly used azo dyes include tartrazine (Yellow No. 5 in the USA, E102 in the EU), sunset yellow (Yellow No. 6 in the USA, E110 in the EU), allura red (Red No. 40 in the USA, E129 in the EU), amaranth (E123), and carmosine (E122) (Fennema 1996). These dyes are frequently added to a variety of foods such as blancmange, marzipan, Swiss rolls, jams, yoghurts, jellies, breadcrumbs, cotton candy, soft drinks, flavored chips, cereals, cake mixes, soups, sauces, rice, ice cream, candy, and chewing gum. The azo compounds, with the functional group N = N and the aromatic rings connected to them are reductively cleaved into aromatic amines, with some of these toxic, mutagenic and carcinogenic aromatic amines (Chung 2000; Zhang & Ma 2013). Tartrazine is particularly controversial, with studies showing both harmful effects, such as DNA binding and toxicity to human lymphocytes (Gao et al., 2011; Axon et al., 2012), and others indicating it is safe at acceptable daily doses (Tanaka *et al*., 2008a; Poul *et al.,* 2009). The European Union has revised its opinion on allura red, suggesting a potential genotoxicity, although some studies contradict this (Carocho *et al.,* 2014). Amaranth, another petroleum-derived dye, has been banned in the United States due to its possible carcinogenicity but remains approved in the EU and other countries. Recent studies have described that it induces colon damage in rats (10 to 100 mg/kg). Additionally, using a somatic mutation and recombination test, amaranth has been shown to induce genotoxicity in human lymphocytes (Mpountoukas *et al.,* 2010; Sarikaya *et al.,* 2012), while others found no evidence of these effects in mouse models (Poul *et al.,* 2009). Similarly, sunset yellow has been linked to genotoxicity and other adverse effects in animal studies, while some research suggests they are safe (Ceyhan *et al.,*

2013; Yadav *et al.,* 2013). Carmoisine, banned in the United States, has been reported to alter biochemical markers in vital organs and affect DNA structure in animal models (Datta *et al.,* 2013; Carocho *et al.,* 2014).

Derivatives of quinophthalone. Quinoline Yellow (E104) is a synthetic quinophthalone dye produced by combining sodium disulfonates, monosulfonates, and trisulfonates. It is commonly used in soft drinks, cakes, cosmetics, and medicines (Al-Shabib *et al*., 2020). However, this compound has been associated with adverse effects such as hives, asthma, skin rashes, and hyperactivity. Additionally, it has been reported to alter the conformation of bovine serum albumin (Carocho *et al.,* 2014).

Triarylmethane group, derived from triphenylmethane, includes several compounds such as bright blue (No. 1 in the USA, E133 in the EU), fast green (E143), patent blue (E131), and bright black (E151). Among these, bright blue and patent blue are the most commonly used additives, although patent blue is banned in the USA and no authorization has been sought for bright black, while fast green is banned in the EU. These dyes are typically added to yogurt, ice cream, drinks, and sweets (Unsal *et al*., 2015). Interestingly, these dyes are not readily absorbed by the human body, with 95% are present in the feces, and there are no reports of deaths due to improper absorption (Gaur *et al.,* 2003). However, some authors suggest that the consumption of lollipops with bright and transparent blue dyes by children could be dangerous due to absorption through the lingual mucosa (Lucová *et al.,* 2013), potentially leading to negative effects on children, who are the main consumers of these products (Carocho *et al.,* 2014).

Xanthene group, consisting of erythrosine (Red No. 3 in USA, E127 in EU), fluorescein, eosin, and rhodamine, is notable for its use in various food products and its health implications. Erythrosine is allowed in both the USA and EU, while fluorescein is banned in the EU. Erythrosine, a poly-iodinated compound, has been linked to impaired infant behavior and thyroid function due to its high iodine content (Carocho *et al*., 2014). Other studies suggest it could induce toxicity on

the testes and alter behavior patterns, and recent findings indicate it may alter DNA structures (Mpountoukas *et al*., 2010). These colorants are commonly found in products such as cocktails, tinned cherries and fruits, biscuits, chocolates, garlic sausages, salmon spreads, scotch eggs, stuffed olives, sweets, bakery items, snack foods, chewing gums, jellies, wines, and ice cream (Tabara *et al*., 2011).

Indigo dyes, known as Blue No. 2 in USA, were originally extracted from the *Indigofera tinctoria* shrub but are now chemically produced. While adverse reactions are rare due to the typical amounts added to food, these dyes have been the subject of extensive studies. One of the most controversial is the "Southampton Study," which found evidence of attention deficit hyperactivity disorder in children consuming a cocktail of food colorings, including indigo dyes. This study was contested by the UK Food Safety Authority's advisory body and EFSA, but led to public advocacy for a voluntary ban (Carocho *et al*., 2014). Indigo dyes are used in various products, including gelatins, ice creams, jelly beans, bubble gums, and candies (Deroco *et al*., 2018).

The conflicting results concerning most food colorants are quite concerning and should prompt relevant institutions to implement regulations to alleviate consumer worries. Despite numerous studies on colorants, few have explored the interactions between dyes and their harmful health effects. Two studies have highlighted the harmful synergistic effects of dyes in neuronal cell lines in vitro (Lau *et al*., 2006; Park *et al*., 2009). The use of dyes in the food industry is controversial, but there is increasing pressure to adopt natural alternatives. Some successful natural colorants, such as carotenoids, anthocyanins, annatto, and paprika, are gradually being approved in both the USA and EU. However, these natural compounds have drawbacks, including instability due to pH and temperature, color loss by oxidation, the need for larger quantities compared to chemical dyes, and higher production costs (Delgado-Vargas *et al*., 2000; Carocho *et al*., 2014). Although colorants are now better regulated and safer, further research is needed to address the shortcomings of dyes, either by improving the safety of approved ones or discovering new alternatives.

1.4.1 Regulatory framework

Worldwide, the two main regulatory bodies for food additives are the European Food Safety Authority (EFSA) and the United States Food and Drug Administration (FDA) (Carocho *et al*., 2014). A significant effort has been made to create a comprehensive database of legal additives for use within the European Union. Regulation 1129 of 2011 lists all approved additives and their Acceptable Daily Intake (ADI) (Council Regulation [EC] 1333/2008; Council Regulation [EC] 1129/2011). In the United States, since 1961, the FDA has mandated that all food components should be labeled as generally recognized as safe (GRAS). In the European Union, food additives are classified into 26 functional groups based on their function in food (Council Regulation, 1333/2008), whereas in the United States, they are ranked into six classes (Carocho *et al.*, 2014). Additionally, additives, including food colorants, can be categorized according to their origin and manufacture into four groups: natural additives (from animal or plant sources), similar to natural additives (mimicking natural ones), modified from natural (natural additives which are then chemically modified), and artificial additives (synthetic compounds) (Carocho *et al*., 2014).

Food colours are classified as additives and must be authorised under EU legislation (Regulation (EC) No 1333/2008) before they can be used in food products. This authorisation must be preceded by complete and rigorous safety assessments. After approval, these substances are still kept under control, and if new information is acquired, the opinion on their use can be reviewed, modified and even suspended. This important control and evaluation action is guaranteed by EFSA at EU level, supported by the "Joint Expert Committee on Food Additives" [JECFA, an international body born from the collaboration between the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO)]. Regulation (EC) No. 1333/2008 mandates that food colorants must meet Food Grade standards to be utilized in food products, aligning with specific characteristics outlined in Commission Regulation (EU) No 231/2012 of 9 March 2012. Details regarding origin, purity criteria, and other necessary information are established at the initial

inclusion of the colorant in the Community lists of Regulation (EC) 1333/2008. A colorant can be authorised for food use if it satisfies the general conditions established for all food additives, in addition to some specific conditions provided for this category. EU food safety legislation delineates the products in which colorants can be employed, alongside the maximum permissible quantities for each food type. Most colorants have an ADI, specifying the amount that can be safely consumed daily. For instance, the ADI for curcumin (E100) is 3 mg per kg of body weight, while for sunset yellow (E110), it is 2.5 mg per kg of body weight. Some colorants, such as chlorophylls, are absent from these tables due to insufficient studies.

1.4.2 Natural food colorant

As for ever-growing trends in the food coloring industry, the use of natural pigments (natural colorants) has increased in foods and beverages as substitutes for their synthetic counterparts. This is mainly due to the growing awareness of environmental risks and potential side effects of chemicals used in the synthesis of food colors (Carocho *et al*., 2014). Additionally, synthetic dyes tend to be replaced in the food industry in order to be in line with current consumer needs. This marketing strategy is in line with the so-called "clean-label" trend.

The classification of natural colorants can become very complex due to the wide variety of pigments. They can be derived from a wide variety of sources and, therefore, natural colorants also exhibit a wide variety of chemical compositions that affect their properties and conditions of use (Sigurdson *et al.,* 2017). They may be classified according to their source (e.g., plants, animals, microorganisms, and minerals) (Sigurdson *et al*., 2017).

Plant-based pigments are the result of biochemical pathways within plants, creating numerous organic compounds with unique physicochemical properties (Miranda *et al*., 2021). These colorful compounds are widespread in nature and fulfill various functions, including participation in photosynthetic processes, attracting pollinators, and providing protection from predators and solar

energy. The main chemical classes of plant pigments are porphyrins, carotenoids, anthocyanins, and betalains, which selectively absorb certain wavelengths of light and reflect others (Sigurdson *et al*., 2017).

Similarly, animals produce distinct chemical compounds that lead to a wide range of pigments throughout the animal kingdom. These pigments perform various functions, including transporting oxygen in the blood, protecting from predators or UV rays and mating (Aberoumand, 2011; Delgado-Vargas *et al*., 2000).

Microorganisms, including bacteria, fungi, and microalgae, produce a wide range of pigments, covering nearly any shade of color. For example, blue pigments (phycocyanins) can be obtained from the microalga Spirulina (*Arthrospira platensis*), while red colorants (carotenoids) are derived from the fungus *Monascus sp.* and the bacterium *Paracoccus carotinifaciens*. The production of pigments from microbial sources is commercially and economically promising due to the relative simplicity of controlling growth conditions and the constant availability of the source (Sigurdson *et al*., 2017; Joshi *et al*., 2003).

Minerals have a long history of use as dyes in food, cosmetics, and art, with their hues depending on their chemical composition and physical structure. Many minerals contain metal cations with electrons in the "d" orbitals that often absorb and reflect visible light (Mason, 2013). For example, viridian, a chromium oxide mineral, is a green pigment used in painting and cosmetics as a certification-free dye (CFR 2016; Sigurdson *et al*., 2017).

Moreover, natural colorants may also be classified according to their chemical structure (Fig 1.4.1):

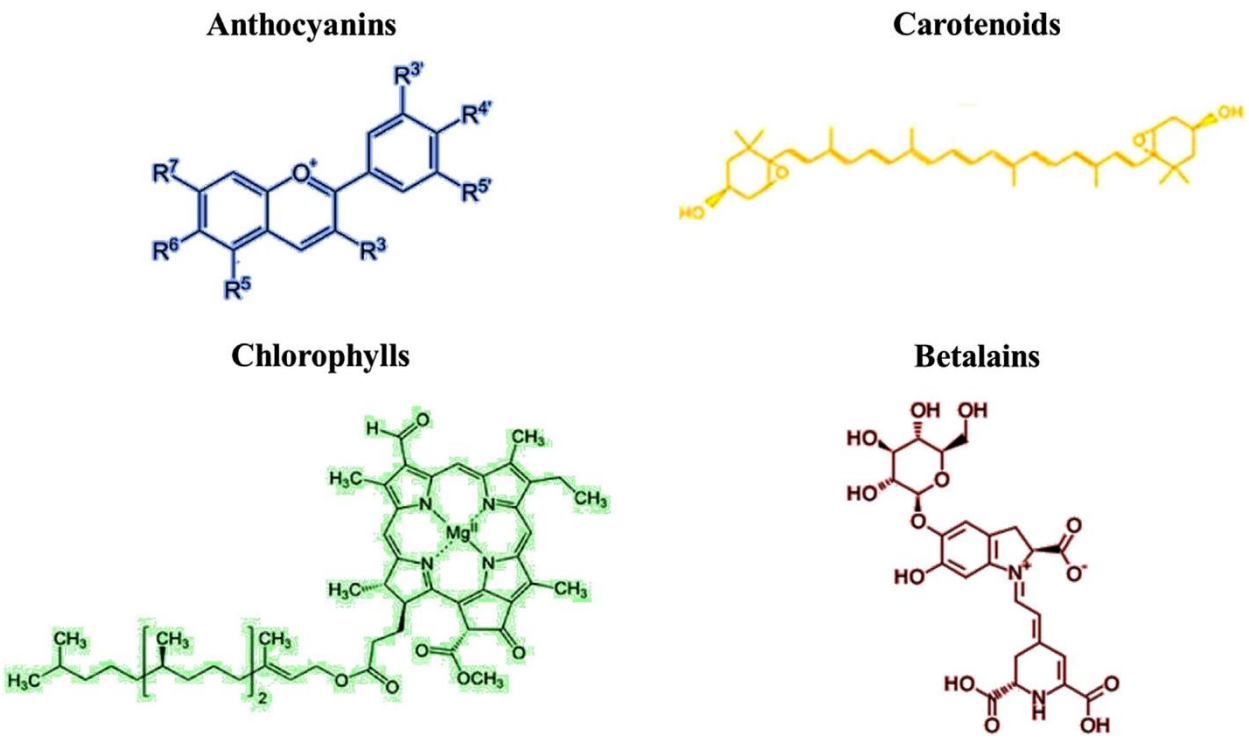

Fig. 1.4.1 Chemical structure of selected natural pigments from vegetable waste. Source: Benucci et al., (2022)

Anthocyanins. Among flavonoids, which are secondary plant metabolites characterized by a carbonaceous skeleton formed by the repetition of the C6-C3-C6 unit, anthocyanins are a significant subgroup of water-soluble pigments that give plants colors ranging from bright red to blue (Obón *et al*., 2009). The aglycone form of anthocyanins, known as anthocyanidin, is commonly found in fruits and vegetables and varies in the degree of hydroxylation and methoxylation. These anthocyanidins can bind to sugars and undergo further acylation with aromatic or aliphatic acids. The stability of anthocyanins increases significantly with both glycosylation and acylation, which is why simple anthocyanins are rarely found in nature (He & Giusti, 2010). Acylated anthocyanins are more prevalent in plants and flowers, while non-acylated ones are typically found in fruits (Wallace & Giusti, 2008). Anthocyanins make up the largest group of natural pigments, with more than 700 different structures identified (Andersen & Jordheim, 2014). However, their poor stability poses a challenge for their use as food colorants, as

they are highly sensitive to light, heat, oxygen, and pH (Dangles & Fenger, 2018). To improve their stability, the food industry is exploring new methods, such as copigmentation and acylation. Currently, anthocyanins are used as food colorants in beverages, yogurts, and dry mixes. In the EU, they are labeled as E163, while in USA, they are exempt from certification (Sigurdson et al., 2017). FW and byproducts from industrial processes, such as wine and juice industry, are considered excellent sources for recovering anthocyanins. The most common sources include blackberries and grape pomace (Sharma *et al*., 2021). In line with Circular Economy concepts, using FW as an alternative source of food colorants has gained attention in recent years. Recently, a natural colorant extract from grape skin was obtained through solvent extraction, demonstrating the typical properties of polyphenols (Gordillo *et al*., 2018).

Carotenoids are a group of fat-soluble pigments that range from yellow to orange to red and are widespread in nature, found in higher plants, bacteria, fungi, yeasts, birds, and insects (Tanaka *et al*., 2008b). Chemically, carotenoids are tetraterpenoids composed of 40 carbon atoms arranged in isoprenoid units connected in a head-to-tail pattern. They can be classified into two categories: carotenes and xanthophylls. Carotenes consist of only polyunsaturated hydrocarbons, while xanthophylls have polyunsaturated hydrocarbons with functional groups containing oxygen. Carotenoids can be further classified based on their structure: acyclic (e.g., lycopene), monocyclic (e.g., γ-carotene), or dicyclic (e.g., lutein). Their conjugated systems, with delocalized π electrons along the entire chain, are responsible for absorbing light in the visible spectrum (Rivera & Canela-Garayoa, 2012). The degree of conjugation affects the intensity of the color, with larger conjugated systems corresponding to redder shades, while shorter chains with at least five double bonds exhibit lower chromaticity. The color is also influenced by the degree of cyclization, explaining why lycopene, β-carotene, and γ-carotene, despite having the same number of double bonds, appear red, orange, and red-orange, respectively (Sigurdson *et al*., 2017). Because of their electron-rich and highly unsaturated chemical structure, carotenoids are particularly prone to

oxidation and isomerization, especially during food processing and storage (Rodriguez-Amaya, 2016). These pigments are mainly utilized as food colorants in cheddar cheese, beverages, and flavored milk drinks (Sigurdson *et al*., 2017). Numerous studies have detailed the recovery of carotenoids from vegetable waste for use as food colorants. Researchers have refined green extraction methods using sources such as carrots, pomegranate peel, paprika leaves, orange peel, pumpkin (Sharma et al., 2021), and unsold tomatoes (Lombardelli *et al*., 2020). Additionally, carotenoids extracted from tomato peel have been successfully stabilized by encapsulation in gelatin nanofibers, maintaining their antioxidant, antimutagenic, antiproliferative, and anti-atherogenic activities (Horuz & Belibağlı, 2018).

Chlorophylls, symmetrical cyclic tetrapyrroles naturally occurring with phytol and magnesium ion at their core, can cover nearly the entire visible spectrum (Newsome *et al*., 2014). These components are crucial for the molecule's photosynthetic and coloring functions (Sigurdson *et al*., 2017). In the EU, these complexes are approved for a wide range of food applications, whereas in the US, they are only allowed in citrus-based dry beverage mixes (CFR, 2016). Chlorophylls extracted from various plant wastes are often used as antioxidants, though their application as food colors is still limited. One example of chlorophyll used as colorant comes from avocado peel (Sharma *et al*., 2021). Additionally, chlorophyll extracts from spinach byproducts can be formulated into nutraceutical or functional foods due to their antioxidant, anti-inflammatory, and antimutagenic properties (Derrien *et al*., 2018)

Betalains, a notable group of nitrogen-heterocyclic compounds, are water-soluble pigments derived from betalamic acid. These pigments impart colors to plants in the *Caryophyllales* order, such as red beets and prickly pears (Strack *et al*., 2003). Betalains are divided into two main groups: the red-purple betacyanins and the yellow-orange betaxanthines (Lombardelli *et al.*, 2021). Betacyanins result from the condensation of betalamic acid with cyclo-dopa, while betaxanthines are formed from the condensation of betalamic acid and amines (Stintzing *et al*., 2002).

Betacyanins can also bind with mono or disaccharides, forming glycosides that may be acylated, creating various beta-cyanide structures. Betalains are stable over a wide pH range (3 to 7) and can be used in low-acid foods such as dairy products (yogurt and ice cream) and in syrups, sausages, and confectioneries (Stintzing & Carle, 2004). In EU, they are labelled as E162, and in USA, they are exempt from certification (Sigurdson *et al*., 2017). Unlike other natural colorants such as anthocyanins, the color of betalains is independent of pH values. However, their stability can be affected by factors such as temperature, light, oxygen, and other food components. Sharma *et al*. (2021) detailed the extraction of betalains from vegetable waste, including red dragon fruit peels and prickly pear. Recently, Lombardelli *et al*. (2021) developed an eco-friendly approach for recovering betalains from unsold beetroot.

1.5 Chlorophylls and their derivatives

Chlorophyll, a liposoluble and plant-derived natural pigment, is present in greater abundance than any other organic pigment in nature (He *et al*., 2019). The term chlorophyll comes from the Greek words "*chloros,*" meaning “green,” and "*phyllon*" for “leaf” (Pareek *et al*., 2017). It is present in the grains of chloroplasts of plant cells, or in prokaryotic organisms that carry out chlorophyll photosynthesis. Chloroplasts are situated in the mesophyll layer in the middle of plant leaves and possess thylakoid membranes containing green chlorophyll pigment (Pareek *et al*., 2017). It was isolated for the first time in 1817 by Joseph Bienaimé Caventou and Pierre-Joseph Pelletier (Humphrey, 1980). Its primary function is to absorb sunlight, maintaining metabolism, and preventing photo-oxidative damage (Das *et al*., 2022), providing the energy necessary for the plant to carry out its vital processes through photosynthesis.

Chlorophyll belongs to the class of compounds known as pyrroles. Pyrroles are organic compounds characterized by a five-membered ring structure composed of four carbon atoms and one nitrogen atom. In biological systems, they play many useful roles due to their ability to form

hydrogen bonds, coordinate metals and interact with other cellular components (Walsh *et al*., 2006). Furthermore, they are the building blocks of many heteroaromatic systems and linear polypyrroles. Tetrapyrrole compounds are able to cover almost the entire chromatic scale of the visible spectrum (Newsome *et al*., 2014). Pyrroles are a crucial structural component of many biological molecules, including chlorophyll, hemoglobin, and certain vitamins. The basic structure of chlorophyll (Fig.1.5.1) is structured around a porphyrin ring, consisting of four pyrrole rings (C_4H_4NH) coordinated by a central magnesium ion and a long hydrophobic alkyl chain (Leite *et al*., 2018). Chlorophylls represent porphyrin ring structures with one reduced double bond, known as chlorins (Fig. 1.5.1). In the chlorin core is magnesium, bonded to the tetrapyrrole ring. These molecules feature a hydrophilic (porphyrin) group head and a lipophilic hydrocarbon tail (phytol group), making them generally insoluble in polar solvents due to their lipophilic phytol tails. These two components are an essential part for the molecule to perform its function and it is, also thanks to them, that chlorophyll can perform the function of coloring in food (Sigurdson *et al*., 2017).

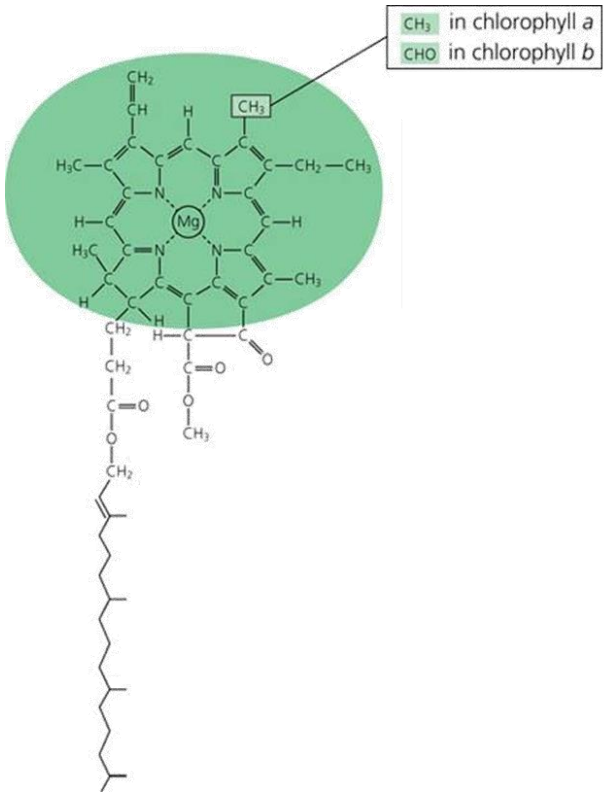

Fig. 1.5.1 Structure of chlorophyll molecules

In nature, various forms of chlorophyll exist. Specifically, in higher plants, chlorophyll *a* and *b* are predominant. Meanwhile, chlorophyll *c*, *d*, and *e*, along with their derivatives, are present in photosynthetic algae and certain other species, including brown, red, and yellow-green algae (Sheer, 1991). In detail, Chlorophyll *a* ($C_{55}H_{72}MgO_5N_4$) has a methyl group ($–CH_3$) at the carbon-7 position, whereas chlorophyll *b* ($C_{55}H_{70}MgN_4O_6$) has an aldehyde group (–CHO) at the same position (Carrillo *et al*., 2022) (Fig. 1.5.2). These distinct chemical structures result in color variations: chlorophyll *a* appears blue-green, while chlorophyll *b* appears blue-yellow. In plants, chlorophyll *a* and *b* coexist in a ratio of 3:1 (Carrillo *et al*., 2022). Studies have shown that plants growing in the shade have a higher proportion of chlorophyll *b* compared to chlorophyll *a*, a trend also observed when plants of the same species are grown under different light intensities (Khoi Nguyen et al., 2021).

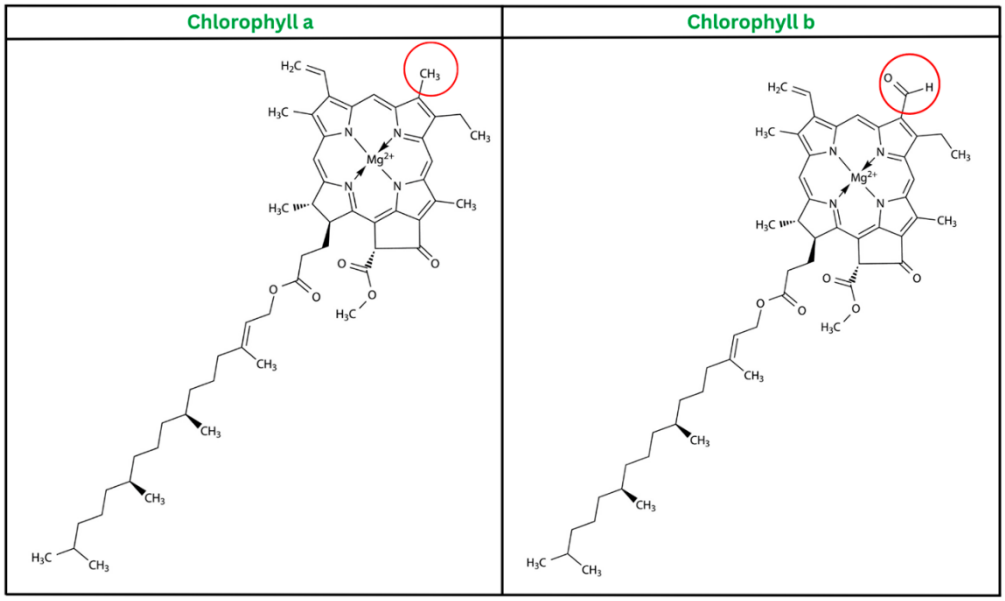

Fig. 1.5.2 The chemical structure of chlorophyll *a* and *b*. The difference is highlighted with a red circle. Source: Ebrahimi (2023)

With alkaline saponification, the phytol bond is broken down, forming chlorophyllin, which changes the pigment from lipophilic to hydrophilic (Humphrey, 2004). Weak acid treatments cause the central displacement of the Mg^{2+} ion, creating phaeophytin, which changes the pigment's color to shades of olive green/gray. Although this reaction is irreversible, strong complexes can form when Cu^{2+} or Zn^{2+} replace the central magnesium ion, restoring the pigment's functions and properties. These ions enhance the pigment's stability and produce more desirable green hues. Cu-chlorophyll complexes can also form naturally, as many plants contain significant levels of copper, and these complexes have been observed in plant extracts obtained with supercritical CO_2 (Humphrey, 2004).

European legislation, specifically Regulation (EC) No 1333/2008 and its amendments, has permitted the use of two natural green colorants, E140 and E141, which are structurally related to chlorophylls. In detail:

- E140 (i): chlorophyll extracted from green plants using organic solvents

- E140 (ii): chlorophyllins, derivatives of chlorophylls obtained by saponification. They are therefore more soluble than E140(i)

However, both colorants are relatively unstable, and their color tends to shift dramatically from green to brown. Consequently, the food industry prefers using colorant E141, created by adding copper to lipid or water-soluble chlorophyll solutions. The incorporation of copper into the chlorophyll molecule stabilizes its structure, ensuring that the green color remains unchanged regardless of processing conditions or storage duration. In detail:

- E141 (i): copper chlorophylls, produced from E140. Magnesium ion replaced with the copper ion. The resulting compound is green and more stable
- E14 (ii): copper chlorophyllins, chlorophyllins that have first undergone the saponification process and subsequently treatment with copper to replace the central ion

The application of chlorophyll, if obtained from edible plants, grass, and nettles, is allowed in Europe (Ebrahimi *et al*., 2023). Green food by-products are abundant in chlorophylls, which provide coloring and health-promoting benefits for foods, cosmetics, and pharmaceuticals, thanks to their bioactive properties. Consequently, chlorophyll extracted from these by-products can be repurposed for various applications (Ebrahimi *et al*., 2023). For example, it may be applied in cosmetics as mouthwash, soap, perfume, skincare due to its bioactive effects, or in pharmaceutics field thanks to its anti-disease effect, or in food industry applied such as food colorant, functional food, food additives and to increase food safety (Ebrahimi *et al*., 2023).

1.5.1 Extraction from plant matrices

Vegetable FW or byproducts are widely available and inexpensive and may be used as a cost-effective alternative source of natural pigments (Benucci *et al*., 2022). Valorizing plant FW could meet the demand for industrial-scale chlorophyll production, with applications in functional foods,

pharmaceuticals, and cosmetics (Sharma *et al*., 2021; Ebrahimi *et al*., 2023) Hence, in the last decades, numerous technologies for the utilization of vegetable FW as a resource in the framework of Circular Economy have been implemented (Benucci *et al*., 2022). This can be achieved through the extraction of colored pigments and other bioactive compounds using conventional techniques (e.g., Soxhlet, maceration) or green methods (e.g., ultrasound, pulsed electric fields, enzyme-assisted extraction) (Das *at al*., 2022). While conventional methods may yield reliable results, they have drawbacks, such as low yield, high cost, and the use of harsh chemicals that can harm the environment and reduce effectiveness (Ebrahimi *et al*., 2022). Therefore, more environmentally friendly techniques are needed to ensure safe extraction (Ebrahimi *et al*., 2023).

1.5.1.1 Conventional methods

Conventional methods like Soxhlet extraction, maceration, and hydrodistillation are simple, cost-effective, and easy to handle (Ngamwonglumlert *et al*., 2017). As a result, these techniques have been extensively employed to extract essential oils, bioactive compounds, and natural pigments from various plant materials.

Soxhlet extraction involves placing a plant sample in a thimble and repeatedly percolating it with condensed solvent vapor until extraction is complete, as indicated by the solvent becoming colorless. Despite its simplicity and ease of use, this method demands a large quantity of solvent, a long extraction period, and often results in pigment degradation due to heat (Veggi *et al*., 2013).

Maceration is a traditional extraction method that can be performed at room temperature, making it suitable for extracting heat-sensitive pigments. For example, Cha *et al*. (2010) found that phaeophytin content, which is an indirect indicator of the degradation of chlorophyll, was lower in a crude extract of green microalgae (*Chlorella vulgaris*) obtained by maceration (using 90% ethanol/water) at 25 °C for 6 hours, compared to Soxhlet extraction (at 100 °C for 2 hours). To extract raw pigments, the plant sample is ground and mixed with an extraction solvent, then stirred

in an extraction vessel. After the process, the liquid is filtered and pressed mechanically or centrifuged to repeat extraction with fresh solvent until the solvent is colorless (Azmir *et al*., 2013). Although maceration is typically performed at room temperature, it requires a long extraction time and a large amount of solvent (Ngamwonglumlert *et al*., 2017).

Hydrodistillation is a traditional method typically used to extract essential oils from plant material. The extraction medium, consisting of hot water and/or steam, is placed directly in contact with the sample to extract the desired compounds (Azmir *et al*., 2013). Since this method uses water for extraction, the resulting extract is free from contamination by organic solvents. However, because hydrodistillation usually operates at a high temperature (around 100 °C), pigment degradation can easily occur

1.5.1.2 Green extraction methods

Despite conventional extraction techniques with organic solvents are user-friendly, cost-effective, and require no sophisticated equipment, they do have several drawbacks. These include the need for a large amount of solvent, lengthy processing times, and potential degradation of the extracted pigment. To address these issues, green or unconventional extraction methods have been developed (Ngamwonglumlert *et al*., 2017). To enhance the yield and chemical stability of natural colorants, it's crucial to select an appropriate extraction procedure for each pigment type. Various innovative techniques, such as those using supercritical fluids, pressurized liquids, microwaves, ultrasounds, pulsed-electric fields, and enzymes, have been explored for extracting new potential food colorants from plant wastes (Ngamwonglumlert *et al*., 2017).

Supercritical fluid extraction (SFE) harnesses the advantages of supercritical fluids, which have properties similar to gases—high diffusivity and low viscosity—and the high solvation power of liquids (Macías-Sánchez *et al*., 2005). These characteristics allow better penetration into the matrix, leading to improved extraction efficiency. Most SFE solvents are non-polar, making this

method ideal for extracting low-polarity pigments like carotenoids and chlorophylls (Ngamwonglumlert *et al*., 2017). Despite requiring small quantities of solvent, SFE demands significant capital and operating costs due to the high pressures used (Veggi *et al*., 2013). This method has been shown to efficiently recover nonpolar pigments, with Derrien *et al*., (2018) reporting higher recovery rates for chlorophyll and lutein from spinach waste using supercritical CO_2 and ethanol compared to conventional methods like acetone extraction.

Pressurized liquid extraction (PLE) uses a liquid solvent at high pressure (10.3–13.8 MPa) and temperatures between 40 and 200 °C for extraction (Antunes *et al.*, 2008; Miranda *et al.*, 2021). The high temperature facilitates better diffusion of the solvent into the sample matrix, destroying plant cells and effectively releasing pigments. High pressure forces the solvent into matrix pores, enhancing contact between the solvent and compounds to be extracted (Ngamwonglumlert *et al*., 2017). Consequently, PLE requires shorter times and fewer solvents (Antunes *et al.*, 2008). PLE can extract both polar and apolar pigments, depending on the solvent used. However, it is not suitable for heat-sensitive pigments due to the high temperatures involved, making it more appropriate for pigments like anthocyanins, carotenoids, and chlorophylls. Despite requiring less solvent and time, PLE cannot effectively recover heat-sensitive pigments (e.g., betalains) due to high temperatures and involves significant capital and operating costs (Ngamwonglumlert *et al*., 2017).

Microwave Assisted Extraction (MAE) is gaining interest as an alternative for extracting both hydrophilic and hydrophobic plant pigments due to its speed and reduced solvent consumption compared to conventional methods (Dahmoune *et al*., 2014). The rapid heating from microwave radiation causes an expansion of the plant cell structure, leading to the breakdown of cell walls. Consequently pigments, can easily migrate from cells, resulting in an increased extraction rate (Ngamwonglumlert *et al*., 2017). Combining MAE with vacuum, leading to vacuum microwave assisted extraction (VMAE), has recently been proposed for extracting heat-sensitive bioactive

compounds and pigments (Hiranvarachat *et al*., 2015). VMAE can reduce pigment degradation by oxidation due to less oxygen availability during the extraction process.

Ultrasound-assisted extraction (UAE) utilises cavitation bubbles generated by ultrasonic waves to enhance extraction efficiency (Rastogi, 2011). When ultrasonic waves pass through a medium, they create alternating cycles of compression and decompression, leading to the formation and expansion of cavitation bubbles. These bubbles eventually collapse when they can no longer be contained by surface tension forces, generating shear forces that break or destroy plant cell walls, thus enhancing the release of intracellular compounds (Pitt *et al*., 2004). UAE is known for its lower extraction efficiency compared to other non-conventional processes like SFE, PLE, MAE, and pulsed-electric field (PEF) extraction (Ngamwonglumlert *et al*., 2017). For instance, Cha *et al*. (2010) found that PLE at 160 °C for 30 minutes yielded higher amounts of carotenoids and chlorophylls from *Chlorella vulgaris* compared to UAE at 25 °C for 360 minutes. However, PLE's higher operating temperatures caused greater chlorophyll degradation, resulting in increased phaeophytin content. To improve efficiency and reduce the time required for MAE, the combined use of UAE and MAE has been proposed, causing more extensive damage to the matrix structure than either method alone (Ngamwonglumlert *et al*., 2017). UAE is suitable for extracting both polar and nonpolar compounds, as well as heat-sensitive compounds since it is an athermic process.

Enzyme-assisted extraction (EAE). This extraction technique will be explored in the next chapter.

1.5.1.3 Enzyme-assisted extraction

Enzyme-assisted extraction of biomolecules from plants is an increasingly alternative to conventional solvent methods due to its efficiency, sustainability, and eco-friendliness (Nadar *et al*., 2018). This technique leverages the unique properties of enzymes, which perform reactions

with high specificity and regioselectivity, under mild conditions, thus preserving the extracted compounds (Puri *et al*., 2012). The core principle of enzyme-assisted extraction involves hydrolysis of the plant cell wall, using enzymes as catalysts under optimal conditions to release intracellular components. The plant cell wall binds to the enzyme's active site, causing the enzyme to change shape and maximize interaction with the substrate. This shape change breaks down the cell wall bonds, releasing the active constituents (Sheldon & van Pelt, 2013).

This environmentally friendly extraction method not only reduces the need for hazardous solvents but also shortens extraction times. Additionally, because the extraction is carried out under controlled temperature conditions, it is highly effective for extracting heat-sensitive molecules such as flavors, pigments, and oils. A variety of carbohydrate-hydrolyzing enzymes are typically used in enzyme-assisted extraction. For example, Özkan & Bilek (2015) studied the recovery of chlorophyll from spinach using the commercial enzyme mix Pectinex Ultra SP-L (containing polygalacturonase, hemicellulase, cellulase, protease and amylase) for the pretreatment of the plant matrix, followed by ethanol extraction. The authors demonstrated that EAE increased the extraction yield by 39%, while allowing a significant reduction in the solvents used. Each of these enzymes has the capability to hydrolyse key components of plant cell walls. Specifically, cellulases break down cellulose into cellobiose and glucose molecules. Hemicellulases degrade hemicelluloses, producing simple sugars or oligosaccharides. Pectinases hydrolyze various types of pectins, releasing uronic acids. Lastly, proteases break down proteins into peptides, amino acids, and other components (Chemat & Vian, 2014).

Many compounds of interest are trapped within the cell, with the primary barrier being cell walls composed of polysaccharides and their derivatives like cellulose, hemicellulose, and pectin, which are connected through hydrophobic interactions and hydrogen bonds (Nadar *et al*., 2018). Additionally, some phenolic acids form ether bonds with lignin via their hydroxyl groups in the aromatic ring and ester bonds with structural carbohydrates and proteins through their carboxyl groups (Fig. 1.5.3).

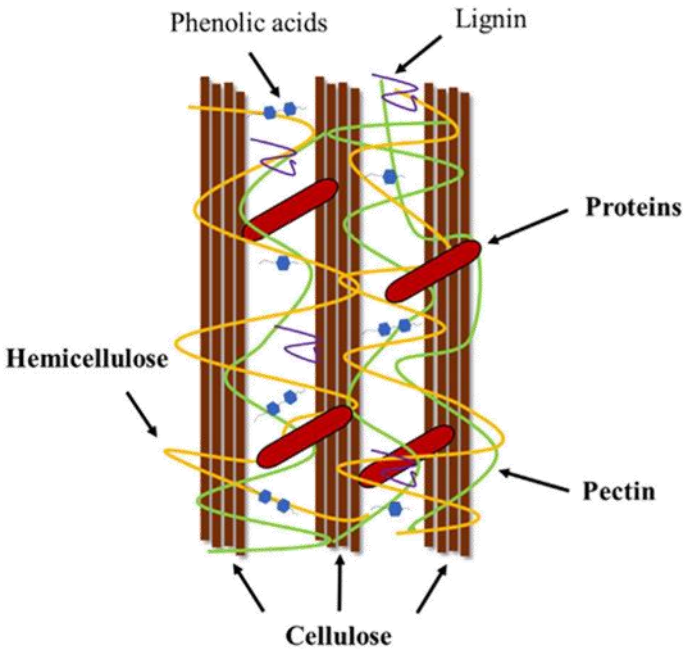

Fig. 1.5.3 Representation of primary vegetable cell wall components

Key factors in the extraction process include reaction temperature, extraction time, system pH and enzyme concentration (M'hiri *et al*., 2014). In fact, selecting the right enzymes and optimizing extraction conditions are crucial. Temperature is a fundamental factor, as high temperatures can gradually reduce enzymatic activity and inactivate proteins and other bioactive compounds (Peterson *et al*., 2007). On the other hand, lower temperatures do not accelerate enzyme actions, resulting in decreased pigment extraction. Each enzyme has an optimal pH for enzymatic hydrolysis, typically within the isoelectric pH range of different proteins (Talley & Alexov, 2010). Since proteins are highly insoluble at this pH, biomolecule release can be delayed. Thus, the optimal extraction pH must be chosen to avoid impeding enzyme functionality (Nadar *et al*., 2017). Extending the contact time between the enzyme and the extract generally enhances the degradation of plant cell wall components. However, this is impractical on a large scale due to the potential for lower product quality and higher energy consumption associated with long incubation periods (Babbar *et al*., 2016). Moreover, extending extraction time may reduce yield due to substrate

exhaustion and/or the formation of enzyme-inhibiting compounds (Nadar *et al*., 2018). The overall enzyme dosage is another critical parameter. High concentrations can enhance enzyme-substrate interactions, facilitating cell wall degradation, but might also lead to the extraction of undesirable substances. Therefore, the optimal dosage should balance enzyme cost with pigment quality (Jiang *et al*., 2010).

Overall, EAE offers significant benefits, including lower energy consumption, faster extraction rates, higher yields, easier recovery with reduced solvent use (Puri *et al*., 2012), reduced risk of residual solvents in the extract, and better retention of target compounds (Ozkan *et al*., 2021). Additionally, this method is highly effective for extracting heat-sensitive molecules, due to its mild temperature conditions. Although its industrial applications are currently limited, numerous studies indicate that enzymes hold great potential for large-scale recovery of high-value molecules from agri-food wastes. However, Sagar *et al*., (2018) highlight several drawbacks of EAE: high enzyme costs for large sample volumes, challenges in industrial feasibility, and the need to optimize parameters like particle size, hydrolysis time, enzyme concentration, and composition. Despite the high cost of enzymes, it's crucial to note that Chemat *et al*., (2014) demonstrated through an economic analysis that EAE can reduce production costs by saving time, equipment, and waste disposal compared to other extraction techniques. Furthermore, most commercial enzyme preparations operate at relatively low temperatures (<50°C), thereby lowering energy requirements (Chemat *et al*., 2014).

1.5.2 Effects on human health

The growing number of epidemiological and experimental studies that associate diets rich in fruit and vegetables with the prevention of chronic diseases, such as cancer, has stimulated interest in the phytochemical compounds present in these matrices as physiologically active dietary components (Ferruzzi & Blakeslee, 2007). Several studies have demonstrated a powerful anticancerogenic action of chlorophyll and its derivatives thanks to the ability to form complex

structures with some substances toxic to the human body, interfering with the gastrointestinal absorption of potential carcinogens and thus decreasing the amount of these substances in susceptible tissues (Ferruzzi & Blakeslee, 2007; Young & Beregi,1980).

This anticancerogenic activity has been observed especially in chlorophyllin, a semi-synthetic derivative that differs from chlorophyll mainly for the replacement of the magnesium atom with a copper atom and for the absence of the hydrophobic tail (phytol). The absence of phytol would seem to increase the effectiveness against carcinogenic and mutagenic agents (Tachino *et al*., 1994). Furthermore, the effectiveness of chlorophyll and its derivatives against some substances dangerous to humans has been demonstrated, such as: aflatoxins and mycotoxins (Jubert *et al*., 2009), polycyclic aromatic hydrocarbons that form following incomplete combustion (Tachino *et al*., 1994) and heterocyclic amines that form with meat cooked at high temperatures (Shaughnessy *et al*., 2011).

Many studies report that chlorophyll and its derivatives have antioxidant properties and therefore help neutralize free radicals, which cause cellular damage (Zhang *et al*., 2008) with a greater effect from pheophytin (Hsu *et al*., 2005), that is, chlorophyll in which the magnesium atom has been replaced with two hydrogen ions. The protective effect against oxidative damage would be due to the promotion by chlorophyll of the expression of heme oxygenase, an enzyme that helps maintain cellular homeostasis and whose deficiency would make the organism more vulnerable to inflammatory stimuli and oxidative stress. Furthermore, the efficacy of chlorophyll in the prevention of colon cancer has been demonstrated and its antimicrobial activity (Larato,1970).

The properties and benefits of chlorophyll for human health are outlined in Fig. 1.5.4.

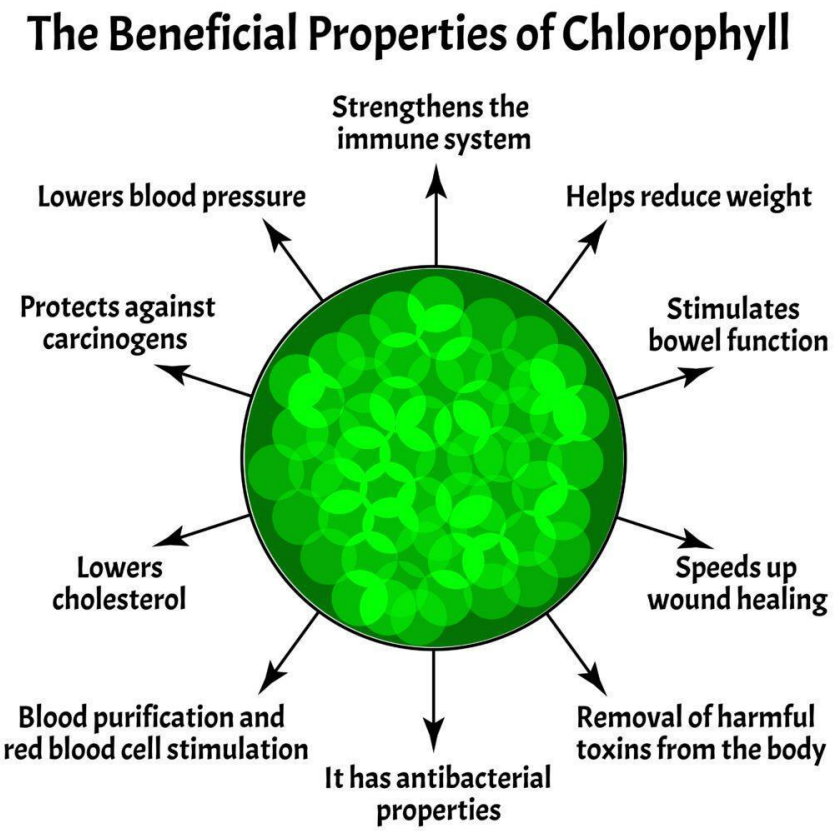

Fig. 1.5.4 Schematic rapresentation of the properties and benefits of chlorophyll for human health

1.5.3 Stability

Under natural and physiological conditions, chlorophylls are stable pigments. However, once extracted or processed, changes in pH and temperature during the processing and storage of green foods can cause chlorophylls to undergo structural alterations. These alterations lead to changes in their color. The most significant reaction involves the replacement of the central magnesium (Mg) ion with two hydrogen ions. This replacement leads to the formation of pheophytin and pyropheophytin *a* (grayish color) and pheophytin and pyropheophytin *b* (brownish color) (Fig. 1.5.5) (Rodriguez-Amaya *et al.,* 2016). A similar reaction occurs in chlorophyllide, obtained by the loss of phytol, from which we arrive at pheophorbide and pyropheophorbide (olive brown color). Consequently, this quick reaction is the main reason for the loss of the original green color during the processing and storage of green foods (Rodriguez-Amaya *et al.,* 2016).

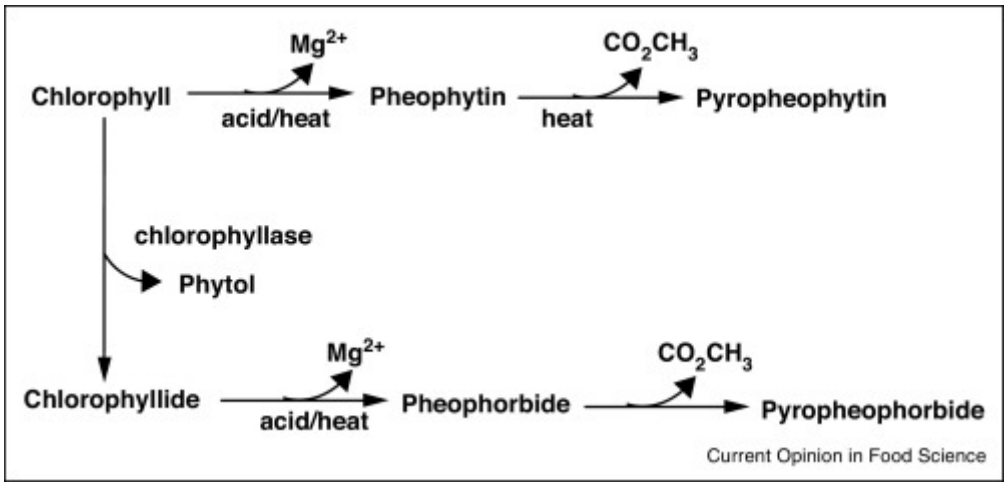

Fig. 1.5.5 Chlorophyll degradation. Source: Rodriguez-Amaya et al., (2016)

Chlorophyll is a stable pigment in nature, but when extracted from plant tissues, it could be degraded due to many factors (Fig. 1.5.6) such as heat, light, oxygen, pH and degradative enzymes (Koca *et al*., 2007).

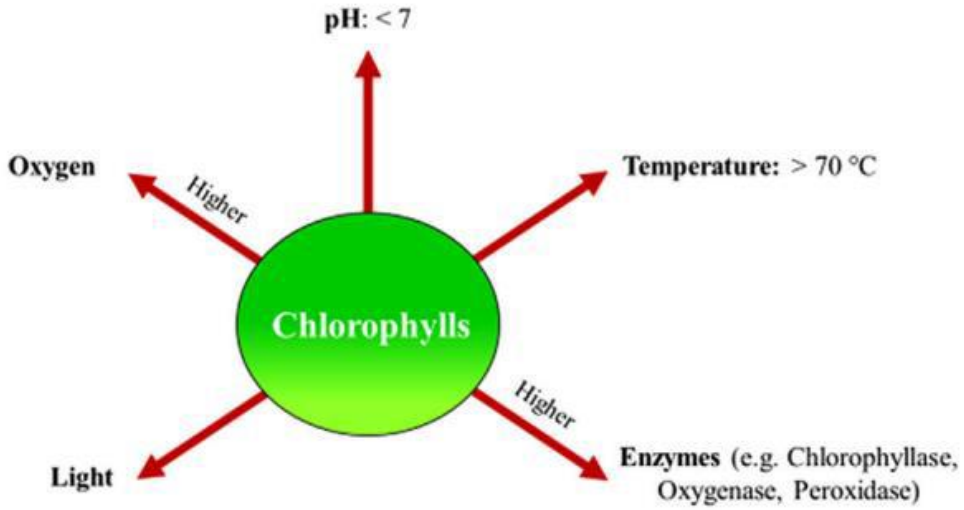

Fig. 1.5.6 Factors affecting chlorophyll stability

Operations such as pasteurization, cooking, drying, freezing and thawing of vegetables rarely cause rapid color degradation, but rather initiate a slow color transformation that becomes evident during shelf-life. Degradation proceeds more rapidly if matrices are subjected to excessive heat

treatments or if they are processed/used in acidic foods, such as aromatic herbs in vinaigrette sauce (Canjura, 1991). For example, Koca *et al*., (2007) studied the effect of various pH (5.5, 6.5,7.5) on chlorophyll degradation and color loss in peas. The results showed an increase in half-life values with increasing pH and an increase in the degradation rate at pH 5.5 compared to pH 7.5 by about 2.7-7.3 times. Consistently, the rate constants of color loss decreased with increasing pH, confirming that green is maintained at higher pH conditions. Another factor to consider is temperature: chlorophyll content present in various products decreases significantly when these are subjected to conditions of strong heat. Pistachios roasted for 60 minutes at 138°C showed a significant increase in the levels of pyropheophytins *a* and *b*, 10/12 times higher than raw pistachios. These compounds, resulting from the degradation of the pigment, cause a color variation; in fact, the fruit underwent a change from bright green to dull green (Pumilia *et al*.,2014). Regarding the effects of light on chlorophyll, studies present sometimes contradictory results: some suggest a positive effect since light can delay the degradation of chlorophyll, while others indicate the opposite. In the work conducted by Ferrante *et al*., (2004), it was observed that rocket (*Eruca sativa Mill*.) exposed directly to light for 8 consecutive days, showed a decrease in chlorophyll content of about 50%, accompanied by a strong yellowing. However, such changes were not found in dark conditions, where the color change was barely observable.

1.5.3.1 Stabilization of Green Color

As mentioned earlier, the reduction or complete loss of the green color is associated by consumers with a decrease in product quality. To address this issue, the food industry has developed various strategies to maintain the initial green color. Strategies to preserve chlorophyll include: heat inactivation of the enzyme chlorophyllase, to minimize the conversion of chlorophyll to pheophytin, the addition of Mg^{++} salts to prevent loss of the ion, control of processing conditions

such as pH, temperature and ionic strength of food products, the addition of antioxidants to prevent oxidation of chlorophyll by light (Heaton & Marangoni, 1996).

Early methods included adding alkalinizing agents (even directly into the can), but these were eventually abandoned due to undesirable side effects like softening of the edible material or the release of off-flavors (Schwartz *et al*., 2017).

An alternative method has involved replacing the central hydrogen atoms with zinc or copper ions to create more stable green metall-chlorophylls, effectively restoring the green hue of the food product. Over the years, the conditions for generating zinc and copper chlorophyll complexes in vegetables during processing have been refined (Jones *at al*., 1977; LaBorde *at al*., 1990). The industrial and commercial significance of this process has led to numerous patents, the most well-known being the "Veri-green" method developed by the former Continental Can Company (Segner *et al*., 1984). This method involved blanching vegetables in brine solutions containing small amounts of Zn^{+2} or Cu^{+2} salts to primarily form zinc or copper pheophytins, thereby making the processed vegetables appear greener (Von Elbe *et al*., 1986).

New strategies are being developed to encapsulate zinc-chlorophylls using various matrices such as gum Arabic, maltodextrin, OSA-modified starch, or whey proteins (Porrarud *et al*., 2010; Özkan *et al*., 2018). Encapsulation is a process of “packaging” active agents inside a carrier material (Fig.1.5.7).

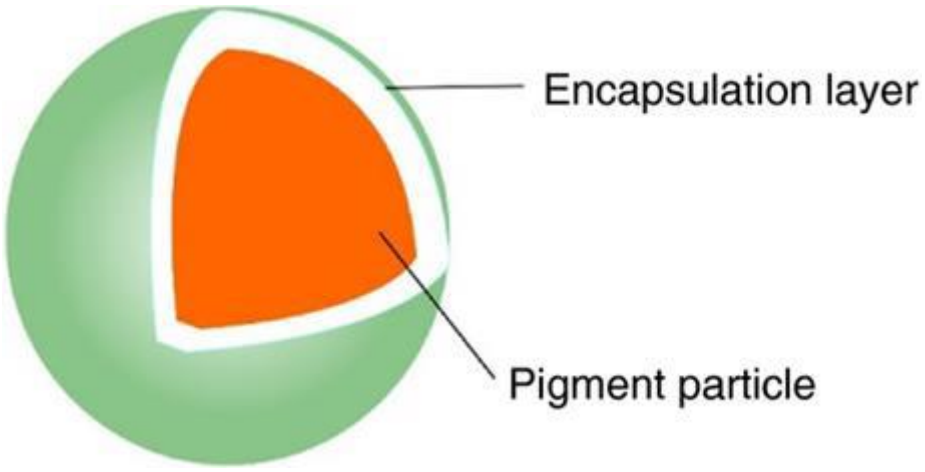

Fig. 1.5.7 Schematic illustration of pigment encapsulation. Source: Tawiah et al.,(2022)

This technique, developed about 60 years ago, has the aim of preserving the stability of bioactive compounds during processing and storage; in recent years it has proved particularly useful in the food sector, as well as in the pharmaceutical sector where it has already been widely used for some time, in the protection of pigments from destabilizing factors such as light, temperature and humidity (Nedovic *et al*., 2011). The materials used for the coating are many, but all must satisfy some requirements:

- be recognized as GRAS "generally recognized as safe"

- provide protection from external factors

- retain the active ingredients within the complex, both during the processing and storage phases

- have good rheological characteristics

- do not react with the encapsulated material

In the case of food products, these materials must be edible, biodegradable, but at the same time stable during the various process phases. To date, the most commonly used are materials of a carbohydrate nature, but also proteins and lipids (Nedovic *et al*., 2011).

Encapsulation can be performed through various techniques:

Spray drying is a relatively old encapsulation technique, yet it remains one of the most widely used methods in the food and pharmaceutical industries. This is because it offers good encapsulation efficiency, allowing for the rapid production of a stable final product at moderate costs. The process involves converting a fluid into powder through several steps. A mixture of wall and core material is properly hydrated and atomized into droplets. These droplets then enter the drying chamber, where hot air circulation enables their drying, resulting in solid particles. Despite its extensive use, the technique has the issue of potentially compromising heat-sensitive ingredients due to the high temperatures employed (Patel *et al*.,2009; Drosou *et a*l.,2017).

Freeze drying is a technique comprising three stages. First, the material is frozen at temperatures ranging from -50°C to -80°C, which is a delicate phase as it risks altering the compounds of interest. This is followed by two drying stages. In the first stage, which occurs very slowly, 95% of the water sublimates under pressures between 0.1 mbar and 0.2 mbar, with heat addition and potential application of partial vacuum. During the secondary drying, non-frozen water molecules are removed, the temperature increases compared to the previous stage, while the pressure remains constant. At the end of the process, the vacuum is broken with inert gas, and the final water content is about 1%. This technique is mainly used for drying heat-sensitive materials and foods prone to oxidation (Kandasamy *et al*., 2022; Shukla *et al*.,2011).

Coacervation can be classified into simple and complex (based on the number of polymers involved). In general, this technique involves three main step (Fig. 1.5.8): i) Preparation of the Dispersion or Emulsion: pigment is dispersed in a cationic aqueous polymer, to which a second, generally anionic, solution is added; ii) Encapsulation of the Chosen Material: the complex forms through agitation, as the wall material deposits onto the particles of the material to be encapsulated. This process is facilitated by the addition of salt, and adjustments in pH and/or temperature; iii) Stabilization of the Encapsulated Material (typically achieved through heat treatment) (Tawiah *et al*.,2022).

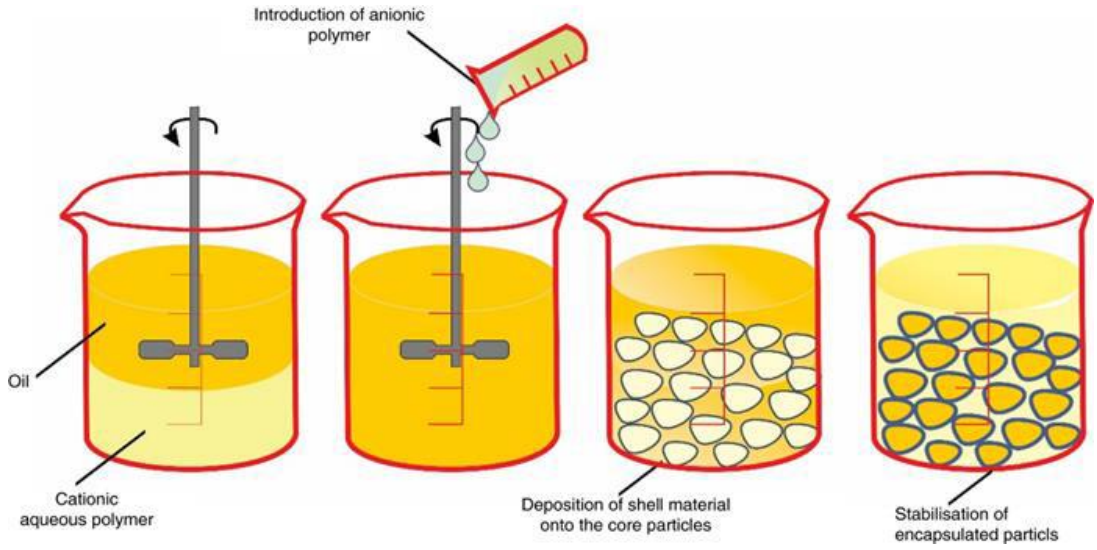

Fig. 1.5.8 Coacervation. Souce:Tawiah et al.,(2022)

Inclusion complexation, also known as molecular inclusion, is an encapsulation technique that uses the properties of host molecule to trap the substance of interest (guest molecule). The success of inclusion is influenced by entropic factors, responsible for the displacement of water molecules, and enthalpic factors, which involve hydrophobic interactions between the host molecule cavity and the included guest molecule. The complex is stabilized by weak bonds, such as Van der Waals forces and hydrogen bonds (Pascarella, 2016). The formation of the complex depends on the size compatibility between the host molecule and the guest molecule (Singh *et al*.,2023; Sawyer & Kontopidis, 2000; Agarry et al., 2022).

Chlorophyll has been successfully encapsulated using different materials and techniques. For example, Agarry *et al*., (2022) have encapsulated chlorophyll using different carrier agents such as zein, casein, and whey protein isolate (WPI). The study showed that they could be effectively used as a carrier agent in chlorophyll encapsulation as they have been shown to be effective in protecting chlorophyll from acid (pH 2 to 6, chlorophyll retention; 39.3 to 97.8%) and from light (chlorophyll retention; 41.6 to 65.5%) condition. Kang *et al*. (2019) used different blends of gum Arabic and maltodextrin for encapsulation of chlorophyll by spray drying, proving that encapsulation with maltodextrin alone showed the highest storage stability (94.7%−97.5%). In a

recent study, soy protein isolate (SPI) and chitosan were used as carriers to encapsulate chlorophyll. The authors demonstrated that the predicted and experimental encapsulation efficiency was 97.95% and 96.21% (using a 1:7 ratio of chitosan:SPI, a 1:14.8 ratio of chlorophyll: SPI, respectively). Furthermore, using the complex coacervation and vibrating nozzle technology, green nanoparticles with satisfactory encapsulation efficiency (94.9–96.1%) and good loading capacity (3.32–4.22%) were obtained (Agarry *et al*., 2022). Zhang *et al*., (2019) used Whey Proteine Isolate (WPI) to improve the solubility and stability of chlorophyll by producing WPI-CH microcapsules by spray drying. The work results showed that the encapsulation efficiency and solubility of chlorophyll were increased by 3.78% and 7.79%.

2. Results and Discussion

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COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

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Natural colorants from vegetable food waste: Recovery, regulatory aspects, and stability—A review

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Abstract: Worldwide, approaches inspired by the Circular Economy model have been increasing steadily, generating new business opportunities such as the recovery of high-added value molecules (e.g., pigments) from vegetable food waste that may be applied as food additives (e.g., colorants). Indeed, food waste is a global problem that does not seem to be decreasing, leading to economic, environmental, and social issues. Moreover, synthetic dyes have been associated with adverse effects on human health, encouraging research to explore much safer, natural, and eco-friendly pigments. This state-of-the-art review gives a brief overview of the regulatory aspects concerning food waste, Circular Economy, and natural versus synthetic colorants. We have critically reviewed the recent advances in pigment recovery from vegetable food waste bringing back the green/unconventional extraction methods. Among them, enzyme-assisted extraction as a depth feature technique is highlighted, given that it allows the recovery of pigments in a mild, selective, efficient, and sustainable way. Furthermore, the stability issue of the different natural colorants has been critically discussed in relation to the extraction and application conditions. Several and tailored stabilization methods have been described and reported for each pigment although additional research is necessary on their long-lasting stabilization and utilization in food matrices.

KEYWORDS
Circular Economy, enzyme-assisted extraction, food waste, pigment from vegetables, sustainable routes

Practical Application

This review focuses on the main types of natural pigments in vegetable food waste, their legislative framework, extraction technologies and strategies to improve the stability , as well as their possible applications.

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1 | INTRODUCTION

Food waste (FW) is a global issue that does not appear to decrease, leading to economic, environmental, and social questions (Teigiserova et al., 2020). This problem has long been neglected, in such degree more and more waste is recorded at all levels of the food chain (Peira et al., 2018). The European Union defines FW as: "all the foodstuffs discarded from the food supply chain for economic or aesthetic reasons or owing to the nearness of the 'use by' date, but which are still perfectly edible and fit for human consumption and, in the absence of any alternative use, are ultimately eliminated and disposed of, generating negative externalities from an environmental point of view, economic costs and a loss of revenue for businesses" (European Parliament Resolution, 2012).

FW represents a loss of edible food that is discarded from the supply chain by free choice and/or negligence by its actors (Peira et al., 2018) throughout distribution and consumption phase (wholesaling, logistic, retailing, market, food service, and household) (Parfitt et al., 2010). Recent studies have reported that global FW is 1.6 billion tons/year, of which 1.3–1.6 billion tons/year is edible, for a business value of US $750 million. A common family produces most of the FW (about 47 million tons/year), succeeded by the processing phase (17 million tons), catering (10.5 million tons), primary production (9.1 million tons), and distribution (4.6 million tons). FW mainly consists of meat and fish (30%), vegetables (19%), and dairy products (17%), amounting to US $27 billion (Peira et al., 2018). It has been assumed that the lack of initiatives to reduce FW could increase it by about 40%, corresponding to 126 million tons/year (Peira et al., 2018). An effective waste management may be achieved only throughout a valuable food chain control and a better use of the limited resources available (Battilani, 2015). Keeping in mind Circular Economy concepts, recent developments have been focused on the utilization of vegetal wastes as source of natural pigments.

2 | CIRCULAR ECONOMY APPROACH ON FOOD WASTE

One of the pillars of sustainable development is the Circular Economy model. The transition from a linear economy model to a circular one means that resources remain in the economy even after the end of life of materials and products. In detail, Circular Economy may be defined as a model of production/consumption, which involves sharing, reusing, and recycling products as long as possible, thus minimizing waste. The Circular Economy can define the background requirements for the management of FW and other kinds of waste, generating new business opportunities. FW may be applied to produce several biomaterials (Mirabella et al., 2014), bioenergy (Dahiya et al., 2018), and high-value products (Galanakis, 2012; Sagar et al., 2018). Taking into account the definition of waste by Circular Economy (waste = secondary resource), EU has approved an action plan to incorporate it into Member States legislation (Directive EU, 2018/851). The FW legislation in Europe has always been unclear. The key EU directive, Waste Framework Directive (European Parliament. (2008), Directive EU, 2008/98/EC), does not distinguish FW from other kinds of bio-waste (such as garden waste) (Teigiserova et al., 2020). In this scenario, a Circular Economy program was drawn up to manage food waste in order to reduce it as much as possible. This model is explained by means of a pyramid chart (Sanchez et al., 2020) which shows actions that should be implemented (Figure 1).

This pyramid describes the optimal strategies, focusing first on prevention actions, followed by the paths of reusing of surplus food suitable for human consumption, the reuse of food no longer intended for human consumption as animal feed, recycling/recovery of high-added value molecules (e.g., natural colorant), nutrient recycling, energy recovery and, as the last option, food waste disposal (Figure 1) (Sanchez et al., 2020; Teigiserova et al., 2020). In particular, regarding the recovery of high-added value molecules, the scheme in Figure 2 shows five distinct phases. The method (general and flexible based on the matrix and the molecule to be extracted) proceeds from the macroscopic to the macromolecular level, followed by purification of the target compounds and subsequently to the extraction of specific micromolecules (Figure 2) (Galanakis, 2012).

Aiming to minimize the environmental impact of waste supporting the Circular Economy concepts, the great challenge for the future is the exploitation of natural additives from FW. The industrial use of such additives (including natural pigments) has been increasing in the last decades due to the consumers' demand for healthy food (Martins et al., 2016).

3 | FOOD ADDITIVES AND COLORANTS

Food additives present a long history of use and more specifically food colorants are a good example. The former are currently defined as "any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its

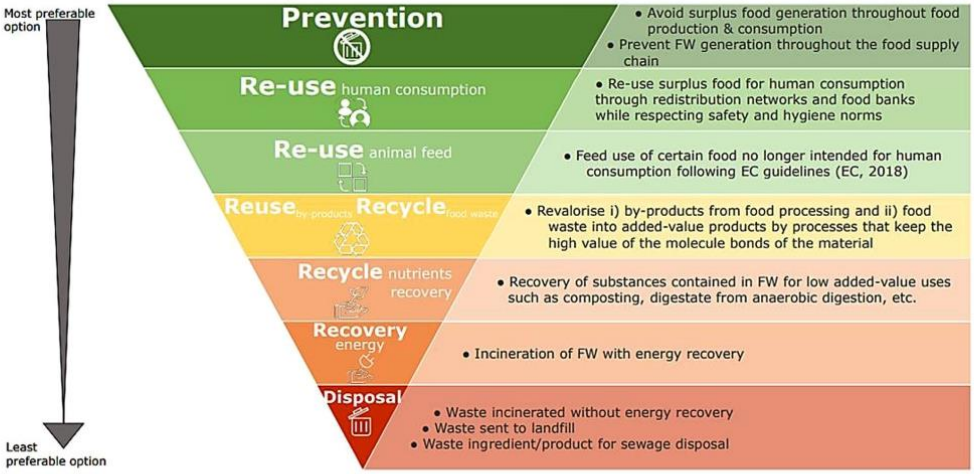

FIGURE 1 Practical application of the waste hierarchy for food. *Source*: Sanchez et al. (2020)

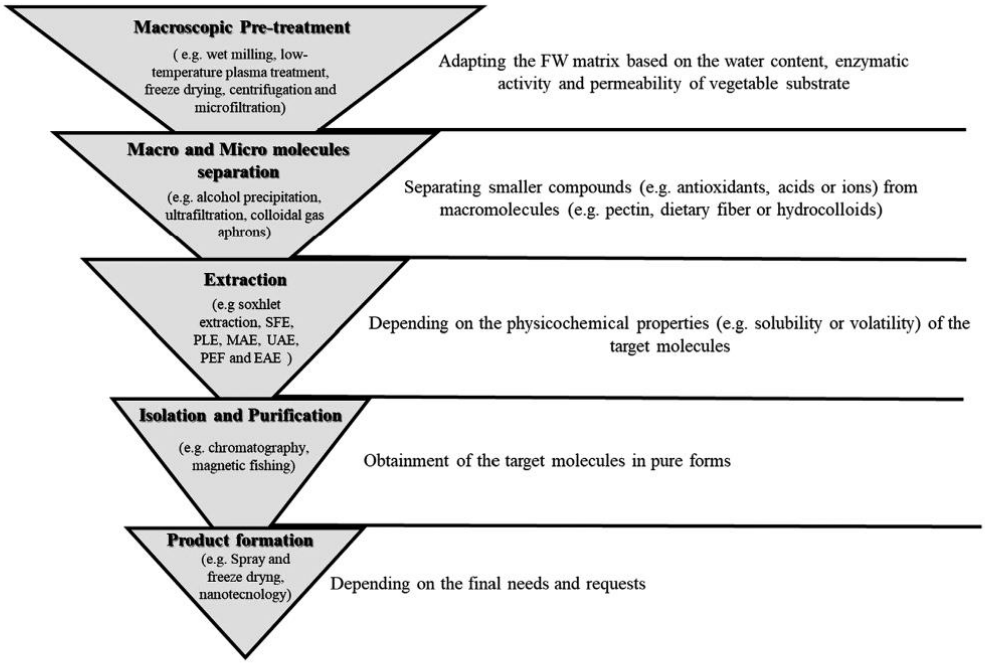

FIGURE 2 Recovery phase of high-added value compounds from food wastes (FW). Abbreviations: EAE, enzyme-assisted extraction; MAE, microwave-assisted extraction; PEF, pulsed electric field; PLE, pressurized liquid extraction; SFE, supercritical fluid extraction; UAE, ultrasound-assisted extraction

by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities" (Codex Alimentarius Commission, 2017). Worldwide, the two main regulatory bodies for food additives are the European Food Safety Authority (EFSA) and the United States Food and Drug Administration (FDA) (Carocho et al., 2014). A huge effort has been made to assemble knowledge for the creation of a unique database of legal additives for use within the European Union. In Regulation 1129 of 2011, all approved additives and their acceptable daily intake (ADI) have been listed (Council Regulation (EC) 1333/2008; Council Regulation [EC] 1129/2011). In the United States, starting from 1961, the FDA ruled that all food components should be labeled as generally recognized as safe (GRAS). In the European Union, food additives are classified into 26 functional groups, according to their function in food (Council Regulation, 1333/2008), whereas in the United States they are ranked in six classes (Carocho et al., 2014). In addition to these classifications, additives, including food colorants, may be categorized according to origin and manufacture, dividing into four groups: natural additives (from animal or plant sources), similar to natural additives (mimicking natural ones), modified from natural (natural additives which are then chemically modified), and artificial additives (synthetic compounds) (Carocho et al., 2014).

In details, food colorant is defined as "any dye, pigment or substance which when added or applied to a food, drug or cosmetic, or to the human body, is capable (alone or through reactions with other substances) of imparting color" (Martins et al., 2016). Legislation regarding food colors differs from country to country.

3.1 | Synthetic food colorants

Colorants or dyes are applied as food additives to change or give color to food, in order to improve its appeal to consumers (Sigurdson et al., 2017). Synthetic colorants may be produced by full chemical synthesis or modification of several natural precursor compounds (E160 carotenoids, E161 xanthophylles, E162 betanine, E163 anthocyans, E140-E141 chlorophylls). Although dyes have long been used by the food industry, there are a number of controversies and disagreements over their potential effects on human health (Branen et al., 2001; Msagati, 2013). Legislation regarding food colors differs from country to country. For example, amaranth (E123), carmosine (E122), and others are banned in the United States but not in the European Union, while fast green (FD&C Green No. 3) is banned in the European Union and lawfully added to food in the United States. Food colorants may be categorized into five groups, according to their chemical structure: azo-compounds, quinophthalone derivatives of quinoline yellow, triarylmethane group, xanthenes, and indigo dyes (Sarikaya et al., 2012).

Among azo compounds, some of the most used are tartrazine, known in the United States as FD&C Yellow No. 5 and in the European Union as E102, sunset yellow (USA: FD&C Yellow No. 6; EU: E110), allura red (USA: FD&C Red No. 40; EU: E129), amaranth (E123), and carmosine (E122) (Carocho et al., 2014). The European Union has revised its opinion on allura red, concluding that there is a possibility that it may be genotoxic; however, some existing studies indicate the opposite (Carocho et al., 2014). Amaranth, another petroleum-derived dye, has been banned in the United States as possibly carcinogenic, but it is approved in the European Union and some other countries. Azo compounds are commonly added in blancmange, marzipan, Swiss roll, jam, yoghurt, jelly, breadcrumb, cotton candy, soft drinks, flavored chips, cereals (corn flakes, muesli, etc.), cake mixes, soups, sauces, some rice, ice cream, candy, and chewing gum (Amin et al., 2010). Quinoline Yellow (E104) is a synthetic quinophthalone dye chemically obtained by mixing sodium disulfonates, monosulfonates, and trisulfonates and is usually used in soft drinks, cakes, cosmetics, and medicines (Al-Shabib et al., 2020). Triarylmethane group includes the following: bright blue (USA: FD&C Blue No. 1; EU: E133), fast green (E143), patent blue (E131), and bright black (E151). Bright blue and patented blue are the most common colorants, although the former is banned in the United States. No authorization has been required for bright black, while fast green is banned in the European Union. These compounds are generally added in yogurt, ice cream, drinks, and sweets (Unsal et al., 2015). Xanthene group is composed of erythrosine (USA: FD&C Red No. 3; EU: E127), fluorescein, eosin, and rhodamine. Erythrosine is permitted in the United States and the European Union, while fluorescein is prohibited in the European Union. They may be found in cocktails, tinned cherries and fruits, biscuits, chocolates, garlic sausages, salmon spreads, scotch eggs, stuffed olives, sweets, bakery items, snack foods, chewing gums, jellies, wines, and ice cream (Tabara et al., 2011). Finally, indigo dyes (FD&C Blue No. 2) are synthetic additives, recovered in the past from the *Indigofera tinctoria* shrub. Nowadays, they are chemically obtained and used in gelatins, ice creams, jelly beans, bubble gums, and candies (Deroco et al., 2018).

3.2 | Food colorants from natural sources

As for ever-growing trends in the food coloring industry, the use of natural pigments (natural colorants) has increased in foods and beverages as substitutes for

their synthetic counterparts. This is mainly due to the growing awareness of environmental risks and potential side effects of chemicals used in the synthesis of food colors (Carocho et al., 2014). Additionally, synthetic dyes tend to be replaced in the food industry in order to be in line with current consumer needs. This marketing strategy is in line with the so-called "clean-label" trend.

The use of natural colorants in the European Union is regulated by the Council Regulation (EC) 1333/2008 (ex 94/36/EC), which includes pigments selectively extracted from a source and intentionally added for the purpose of coloring in the preparation of the final food (Reinhart, 2014).

For example, it is possible to talk about the curcumin dye (E100) only when, after selective extraction from the turmeric root, cellulose, waxes, vegetable lipids, and volatile oily compounds are eliminated. However, some natural pigments, in addition to having coloring properties, may have further physiological (e.g., antioxidant) or flavoring characteristics. For this reason, their classification with respect to food law is not always easy; mainly because the requirements for premarketing approval depend on the classification of the substance (Reinhart, 2014). To solve this problem, the EU Council Regulation excludes from the legislation on food additives those pigments that are explicitly used for their aromatic, savory, or nutritional purpose, and when their coloring effect in special food is only secondary (e.g., butter, which may be colored exclusively with carotenes).

In the United States, dyes are regulated through the Federal Food, Drug and Cosmetic Act (FFDCA). Unlike the European legislation, American legislation does not distinguish between colors produced by synthesis and pigments extracted or isolated with or without modifications from vegetable, animal, mineral, or other sources. However, the approved colors may be divided into two groups: certified colors (which are all artificial) and those exempt from certification (which also contain pigments obtained from natural sources, some of which may also be produced by synthesis) (Lehto et al., 2017).

Commonly used natural food pigments include anthocyanins, carotenoids, betalaines, chlorophylls (Figure 3), among others (Miranda et al., 2021). In addition to their dietary application, the consumption of these naturally colored compounds has been associated with a reduction in noncommunicable diseases such as cancer, diabetes, and obesity (Cortez et al., 2017).

Another possible classification criterion of natural colorants is based on their source (e.g., plants, animals, microorganisms, and minerals) (Sigurdson et al., 2017).

Plant-based pigments are the result of biochemical pathways that take place within the plant, resulting in a large number of organic compounds with unique physicochemical properties (Miranda et al., 2021). These colorful compounds are widespread in nature and perform different functions, participating in photosynthetic processes, attracting pollinators and providing protection from predators and solar energy. Porphyrins, carotenoids, anthocyanins, and betalains are the main chemical classes of plant pigments, which selectively absorb some wavelengths of light and reflect others (Sigurdson et al., 2017). As the size of the natural pigment market is growing rapidly, a more sustainable production is needed. For this reason, research has moved toward the enhancement of vegetable waste (fruit and vegetable) and their byproducts (e.g., skins, seeds, or pomace) for the recovery of natural pigments that find industrial application in food, pharmaceutical, and cosmeceutical sectors (Sharma et al., 2021).

Likewise, a wide range of pigments are obtained from different animals, where they perform various functions (e.g., transporting oxygen in the blood, protecting from predators or UV rays and mating) (Aberoumand, 2011).

Microorganisms (bacteria, fungi, and microalgae) produce a variety of different pigments, able to cover almost any shade of the color. For example, blue pigment (phycocyanins) may be recovered by microalga spirulina (*Arthrospira platensis*) and red colorant (carotenoids) by the fungus *Monascus sp*. and bacterium (*Paracoccus carotinifaciens*). Obtaining pigments from a microbial source is commercially and economically promising, due to the relative simplicity in controlling growth conditions and the source availability (Sigurdson et al., 2017).

Minerals have long been used as dyes in food, cosmetics, and art (e.g., viridian). Their hue depends on the chemicophysical properties (CFR, 2016; Sigurdson et al., 2017).

Moreover, natural colorants may also be classified according to their chemical structure (more thoroughly discussed below).

3.2.1 | Anthocyanins

Among flavonoids (secondary plant metabolites characterized by a carbonaceous skeleton formed by the repetition of the C6-C3-C6 unit), anthocyanins (Figure 3) are important water-soluble subgroup pigments that give plants color ranging from bright red to blue (Obón et al., 2009). Their aglycon form, anthocyanidin, is commonly found in fruits and vegetables and differs in the degree of hydroxylation and methoxylation. Anthocyanidins can be bound to sugars and undergo further acylation processes (with aromatic or aliphatic acids).

The stability of anthocyanins considerably increases both with the degree of glycosylation and with acylation, which explains why simple anthocyanins are rarely found

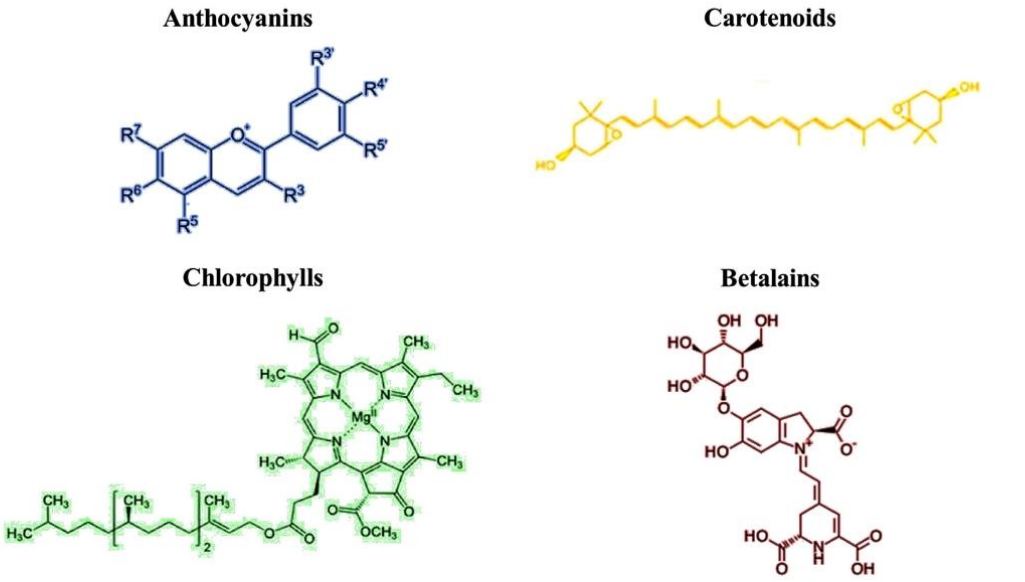

FIGURE 3 Chemical structure of selected natural pigments from vegetable waste

in nature (He & Giusti, 2010). Acylated anthocyanins are more frequent in plants and flowers, while the nonacylated ones are prevalent in fruits (Wallace & Giusti, 2008). Anthocyanins constitute the largest group of natural pigments, and more than 700 different structures have been categorized (Andersen & Jordheim, 2014). Their poor stability makes their application as food colorant challenging. Since these pigments are very sensitive to light, heat, oxygen, and pH (Dangles & Fenger, 2018), the food industry is looking for new ways to improve their stability (e.g., copigmentation, acylation).

Nowadays, anthocyanins as food colorant have been applied to beverages, yogurts, and dry mixes. In the European Union, they are labeled as E163, while in the United States they are exempted from certification (Sigurdson et al., 2017). The FW and byproducts deriving from industrial processes (e.g., wine and juice industry) may be considered excellent sources for the recovery of anthocyanins. The most common FW sources from which they may be extracted are blackberries and grape pomace (Sharma et al., 2021). In this sense, and having the Circular Economy concepts in mind, the use of FW as alternative source of food colorants has gained increased attention in the last few years. Recently, a natural colorant extract from grape skin has been obtained by solvent extraction exhibiting the common properties of polyphenols (Gordillo et al., 2018).

3.2.2 | Carotenoids

Carotenoids are a fat-soluble group of pigments (Figure 3), which vary from yellow to orange to red and they are widespread in nature (e.g., higher plants, bacteria, fungi, yeasts, birds, and insects) (Tanaka et al., 2008). Chemically, carotenoids are tetraterpenoids with 40 carbon atoms made up of isoprenoid units joined in a typical head-to-tail pattern. They can be organized into two classes: carotenes and xanthophylls. The former contains only polyunsaturated hydrocarbons, while the latter has polyunsaturated hydrocarbons with some functional groups containing oxygen. Carotenoids may be classified as acyclic (e.g., lycopene), monocyclic (e.g., γ-carotene), or dicyclic (e.g., lutein). Conjugated carotenoid systems, having delocalized π electrons along the entire chain, are accountable for their absorbance in the visible spectrum (Rivera & Canela-Garayoa, 2012). Depending on the level of conjugation, carotenoids may have more or less intense colors, for example, the larger conjugate systems correspond to the reddest shades, while short chains with a minimum of five double bonds show poor chromaticity. The color is also affected by the degree of cyclization, which explains why lycopene, β-carotene, and γ-carotene, despite having the same number of double bonds, show red, orange, and red-orange colors, respectively (Sigurdson et al., 2017).

Due to their electron-rich and highly unsaturated chemical structure, carotenoids are very sensitive to oxidation and isomerization, especially during food processing and storage (Rodriguez-Amaya, 2016).

These pigments are mainly used as food colors in cheddar cheese, beverages, and flavored milk drinks (Sigurdson et al., 2017). Numerous studies described the recovery of carotenoids from vegetable waste to be applied as food colorants. Many researchers fine-tune green extraction methods, using carrots, pomegranate peel, paprika leaves, orange peel, pumpkin (Sharma et al., 2021), and unsold tomatoes (Lombardelli et al., 2020) as a source. Moreover, carotenoids from tomato peel have been successfully stabilized by means of encapsulation into gelatin nanofibers, retaining antioxidant, antimutagenic, antiproliferative and anti-atherogenic activities (Horuz & Belibağlı, 2018).

3.2.3 | Chlorophylls

Clorophylls (symmetrical cyclic tetrapyrrole, occurring in nature with a centralized attack of phytol and magnesium ion) are able to cover almost the entire chromatic scale of the visible spectrum (Newsome et al., 2014). These two components are an essential part of the molecule to perform its photosynthetic and coloring functions (Figure 3) (Sigurdson et al., 2017). Treatments with weak acids cause centralized displacement of the Mg^{2+} ion, giving rise to phaeophytin, which results in an olive green/gray color shift. Even if this process is permanent, robust complexes may be produced using Cu^{2+} or Zn^{2+} to replace Mg^{2+}, thus restoring chromophore functions and properties. It has been proved that these ions are able to increase the pigment stability, also causing the appearance of more attractive green colors. These complexes are approved for a wide range of application in foods in the European Union, meanwhile in the United States they are allowed only in citrus-based dry beverage mixes (CFR, 2016).

Chlorophylls extracted from various plant waste are commonly used as antioxidants, but their application as food colors is still limited. The only example of chlorophyll used as dye is from avocado peel (Sharma et al., 2021). Furthermore, chlorophyll extracts from spinach byproducts may be used in the formulation of nutraceutical or functional foods due to their antioxidant, anti-inflammatory, and antimutagenic properties (Derrien et al., 2018).

3.2.4 | Betalains

Among nitrogen-heterocyclic compounds, betalains are of particular interest (Figure 3). They are a group of water-soluble pigments, deriving from betalamic acid, that give colors to plants of the order Caryophyllales, such as red beets and prickly pears (Strack et al., 2003). They are composed of two main groups: the red-purple betacyanins and the yellow-orange betaxanthines (Lombardelli et al., 2021a). The former are the products of the condensation of betalamic acid with cyclo-dopa, while the latter are derived from the condensation of betalamic acid and amines (Stintzing et al., 2002). Betacyanins can also be linked with mono or disaccharides, and the resulting glycosides may be acylated, forming several beta-cyanide structures.

Being stable over a wide pH range between 3 and 7, betalains may be used in low-acid food such as dairy products (yogurt and ice cream) (Stintzing & Carle, 2004) and also in syrups, sausages, and confectionaries. In the European Union, they are labeled as E162, and in the United States they are exempted from certification (Sigurdson et al., 2017). Furthermore, the color of betalains is absolutely independent from the pH values compared to other natural colorants, such as anthocyanins. The stability of betalains is affected by several factors, for example, temperature, light, oxygen, and other food components. Sharma et al. (2021) described in depth the betalains extraction from vegetable waste, such as red dragon fruit peels and prickly pear. Recently, Lombardelli et al. (2021a) developed an eco-friendly approach for the recovery of betalain from unsold beetroot. Vulić et al. (2014) showed the antioxidant, antiproliferative, and free radical scavenging activities of betalains recovered from beetroot peel and pomace by ultrasound-assisted extraction (UAE). Likewise, the extracts have proved to be useful in preventing active oxygen-induced and free radical-mediated oxidation of biological molecules.

4 | PIGMENTS EXTRACTION FROM VEGETABLE FOOD WASTE

Vegetable food wastes or byproducts are widely available and inexpensive and may be used as a cost-effective alternative source of natural pigments. Hence, in the last decades, numerous technologies for the utilization of vegetable FW as a resource in the framework of Circular Economy have been implemented.

The main conventional technique applied for pigments recovery from vegetable FW is the solvent extraction (SE). This process consists of a first step of solid–liquid extraction or maceration (Manzoor et al., 2021), and a second step for solvent removal, usually carried out by means of Soxhlet method (Sharma et al., 2021). The selection of the most appropriate organic solvent is crucial, and it depends on its compatibility with respect to target pigment, toxicity, cost, and recovery (de Souza Mesquita et al., 2021). The

most used solvents are hexane (carotenoids from Gac fruit peel, Chuyen et al., 2017), acetone (lycopene from tomato processing waste, Poojary & Passamonti, 2015), methanol (anthocyanins from eggplant peel, Boulekbache-Makhlouf et al., 2013), and trifluoroacetic acid (betalains from pitaya fruits peel, García-Cruz et al., 2017) (Table 1).

Although the conventional extraction techniques with organic solvents are easy to use, economical, and do not require sophisticated equipment, they have numerous disadvantages including the need of a large amount of solvent, a long time, and possible degradation of the extracted pigment. To overcome these limitations, green/unconventional extraction methods have been developed (Figure 4) (Ngamwonglumlert et al., 2017). In order to improve the yield of natural colorants, as well as their chemical stability, the selection of a suitable extraction procedure for each type of pigment is essential. Various innovative techniques of extraction based on supercritical fluids, pressurized liquids, microwaves, ultrasounds, pulsed-electric fields, and enzymes have been explored for the extraction of new potential food colorants from vegetal wastes (Figure 4).

4.1 | Supercritical fluid extraction

It has the benefits of supercritical fluids (which have properties similar to gases) and may lead to an improvement of extraction (Macıas-Sánchez et al., 2005) as these fluids can more easily penetrate the matrix. As shown in Table 1, this method is suitable for the recovery of nonpolar pigments (e.g., carotenoids, chlorophylls), rather than polar ones (e.g., betalains, anthocyanins), since most of the solvents used for supercritical fluid extraction (SFE; e.g., carbon dioxide, organic solvent) are nonpolar (Ngamwonglumlert et al., 2017). Derrien et al. (2018) applied supercritical CO_2 using ethanol for the recovery of chlorophyll and lutein from spinach wastes confirming higher recovery of phytopigments (70% for lutein and 96% for chlorophylls) compared with conventional extraction technique (e.g., acetone) (Table 1).

Despite the need of small quantities of solvent, this approach requires great capital and operating costs due to the high pressures used (Veggi et al., 2013).

4.2 | Pressurized liquid extraction

It uses a liquid solvent at high pressure (10.3–13.8 MPa) at 40–200°C (Miranda et al., 2021). The high temperature facilitates the diffusion of solvent in the matrix and accelerates the degradation of the plant cells, enabling the pigments recovery, thus increasing the extraction yield. Pressurized liquid extraction (PLE) may be applied for the extraction of polar and apolar pigments, depending on the solvent chosen.

Strati et al. (2015) utilized PLE (700 MPa/10 min) assisted with ethanol on tomato wastes, recovering a higher content of total carotenoids (9.30 mg/kg dw) and lycopene (7.04 mg/kg dw) compared to conventional extraction (total carotenoids −3.63 mg/kg dw, lycopene −2.47 mg/kg dw) (Table 1).

Although PLE needs short times and a low number of solvents, it cannot be effectively applied for the recovery of heat sensitive pigments (e.g., betalains) due to the high temperature used (Miranda et al., 2021) and the high capital/operating costs (Ngamwonglumlert et al., 2017).

4.3 | Microwave-assisted extraction

It is a very promising innovative method for the recovery of polar and nonpolar plant pigments (Table 1) due to its high extraction rate and reduced solvent consumption compared to conventional techniques (Dahmoune et al., 2014). This approach is based on fast heating by microwave radiation causing the breakdown of the plant cell walls, due to the expansion of the cell structure. As a result, pigments migrate more easily outside the cells, resulting in an increase of extraction speed and yield (Ngamwonglumlert et al., 2017). Microwave-assisted extraction (MAE) could reduce the time and solvent volume required during the extraction of pigments, but it is not very selective as analytes are extracted in a wide spectrum of polarity (Miranda et al., 2021). The combination of MAE with vacuum (vacuum microwave-assisted extraction) has been suggested for the recovery of heat sensitive bioactive compounds and pigments (Hiranvarachat et al., 2015).

Elik et al. (2020) recovered carotenoids (77.48%) from carrot juice processing waste using MAE at the optimal process conditions (165 W of microwave power, 9.39 min of extraction time and 8.06:1 g/g of oil to waste ratio) (Table 1).

4.4 | Ultrasound-assisted extraction

It uses ultrasonic waves to generate cavitation bubbles that improve extraction efficiency (Rastogi, 2011). Ultrasonic waves passing through a medium create alternating cycles of compression and decompression, which in turn cause the formed cavitation bubbles to compress and expand. As the bubbles grow larger, they can no longer be contained by the surface tension force and therefore collapse. In this way, shear forces are generated and they are capable of breaking the vegetable cell walls, facilitating the release of intracellular compounds (Pitt et al., 2004). As reported in Table 1, UAE is a technique suitable for the

TABLE 1 Natural pigments extracted from vegetal wastes

Pigment	Sources	Method of extraction	Extraction yield	Reference
Carotenoids	Carrot peels	SFE (SC-CO_2)	5.31% $_{\text{dry basis}}$	(de Andrade Lima et al., 2018)
	Apricot pomace	SFE (SC-CO_2 + ethanol)	100.4 μg/g $_{\text{dry pomace}}$	(Şanal et al., 2005)
	Pumpkin	SFE (SC-CO_2)	109.6 μg/g	(Shi et al., 2010)
	Red pepper byproducts	SFE (SC-CO_2)	68.1% $_{\text{dry basis}}$	(Romo-Hualde et al., 2012)
	Tomato skin	SFE (SC-CO_2)	33.0% $_{\text{dry basis}}$	(Kassama et al., 2008)
	Tomato peels	SFE (SC-CO_2)	60.85% (Lyc, $_{\text{dry basis}}$)	(Kehili et al., 2017)
	Pumpkin rind	SFE (SC-CO_2)	20.50 mg/100 g	(Wang et al., 2017)
	Tomato skin and seeds	PLE	9.30 mg/kg $_{\text{dry basis}}$	(Strati et al., 2015)
	Lemon waste	MAE	3.2–6.2% w/w	(Martínez-Abad et al., 2020)
	Gac peel	MAE	262 mg/100 g $_{\text{dry weight}}$	(Chuyen et al., 2018
	Carrot juice waste	MAE	77.48%	(Elik et al., 2020)
	Pomegranate peels	UAE	0.61 to 0.67 mg $_{\text{carotenoids}}$/100 g $_{\text{dry peels}}$	(Goula et al., 2017)
	Mandarin epicarp	UAE	140.70 mg $_{\beta\text{-carotene}}$/100 g $_{\text{dry sample}}$	(Ordóñez-Santos et al., 2021)
	Carrot waste	UAE	83.32%	(Purohit & Gogate, 2015)
	Guava pulp	UAE	135.0 mg$_{\text{Lyc}}$/100 g	(da Silva Lima et al., 2020)
	Gac peel	SE	271 mg/100 g $_{\text{dry weight}}$	(Chuyen et al., 2017)
	Tomato pulp waste	SE	94.7%	(Poojary & Passamonti, 2015)
Anthocyanins	Blackberry residues	PLE	1.02 mg $_{\text{C3GE}}$/g $_{\text{fresh residue}}$	(Machado et al., 2015)
	Cranberry pomace	SFE + PLE	11.10%	(Tamkutė et al., 2020)
	Black currant marc	MAE	20.4 mg/g	(Pap et al., 2013)
	Acerola pulp	UAE + ethanol	2.00–11.16 mg $_{\text{TA}}$/100 g	(Rezende et al., 2018)
	Purple potato peel	UAE	6.84 mg/100 g	(Albishi et al., 2013)
	Sweet cherries skin	UAE + ethanol	12.2 mg/g	(Milea et al., 2019)
	Black carrot pomace	UAE (thermal)	60.85–74.22 mg/L $_{\text{C3XGGF}}$	(Agcam et al., 2017)
	Blueberry byproducts	PEF	55%	(Pataro et al., 2017)
	Blueberry waste	PEF	223.13 mg/L	(Zhou et al., 2015)
	Elderberry pomace	SFE + SE	24.2%	(Seabra et al., 2010)
	Blackcurrant waste	SE	2%–3% w/w	(Rose et al., 2018)
	Eggplant peel	SE	82.83 mg$_{\text{DGE}}$/100 g $_{\text{dry powder}}$	(Boulekbache-(Makhlouf et al., 2013)
	Red grape pomace	MAE, UAE, SE	34188 ppm$_{\text{GAE dry extract}}$	(Drosou et al., 2015)
	Grape byproducts	UAE, PEF	+10% and +17%	(Corrales et al., 2008)
Betalains	Beetroot pomace	UAE	37.22 mg/100 g $_{\text{dry weight}}$	(Vulić et al., 2014)
	Pitaya peel	UAE	101.04 mg $_{\text{betanin}}$/ 100 g	(Mello et al., 2014)
	Pitaya peel	MAE	–	(Cunha et al., 2018)
	Red beetroots	PEF	90%	(López et al., 2009)
	Red beetroot Peels	UAE	9.81 mg $_{\text{betanin}}$/g $_{\text{dry basis}}$	(Šeremet et al., 2020)
	Beetroot peels	MAE	472.11 mg $_{\text{betanin}}$/L	(Singh et al., 2017)
	Pitaya peel	SE	22,053.6 mg/g $_{\text{dry sample}}$	(García-Cruz et al., 2017)
	Beetroot peel	SE	952.5 mg L^{-1} (Bx) and 1361 mg L^{-1} (Bc)	(Zin et al., 2020)
Chlorophylls	Spinach byproducts	SFE	71.0% w/w	(Derrien et al., 2018)

(Continues)

TABLE 1 (Continued)

Pigment	Sources	Method of extraction	Extraction yield	Reference
	Kiwi pomace	MAE	5.9 mg TCC/100 g_{pulp}	(Carbone et al., 2020)
	Green beans	SE	13.3 mg/100 $g_{green\ beans}$	(Cubas et al., 2008)

Abbreviations: Bc, betacyanins; Bx, betaxanthin; C3GE, cyanidin-3-O-glucoside equivalent; C3XGGF, cyanidin-3-xyloside-galactoside-glucoside-ferrulic acid; DGE, delphinidin-3-glucoside equivalent; GAE, gallic acid equivalents; MAE, microwave-assisted extraction; PEF, pulsed electric field; PLE, pressurized liquid extraction; SE, solvent extraction; SFE, supercritical fluid extraction; TA, total anthocyanins; TCC, total chlorophyll content; UAE, ultrasound-assisted extraction.

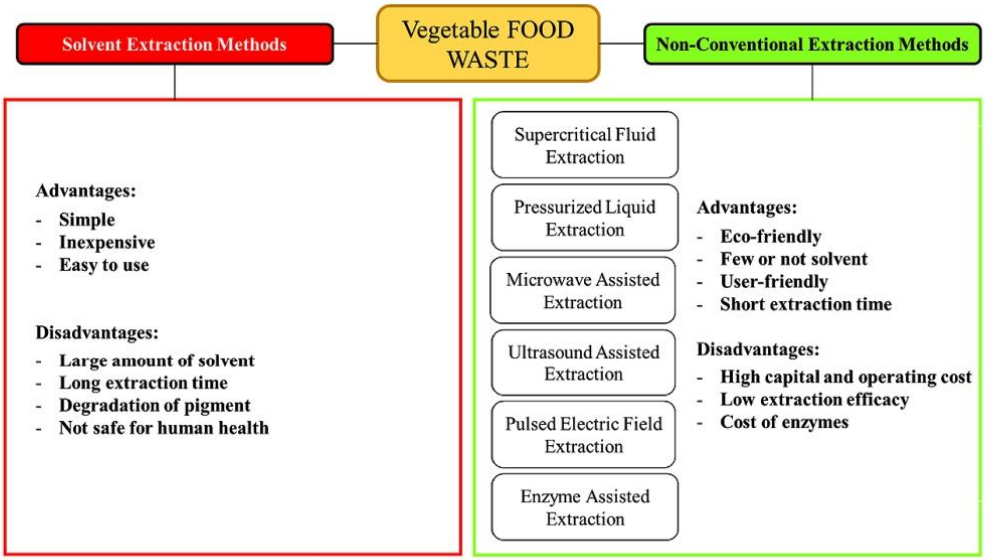

FIGURE 4 Different methods for extraction of pigments from food wastes including advantages and disadvantages

extraction of both polar and nonpolar compounds and also for those sensitive to heat as it is an athermic process, although known for its lower extraction efficiency compared to other nonconventional processes (e.g., SFE and PLE) (Ngamwonglumlert et al., 2017). Drosou et al. (2015) tested three different extraction methods (Soxhlet, MAE, and UAE) for the recovery of anthocyanins from red grapes pomace, revealing the highest procyanidin (43469 ppm) and anthocyanin contents (34188 ppm) in UAE-assisted treatment combined with solvents (Table 1).

4.5 | Pulsed electric field

Pulsed electric field (PEF), using high voltage electrical pulses for short periods, leads to the formation of pores in the plant cell walls allowing more effective extraction (Luzardo-Ocampo et al., 2021). To recover polar pigments (e.g., betalain and anthocyanins), mainly polar solvents with high electrical conductivity are used. On the other hand, PEF cannot be used for the extraction of nonpolar pigments (e.g., carotenoids) since the electric field cannot pass through the nonpolar solvents required for this type of molecules (Ngamwonglumlert et al., 2017). Nevertheless, PEF may be used as a pretreatment to degrade membranes, also enhancing the recovery of apolar molecules (Table 1).

Corrales et al. (2008) compared PEF with other techniques for the recovery of anthocyanins from grape byproducts, observing an improvement in the extraction efficiency (+10% and +17%) with respect to high hydrostatic pressure and conventional solvent extraction (Table 1).

4.6 | Enzyme-assisted extraction

Enzyme-assisted extraction (EAE) of pigments from plants is gaining more attention as it is a useful, mild, green, and

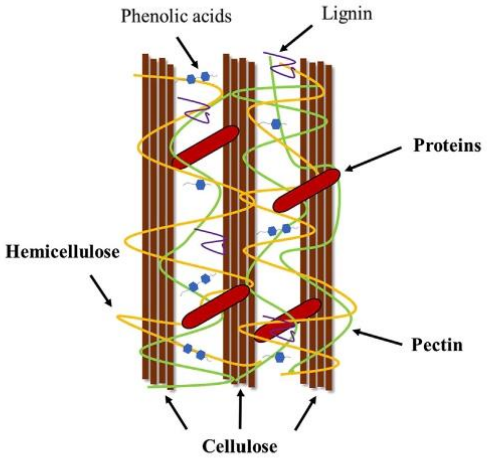

FIGURE 5 Representation of primary vegetable cell wall components

environmental friendly extraction approach, which may be considered a viable alternative to conventional methods (Figure 4) (Nadar et al., 2018). EAE exploits the intrinsic characteristic of enzymes to catalyze reactions with high specificity, region-selectivity, and the ability to conduct such reactions under mild conditions, ensuring the stability of the recovered pigments (Puri et al., 2012). This approach is based on the breakdown of the plant cell wall (Figure 5) by enzymatic hydrolysis. Most of the targeted compounds are trapped inside the cell, and the main obstacle is represented by cell wall consisting of polysaccharides and their derivatives such as cellulose, hemicellulose, and pectin which are linked by hydrophobic interactions and hydrogen bonds. Other compounds, such as phenolic acids, form ether bonds with lignin through their hydroxyl groups in the aromatic ring and ester bonds with structural carbohydrates and proteins through their carboxyl groups (Nadar et al., 2018) (Figure 5).

Hence, enzymes such as hydrolases are employed to solubilize the plant cell wall thereby accelerating the release of intracellular biomolecules. These biocatalysts randomly attack the internal sites of the amorphous region of the polysaccharide chains. This leads to the generation of small oligosaccharides with varied lengths which facilitate easy release of entrapped molecules (Nadar et al., 2018).

Enzymes (e.g., cellulase, hemicellulase, protease, pectinase and xylanase) may be applied alone or in combination as a tailored mix to enhance the extraction yield (Lombardelli et al., 2020).

Among these enzymes, all of them have the ability to hydrolyze some of the main constituents of plant cell walls. In detail, cellulases hydrolyze cellulose releasing cellobiose and glucose molecules when the hydrolysis is complete. Hemicellulases degrade hemicelluloses producing simple sugars or oligosaccharides. Pectinases are able to hydrolyze different types of pectins, releasing uronic acids. Finally, proteases break down proteins in peptides, amino acids, and so forth (Chemat & Vian, 2014).

In order to maximize the enzymatic activities applied, the optimization of all extraction conditions (reaction temperature, extraction time, pH, enzyme concentration, and substrate particle size) is crucial (M'hiri et al., 2014).

The extraction efficiency depends on temperature, pH, treatment duration, enzymatic dosage, and availability of the substrate. As explained above, EAE is very promising in pigment isolation, but the selection of enzymes and the optimization of extraction conditions are very important.

Temperature is a fundamental parameter to consider during extraction since high temperature causes a gradual loss of enzymatic activity and the inactivation of proteins and other bioactive compounds (Peterson et al., 2007). On the contrary, a lower temperature does not accelerate the action of enzymes, leading to a decreased pigment extraction.

The optimal pH for enzymatic hydrolysis differs for each enzyme and it falls within the range of the isoelectric pH of the different proteins (Talley & Alexov, 2010). Since proteins are highly insoluble in this pH range, the release of the biomolecule can be hindered. Therefore, the choice of the optimal extraction pH must be made in such a way as not to hinder the functioning of the enzymes (Nadar et al., 2017).

Considering the treatment time, in general, by extending the contact duration between the enzyme and the extract, it is possible to enhance the degradation of plant cell wall components. However, this is hardly applicable on a large scale as a long incubation time would result in lower product quality and higher energy consumption (Babbar et al., 2016). Furthermore, it has been shown that an extension of the extraction time may lead to a reduction in yield due to the exhaustion of substrates and/or the formation of compounds that inhibit enzymes (Nadar et al., 2018).

Another parameter to be optimized is the overall dosage of the enzyme. High concentrations can ensure a better interaction between enzyme and substrate, facilitating the degradation of the cell wall, but can also lead to the extraction of undesirable substances. The optimal dosage should be a compromise between the cost of the enzyme and the quality of the pigment to be extracted (Jiang et al., 2010).

EAE has been widely applied by using commercially available enzymes (e.g., cellulase, amylase, and pectinase) (Table 2). Prokopov et al. (2017) proved an enhanced recovery yield of carotenoids from tomato peels by an enzymatic mix (cellulase 100 U g^{-1} and hemicellulase 400

TABLE 2 Enzyme-assisted extraction of pigments from food waste

Pigment	Sources	Enzyme	Extraction yield	Reference
Carotenoids	Tomato peel	Cellulase, pectinase, protease, endo-xylanase	55.15 mg/100 g $_{\text{dry basis}}$	(Prokopov et al., 2017)
	Tomato paste	Pectinex Ultra SP-L, Celluclast, Viscozyme L	11.5 mg lycopene/g	(Catalkaya & Kahveci., 2019)
	Red capsicum	Viscozyme L, pectinase, cellulase	41.72–279.83 mg/100 g	(Nath et al., 2016)
	Orange peel	Cellulase, Pectinase	15.0%	(Kumar et al., 2016)
	Pumpkin	Pectinex Ultra SP	2 mg/100 g	(Dinkova et al., 2014)
	Unsold tomato	Polygalacturonase, Cellulase, Xylanase	4.30 mg_{Lyc}/Kg_{tomato})/U	(Lombardelli et al., 2020)
	Tomato peel	Peclyve LI, Cellulyve 50LC, Prolyve 1000	8%–10% w/w $_{\text{dry basis}}$	(Cuccolini et al., 2013)
	Tomato paste	Citrozym CEO and Ultra L, Peclyve EP and L	32.6 $mg_{Lyc}/100\,g_{tomato}$	(Zuorro & Lavecchia, 2010)
	Tomato waste	Peclyve PR, Cellulyve 50LC	72.3%	(Zuorro et al., 2011
	Blueberries	Cellulase, Pectinase	23.5%	(Xu et al., 2016)
	Sohiong fruit	α-Amylase, cellulase, pectinase, protease	984.4 mg	(Swer et al., 2016)
	Blackberries	Klerzyme-150	639 g/L	(Gong et al., 2014)
	Bilberries	Pectinex, Panzym Pro Color, Panzym BE XXL	622, 705, 811 mg $_{\text{MGE}}$/L	(Dinkova et al., 2014)
	Grape skins	Pectinase	3.01 mg/g	(Tan et al., 2020)
Anthocyanins	Bilberry pomace	Viscozyme L	56.15 g/100 g $_{\text{dry basis}}$	(Syrpas et al., 2021)
	Sweet cherry pomace	Depol 740L, Promod 439L, Pectinase 62L	1.75 mg_{GAE}/g	(Domínguez-Rodríguez et al., 2021)
	Raspberry pomace	Pectinex Ultra SP-L, Pectinex Yield Mash, Ultrazym AFP-L	0.32 mg/g	(Szymanowska & Baraniak, 2019)
	Purple corn	Multifect neutral enzyme, Enzeco fungal acid protease	0.94 g/100 g $_{\text{dry basis}}$	(Jing & Giusti, 2007)
Betalains	Unsold beetroot	Polygalacturonase, Cellulase, Xylanase	11.37–14.67 mg/mL U	(Lombardelli et al., 2021a)
	Dragon fruit	Pectinex Ultra SP–L	550 mg/L	(Naderi et al., 2010)
	Red beetroot waste	Celluclast 1.5 L, Pectinex Ultra Mash	3.06 mg/g $_{\text{dry basis}}$	(Fernando et al., 2021)
Chlorophylls	Spinach	Pectinex Ultra SP-L	50.7 mg TCC/100 g_{pulp}	(Özkan & Bilek, 2015)
	Pandan leaf	Pectinex Ultra SP-L	17.82 mg/g $_{\text{fresh weight}}$	(Senklang & Anprung, 2010)

Abbreviations: GAE, gallic acid equivalents; MGE, malvidin glucoside equivalent; TCC total chlorophyll content.

U g^{-1}). In detail, series of experiments were carried out varying enzyme concentrations (100–500 U g^{-1}) in order to establish the influence of enzymatic treatment on the carotenoid extraction yield at a constant temperature (20 or 50°C) and optimum pH. The extraction time was changed from 1 to 5 h. The EAE of carotenoids using mixed cellulase and hemicellulose for 4 h at 50°C resulted in an enhanced recovery yield (1.6 times greater).

Catalkaya and Kahveci (2019) described that lycopene-rich oleoresins may be produced through a treatment of waste by a combination of cellulolytic and pectinolytic enzymes, resulting in a deeper red color extract (Table 2). In depth, central composite design (CCD) was used for the optimization study and response variable was chosen as the lycopene content of the oleoresins (mg/g oleoresin). Enzymatic reaction temperature (40–60 °C) and time (1–5 h), enzyme:substrate ratio (0.2–2 v/w), solvent:substrate ratio (5–30 v/w), extraction time (1–4 h), and enzyme:enzyme ratio (1–3 v/v) were the factors investigated. Optimized conditions were chosen to be as follows: enzymatic reaction temperature = 40°C, enzymatic reaction time = 5 h, enzyme:substrate ratio = 0.2 ml/g, solvent:substrate ratio = 5 ml/g, extraction time = 1 h, enzyme:enzyme ratio = 1. Oleoresin with a concentration of 11.5 mg lycopene/g was obtained at the optimum conditions.

The EAE (cellulase and pectinase) applied to tomato waste increased the carotenoid (sixfold) and lycopene yield (10-fold) compared with nonenzyme-treated sample (Strati et al., 2015). In this study, in the first series of experiments, incubation time varied from 30 to 240 min, while the enzyme concentration was kept constant and equal to 70 and 122.5 U/g for pectinase and cellulase, respectively. The highest carotenoid yield for both enzymes was observed after 180 min of incubation. In the second series of experiments, the incubation time was kept constant (180 min) and the concentration of pectinase and cellulase enzymes varied from 35 to 140 U/g of tomato waste, and 61.25 to 350 U/g of tomato waste, respectively. The highest carotenoid yield was obtained at pectinase and cellulase concentrations of 70 and 122.5 U/g, respectively.

Recently, a tailored EAE protocol for the recovery of carotenoid-containing chromoplasts from unsold tomatoes has been developed. In this study, the specific enzymatic mix (polygalacturonase, pectin lyase, cellulase, and xylanase) was selected taking into account the polysaccharides composition of tomatoes cell wall (Table 2) (Lombardelli et al., 2020). The optimal process conditions for enhancing the recovery of carotenoids from tomatoes (temperature [20–70°C], pH [2.50–8.0], total dosage of enzymatic mix [10–250 U/g] and processing time [20–300 min]) have been investigated. The treatment carried out using the total dosage of 25 U/g for 180 min (45°C, pH 5.5) was the most convenient for maximizing the carotenoids recovery yield.

Dinkova et al. (2014) extracted anthocyanins from bilberry peel by using different commercial enzymes (Table 2), revealing an enhanced yield as well as an extended microbiological juice shelf-life. The extraction was carried out at 50°C for 2 h. Swer et al. (2016) treated *Prunus nepalensis L.* to extract anthocyanins with different enzymes (cellulase, pectinase, protease, and α-amylase), and the highest anthocyanin amount was obtained using cellulase compared to other enzymes (Table 2). In detail, the effect of enzyme concentration, reaction time (30—480 min), and temperature (20—60°C) were evaluated subsequently in several experiments. Cellulase treatment (10% E/S) for 180 min at 4°C exhibited highest anthocyanin yield (Table 2) compared to conventional method.

Lombardelli et al. (2021a) recovered betalain from unsold red-beets applying a tailored enzymatic mix based on the polysaccharide composition of the red-beet cell wall. The process was conducted at 25 and 45°C. Although the pigment extraction at higher temperatures was faster, a comparable recovery yield was achieved at room temperature, also better preserving the color attributes (Table 2). The EAE protocol was optimized, and the most suitable conditions (in terms of pigment yield and color attributes) for the recovery of betalains from unsold beets appeared to be 25 U/g total dose of enzymatic mix, temperature 25°C, and processing time 240 min.

In Özkan and Bilek (2015), Pectinex Ultra SP-L was selected for EAE of zinc–chlorophyll derivatives from spinach pulp, resulting in 39% increase of the extraction yield (Table 2). In particular, the effect of enzyme concentration (1%—9%), treatment temperature (30—60°C), and time (30—210 min) on total chlorophyll content (TCC) was optimized using response surface methodology. Optimum treatment conditions were as follows: 8% enzyme concentration, 45°C, and 30 min, leading to a yield of 50.747 mg TCC/100 $g_{\text{spinach pulp}}$.

Overall, EAE has huge advantages including lower energy consumption, faster extraction rate, higher extraction yield, easier recovery with reduced solvent usage (Puri et al., 2012), reduced risk of their residual in the extract, and higher retention of targeted compounds (K. Ozkan et al., 2021). Furthermore, since this extraction is carried out under mild-controlled temperature conditions, it is very useful for the extraction of heat-sensitive molecules such as flavors and pigments.

Although their use in industrial processes is still limited, several reports in the scientific literature suggest that enzymes have huge potential to be implemented in the advanced large-scale recovery of high-added molecules from agri-food wastes.

Other authors (Sagar et al., 2018) pointed out that the main disadvantages linked to the EAE technique are as follows: (i) high enzyme cost for large volumes of samples, (ii) hardly feasible at industrial level, and (iii) the need to optimize process parameters (e.g., particle size, hydrolysis time, enzyme concentration, and composition).

Despite the price of enzymes represents the bottleneck of this technology, it is certainly important to emphasize that Chemat et al. (2014) have shown, through an economic analysis, that EAE can lead to reduced production costs in terms of savings in time, equipment, and waste disposal compared with other extraction technique. In addition, it has been shown that most commercial enzyme preparations require relatively low application temperatures (<50°C) resulting in a reduction of energy requirements (Chemat et al., 2014).

5 | STABILITY OF NATURAL FOOD COLORANTS

As mentioned above, it has been shown that vegetable FW has the potential to produce very valuable products for further application in the food industries, such as natural colorants.

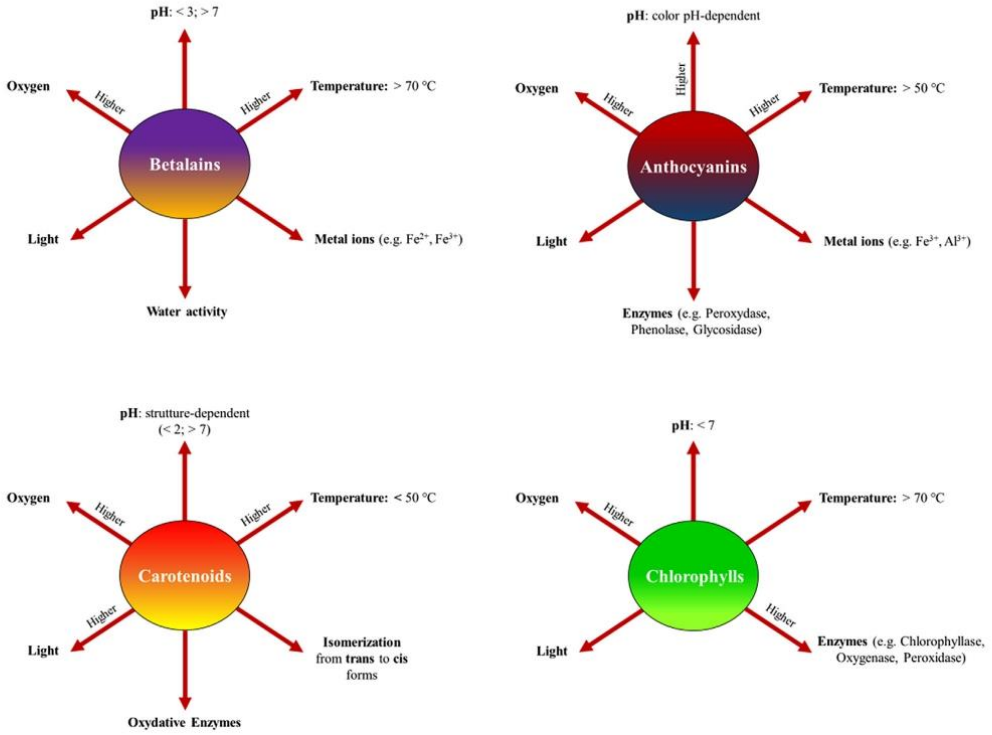

FIGURE 6 Physicochemical factors affecting the stability of pigments

The use of a natural colorant in food requires a detailed knowledge of its stability against potential degradation processes in order to retain as much color as possible throughout production, transport, and storage (Cerreti et al., 2020; Lombardelli, Benucci, & Esti, 2021). Indeed, natural pigments recovered from vegetable waste are highly unstable and susceptible to degradation phenomena caused by external (e.g., processing conditions) and internal factors (e.g., pigment concentration) (Sharma et al., 2021).

Figure 6 shows the main physicochemical parameters which provide instability to pigments, as affected by manufacturing and storage alteration factors.

5.1 | Carotenoids

With a view to the Circular Economy, several studies have been conducted to optimize the extraction of lycopene from tomato processing waste, obtaining a maximum recovery of 95% and reaching a high degree of purity (98%) (Poojary & Passamonti, 2015). As with most carotenoids, the stability of lycopene during food processing and storage is mainly affected by geometric isomerization (cis/trans), oxidation, and fragmentation into smaller volatile molecules (Figure 6) (Rodriguez-Amaya, 2016). The trans isomers of lycopene are commonly found in fruits and vegetables and are thermodynamically stable. However, when lycopene is subjected to heating or irradiation processes, it isomerizes into the cis form, with a consequent reduction in color intensity (Rodriguez-Amaya, 2016). Lycopene oxidation leads to the formation of undesirable small molecules such as acetone, methileptenone, laevulinic aldehyde, and probably glyoxal. In fact, the loss of color is associated with unpleasant tastes due to the presence of aldehydes (Galaffu et al., 2015). The most important degradation factors during the processing of food containing carotenoids are heat, light, and oxygen (Figure 6) (Rodriguez-Amaya, 2016).

The medium in which these reactions occur also affects the stability of carotenoids. Adding antioxidants in the formulation could be a solution for stabilizing such pigments at acidic pH. In this regard, Bou et al. (2011) proved that α-tocopherol was more effective than the other tested compounds (e.g., EDTA, citric acid, tripolyphosphate, propyl

gallate, and gallic acid) in stabilizing lycopene. Encapsulation strategies have been applied to provide stability to carotenoid-rich extracts under food processing and storage conditions (Corręa et al., 2019). It was observed that supercritical CO_2 extraction followed by encapsulation with β-cyclodextrins resulted in more stable forms of lycopene which were stable for 6 months (Blanch et al., 2007). Lobo et al. (2018) developed several inclusion complexes using yellow pepper pigments and β-cyclodextrin with two different procedures. Color stability in all isotonic beverage samples stored in the presence (1400 lux) and absence of light was assessed by the colorimetric method. The values of the coordinates L^*, a^*, and b^* demonstrated a higher color stability of the isotonic drinks added with the inclusion complexes compared to the colored drink with only the raw yellow pepper extract, proving that the inclusion may be a valid alternative for the application of natural additives in food. A significant improvement in the stability of lycopene was shown by Durante et al. (2020) following encapsulation in β-cyclodextrin, as it appeared by the increased stability of carotenoids over a period of 90 days at 25 and 4°C. The results showed that encapsulation significantly improves the stability of lycopene. de Freitas Santos et al. (2021) comprehensively reviewed the materials applied for the production of carotenoid-rich microparticles by spray drying, including carbohydrates (e.g., modified starch, maltodextrin, β-glucan, carboxy-methylcellulose, inulin, and pectin), proteins (e.g., chickpea protein, gelatin, whey protein isolate, and concentrate), and gum arabic (GA). They argue that the choice of extraction solvent, as well as the encapsulation technique and materials may contribute to the successful preservation of carotenoids. Indeed, as reported by Álvarez-Henao et al. (2018), lutein degradation after spray drying decreased from 97.62% to 8.06% when modified starch was substituted by GA as a carrier agent.

Nanoencapsulation seeks to form structures or particles that function as nano-carriers, which implies that at least one of the characteristic dimensions of the capsules is smaller than 100 nm. The most widely used nanoencapsulation methodology applied to carotenoids is nanoprecipitation (Fuenmayor et al., 2021). Afonso et al. (2020) reported that this technique has been used to encapsulate β-carotene in polymers as ethylcellulose (particle size: 60 nm, encapsulation efficiency: 74%) and zein (particle size: 83 nm, encapsulation efficiency: 93%).

Recently, the enzymatic recovery of carotenoids, keeping them inside chromoplasts, allowed us to obtain stable formulations without the need of further stabilization techniques (Lombardelli, Benucci, & Esti, 2021).

5.2 | Anthocyanins

One of the most interesting properties of anthocyanins is their ability to cover a broad spectrum of colors, ranging from red (at acid pH) to purple and blue (at neutral pH) (Figure 6). Red anthocyanins may be used to color soft drinks below pH 3. Also, some anthocyanin-based extracts are widely applied to dairy products to enhance the color of yogurt (pH 4.5). Selecting the right type of anthocyanin is essential for limiting color loss. For example, anthocyanins with particular substituents on the flavyl nucleus (e.g., sugar groups esterified with phenolic acids) maintain a better stability to heat and light (Pina et al., 2012). It may be explained by the fact that the substituted anthocyanins are reorganized in a secondary structural configuration that is able to protect the pigment from the hydration reaction, which normally determines the formation of colorless chalcones. This mechanism has been reported by numerous authors as a co-pigmentation in which a noncovalent interaction occurs between anthocyanin and a co-pigment (other colored or colorless molecules, metal ions or mixtures of these) (Cavalcanti et al., 2011). The resulting effect can generally be expressed as a bathochromic shift and is sometimes accompanied by a hyperchromic effect (Pina et al., 2012). In light of this, co-pigmentation is one of the most effective methods for obtaining stable anthocyanins for food applications (Galaffu et al., 2015). Furthermore, when anthocyanins are in the presence of transition metal salts (e.g., in nutritionally fortified products), important color changes may be observed. Although this mechanism is exploited in nature in flowers (Yoshida et al., 2009), it may represent a problem for the food industry (e.g., fruit-based preparations used in yogurt lose their characteristic color). One possible solution involves the use of chelating agents (EDTA), although their addition is not allowed in all countries (Galaffu et al., 2015). Recently, encapsulation by spray drying has been applied in order to enhance anthocyanins stability throughout storage, promoting their controlled release in food (Cortez et al., 2017). The microencapsulation of anthocyanins has been performed by using different coating materials. Correa et al. (2019) successfully tested maltodextrin, whereas Garcıa-Tejeda et al. (2015) used normal, modified, and waxy maize starches, showing the highest encapsulation efficiency when starch derivatives were applied. Guo et al. (2018) used alginate-pectin hydrogel particles to encapsulate anthocyanins extracted from blueberry and purple corn. The greatest encapsulation efficiency was observed for blueberry anthocyanins (116%) compared to purple corn anthocyanins (65%). The photodegradation rate was reduced in both encapsulates and at the same time the retention rate of anthocyanins during storage was increased, thus improving their

shelf life in low pH beverages (e.g., fruit juices or lemonade).

Liposomes may also be used to encapsulate anthocyanins due to their good ability to protect hydrophilic substances (Enaru et al., 2021). Gültekin-Özgüven et al. (2016) evaluated the encapsulation of spray-dried black mulberry in chitosan-coated nanoliposomes in dark chocolate. Anthocyanins were added to chocolates by varying the degrees of alkalinization (pH 4.5, 6, and 7.5) and the conching temperatures (40, 60, and 80°C). The results showed that, compared to the spray dried extract, the chitosan-coated liposomal powders provided better protection of the anthocyanin content when both temperature and pH increased.

Furthermore, anthocyanins can be easily incorporated into the dispersed phase of an emulsion to be protected in its continuous phase from harmful external environmental conditions, mechanical, chemical, or enzymatic degradation (Souza Simões et al., 2017). Almeida Paula et al. (2018) added a commercial anthocyanin extract and 1.25% guar gum, in the aqueous phase of a double emulsion and observed a high encapsulation efficiency (90.6%), an increase in thermal stability and the protection of anthocyanin molecules from degradation. Furthermore, the addition of guar gum increased the color stability.

5.3 | Betalains

Unlike anthocyanins, betalains are more stable to pH (Figure 6) and changes from 3.5 to 7 do not induce color alteration or hydrolytic cleavage. However, far from this range, degradation occurs rapidly (Rodriguez-Amaya, 2016). It has been observed that betalains are unstable in the presence of oxygen and that some antioxidants (e.g., ascorbic acid and tocopherol) seem to protect them only marginally from oxidation (Castro-Enríquez et al., 2020).

Exposure to light also has a negative effect on their stability. Indeed, it accelerates the oxidation reaction of betalains due to the excitation of electrons toward the orbitals at higher energy levels, resulting in a greater molecular reactivity. Heat treatment remains the most significant problem that needs to be addressed in the use of betalains in food. A significant loss of color begins to occur even below the pasteurization conditions of 70–80°C (Castro-Enrìquez et al., 2020).

Lombardelli et al. (2021b) contributed to increase knowledge about the arranged effect of heat and UV-light on the visual color of betalains recovered from unsold beetroot, proving that high temperature (40°C) leads to a greater pigment degradation especially under UV-light exposure.

To overcome these problems, a solution could be the addition of colorant in food matrices at the end of the process (Emerton, 2008).

Therefore, in order to prevent or limit the loss of betalains color during heat treatment, the absence of metal ions, light, and oxygen is necessary, thus requiring in turn the systematic use of antioxidants and chelating agents. Unlike anthocyanins, betalains may offer red colors at neutral pH conditions, and this property may be exploited to create more expensive and regulated color alternatives such as carmine (Herbach et al., 2006). Encapsulation of betalains has been performed by using different techniques and matrices (e.g., polysaccharides, proteins or in combination with polysaccharides) (Albuquerque et al., 2021; Castro-Enríquez et al., 2020). Ravichandran et al. (2014) reported the encapsulation of betalains from red beet in maltodextrin and its combination with guar gum, GA, xanthan gum, and pectin. Interestingly, the xanthan gum combination allowed to increase the stability (+21%) with respect to the control. Chranioti et al. (2015) encapsulated beet and saffron dye in maltodextrin, GA, modified starch (MS), chitosan and in combination, proving that encapsulation is suitable for color stabilization. The powders produced were evaluated in terms of color strength during storage at 40°C for 10 weeks. Chewing gum samples produced with color extracts encapsulated in GA–MS showed the highest values of a^* (for beet) and b^* (for saffron) indicating better protection. Vargas-Campos et al. (2018) studied the encapsulation of refined betalain extracts using native potato starch and its modification through phosphorylation or succinylation. The microcapsules were produced by spray drying and their stability was evaluated at 40°C for 39 days and using them as yoghurt pigmentants (pH 4.6) at 4°C for 32 days. Succinylated potato starch proved to be the best alternative for stabilizing betalains. Antigo et al. (2017) evaluated the stability of red beet extract microcapsules, dried by freeze-drying and spray drying and stored at room temperature as the pH varied (3–6). The microcapsules were manufactured using a combination of maltodextrin and xanthan gum as encapsulating agents and stored for 7 days. All microcapsules were characterized by a greater stability in terms of betanine content, phenolic compounds, and color parameters during storage at different pH.

5.4 | Chlorophylls and their derivatives

Given that in the culinary field the green color is considered a freshness index, the stabilization of green pigments (present in ingredients such as vegetables or spices) is a fundamental goal to be achieved. Chlorophylls and their derivatives (e.g., Cu-complexes) represent the main

natural green pigments, and their stability, as for other colorants, is affected by temperature and pH (Figure 6) (Galaffu et al., 2015). When the central magnesium ion is replaced by two protons to form phaeophytin, an undesirable color change occurs, which shifts toward grayish shades. The speed of this reaction is strongly affected by the acidity of the matrix and slows down to pH values around neutrality. Hence, chlorophyll stability is strongly affected by the control of this reaction. Operation processes do not cause instant color degradation, but rather initiate a slow color transformation which is observed at the beginning of the shelf-life (Galaffu et al., 2015). Several methods have been patented to prevent the degradation of chlorophyll, but they cannot be applied everywhere due to food legislation of each country. For example, the use of zinc to stabilize the green color of chlorophyll is only allowed in the United States for green beans and peas. In the European Union, the use of copper is allowed (Galaffu et al., 2015). These metal ions, replacing the magnesium of the central structure of chlorophyll, are able to produce more pH-stable green colorants (G. Ozkan & Bilek, 2015). Only few recent studies have been conducted on the stabilization methods of chlorophylls after extraction. Kang et al. (2019) encapsulated chlorophylls by using different blends of GA and maltodextrin by spray-drying, demonstrating that microcapsules coated with maltodextrin alone showed the highest storage stability (94.7%–97.5%). Zhang et al. (2019) produced cabbage leaf chlorophyll microcapsules and whey protein isolate (WPI-CH), in order to improve chlorophyll solubility and stability. The results proved that the encapsulation efficiency and chlorophyll solubility were increased by 3.78% and 7.79%. Raei et al. (2017) evaluated the effects of alfalfa chlorophyll microencapsulation on its stability with respect to high temperatures (80–100°C) and acidic pH (6.5), as well as its application in heated food. The results of this study presented a suitable way to improve the green pigment stability of alfalfa as a valuable and cost-effective source of chlorophylls.

6 | CONCLUSIONS

Emerging advances, innovations, and challenges in the extraction and application of natural pigments are the core focus of this review. Within the Circular Economy framework, the food industry needs to adopt measures to enhance vegetable waste, as a valuable source of natural pigments. In the modern food and biotechnology industry, the application of enzymes for the recovery of natural food colorants has been highlighted as a useful alternative to conventional techniques, being mild, eco-friendly, and economically efficient. The great challenge for the future is the exploitation of natural and health benefiting pigments from food waste that allows to minimize the environmental impact of waste supporting the Circular Economy concepts, also contributing to the sector company profit. Finally, more in-depth studies will be needed to further enhance the stability of recovered natural pigments to enable their usability as food colorants.

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AUTHOR CONTRIBUTIONS

Data curation, writing-original draft preparation, and investigation: Claudio Lombardelli, Caterina Mazzocchi. *Conceptualization, methodology, supervision, validation, writing-reviewing, and editing*: Ilaria Benucci, Marco Esti.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Enzyme-Assisted Extraction for the Recovery of Food-Grade Chlorophyll-Based Green Colorant

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Abstract: The aim of the study was to develop a biotechnological approach for the green recovery of chlorophyll from spinach, to be used as a natural food colorant. The plant matrix was characterized in terms of cell wall polysaccharide composition, and a tailored enzymatic mix based on cellulase (40%) xylanase (41%) and polygalacturonase (19%) was formulated. The process variables (temperature (°C), time (h), enzyme mix dose (U/g), zinc concentration (ppm), and buffer/substrate ratio (B/S)) and their interactions were studied by response surface methodology. The overlay plot made it possible to identify the process conditions (T: 25 °C, Zn: 150 ppm e B/S: 17.5, t: <2 h and enzyme mix dose between 12 and 45 U/g) to maximize the amount of chlorophyll, and concurrently, the quality of the green color of the extract. Finally, the novel colorant was applied in the production of a real food.

Keywords: solvent-free extraction; circular economy; zinc; natural pigment recovery; response surface methodology

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1. Introduction

It has been estimated that about 46 tons of food per year remain unsold on the shelves of large-scale retail trade, and only a small percentage of that is intended for food banks and charity organizations [1]. In order to better manage food loss and food waste, the European Union adopted a Directive in 2018 [2] favoring the circular economy (CE) model. Indeed, the CE may contribute to food waste reduction through valorization in accordance with the principles of reusing, reducing, and recycling [3,4]. In this scenario, CE encourages the use of unsold food (especially fruit and vegetables) as a cheap and valuable source for the recovery of high-added-value molecules [5].

In this context, there is a growing interest in the extraction of colorants from natural sources [6–9]. Among natural pigments, chlorophyll (Chl) is widely distributed in fruits and vegetables (also in algae and cyanobacteria) and may be used as an alternative to synthetic dyes. The following green colorants are currently available on the market, all recovered using organic solvents: E140(i) chlorophyll extracted from plants; E140(ii) chlorophyllins, obtained by saponification of E140(i); E141(i) copper chlorophylls, obtained by substitution of the magnesium (Mg^{2+}) with the copper (Cu^{2+}) ion; and E141(ii) copper chlorophyllins [10]. The main sources of Chl are leafy vegetables such as spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*), and broccoli (*Brassica oleracea*), among others [11]. In detail, spinach is a highly consumed vegetable worldwide, and its production led to 25% of waste, from which high-added-value molecules can be extracted. In fact, spinach by-products may contain high amounts of lutein (3.9–9.5 mg/100 g_{FW}) and chlorophyll (about 130 mg/100 g_{FW}). Recently, these extracts have been used to develop nutraceuticals or functional food products [12–16].

Chl is an oil-soluble pigment and occurs in nature in two different structures: chlorophyll-a (blue-green color), if in the 7-carbon position it has a methyl group, and chlorophyll-b (blue-yellow color), if it shows an aldehyde group in the same position [11,17]. Chl is a

stable pigment in nature, but factors such as oxygen, pH, light, temperature, and chlorophyllase enzymes may lead to degradation phenomena when the pigment is extracted from its natural environment [18]. All these factors may cause a centralized shift of the Mg^{2+} ion in the central position, giving rise to pheophytin, which causes a color change towards an olive green/gray tone. It is possible to produce more stable complexes by substituting Mg^{2+} with Cu^{2+} or Zn^{2+}, thus restoring the functions and properties of the molecule and producing a greening effect [19]. Although Cu^{2+} is more easily inserted into the structure of the molecule than Zn^{2+}, the toxicity of Cu^{2+} is greater than that of Zn^{2+} [7].

Commonly, Chl is recovered from plant matrices through various conventional methods such as maceration and Soxhlet extraction techniques with organic or inorganic solvents. These non-environmentally friendly and cost-intensive methodologies have several disadvantages, including low extraction yield and long process times [11]. For these reasons, "green" extraction techniques have been developed (such as supercritical fluid extraction, ultrasound assisted extraction, microwave assisted extraction, pulsed-electric field-assisted extraction, and enzyme-assisted extraction) [20–22]. Enzyme-assisted extraction (EAE) of pigments is based on the breakdown of the plant cell wall by enzymatic hydrolysis [19,23]. Cell-wall-degrading enzymes have been used successfully for the recovery of a variety of constituents, including anthocyanins [24], lycopene [25], β-carotene [26], carotenoids [4] and betalains [27] from plant tissues. Only a few studies investigated the application of EAE for the recovery of Chl via commercial standard preparations [7,28]. Özkan et al. [7] described the extraction of chlorophyll from spinach using Pectinex Ultra SP-L, which was not able to completely hydrolyze the plant cell wall. This is likely because the provided enzymatic activities are not flexible in relation to the vegetable tissue composition.

Therefore, in this study, a tailor-made EAE protocol has been formulated for the recovery of Chl from unsold spinach, considering its polysaccharide cell wall composition and concurrently optimizing the process parameters (such as temperature, time, total enzyme mix dose, zinc concentration, and buffer/substrate ratio) to maximize the amount of Chl extracted. Finally, the green Chl-based extract obtained by EAE was incorporated in the formulation of a real food (Baghrir) to evaluate its tinting strength.

2. Materials and Methods

2.1. Plant Material, Enzymes and Chemicals

Unsold spinach (*Spinacia oleracea*) was supplied by Unicoop Tirreno S.C. (Viterbo, Lazio, Italy) and stored at 4 °C until use.

The enzyme preparations (Merck, Milan, Italy) used were as follows: cellulase (CL, 0.8 U/mg) from *Aspergillus niger*, xylanase (XL, ≥2500 units/mg) from *Aspergillus oryzae*, and polygalacturonase (PG, ≥0.3 U/mg) from *Aspergillus niger*. All the reagents used, including enzyme substrates (polygalacturonic acid, carboxymethylcellulose, and xylan), were purchased from Merck (Milan, Italy).

2.2. Plant Material Characterization

2.2.1. Moisture and Dry Matter

Moisture content (%) and dry matter (DM, %) were evaluated after drying spinach samples (100 g) in an air-forced oven (Venticell, MMM Medcenter Einrichtungen GmbH, Planegg/München, Germany) at 30 °C for 5 days [29].

DM was determined through the following equation (Equation (1)):

$$DM = d_w/f_w \times 100 \quad (1)$$

where: DM: dry matter, f_w: fresh weight (g), d_w: dry weight (g).

2.2.2. pH

The pH was determined according to the Official Methods of Analysis of AOAC INTERNATIONAL [30] by means of a pH-meter (HANNA Instruments, Woonsocket, RI, USA) after blending spinach with 20% distilled water (spinach/water).

2.2.3. Cell Wall Polysaccharides Composition

The content of cellulose, hemicellulose (xylans), and pectin was determined as described by Ververis et al. [29] and Lombardelli et al. [4]. Sugars released following acid hydrolysis (0.7 g d_w sample boiled with 5 mL H_2SO_4 solution 72% v/v for 4 h) were determined by using enzymatic kits (Megazyme International, Bray, Ireland) of D-fructose/D-glucose, D-xylose and D-glucuronic acid/D-galacturonic acid. Carbohydrate concentration was obtained by means of appropriate calibration curves.

2.3. Characterization of Enzyme Preparations

Enzyme preparations (CL, XL and PG) were characterized in terms of protein content, electrophoretic profile, and kinetic parameters. In detail, protein content was determined using the Bradford method [31], and the Abs was spectrophotometrically detected at 595 nm (Shimadzu UV 2450, Milan, Italy). The calibration curve was obtained using Bovin Serum Albumin (BSA) as standard. The electrophoretic profile was analyzed by SDS-PAGE gel electrophoresis under reducing conditions as reported by Lombardelli et al. [4].

The activities of CL, XL, and PG were determined in 0.1 M McIlvaine buffer, pH 5.0 at 45 °C. The resulting reaction products (glucose, xylose, and galacturonic acid) were detected by the 3′,5′-dinitrosalicylic method (DNS), reading the Abs at 530 nm [32]. The specific activity (S.A.) was indicated as I.U./mg protein expressed as BSA_{eq} (I.U./mg_{BSAeq}).

For each enzyme, the kinetic characterization was carried out under the test conditions described above, varying the substrate concentration for each enzyme preparation: CL (carboxymethylcellulose: 1.55–12.75 mg/mL), XL (xylan: 0.066–0.1 mg/mL), and PG (polygalacturonic acid: 0.2–1 mg/mL). The Michaelis-Menten equation was applied for the estimation of kinetic parameters V_{max} (I.U./mg_{BSAeq}) and K_M (mg/mL) using a nonlinear regression procedure (GraphPad Prism 5.01, GraphPad Software, Inc., La Jolla, CA, USA.). Furthermore, the catalytic constant (k_{cat}, min^{-1}) and the affinity constant (K_a, min^{-1}/mg mL^{-1}) were calculated.

2.4. Extraction Process Optimization—Response Surface Methodology

The process conditions (temperature (T, °C), time (t, h), total enzyme dose (enzyme mix, U/g), $ZnCl_2$ concentration (Zn, ppm) and buffer/substrate ratio (B/S)) were optimized for maximizing the amount of recovered Chl (μg/g). An experimental design based on the statistical model of Design of Experiment (DOE) was used [4]. The variance for each factor evaluated was divided into linear, quadratic, and interactive components, and was represented using the following polynomial function (Equation (2)):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{55}x_5^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{34}x_3x_4 + b_{35}x_3x_5 + b_{45}x_4x_5 \quad (2)$$

where, the dependent variable (y) represents the amount of extracted Chl. The coefficients of the polynomial are represented by b_0 (constant term), b_1, b_2, b_3, b_4, b_5 (linear coefficients), b_{11}, b_{22}, b_{33}, b_{44}, b_{55} (square coefficients), b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, b_{45} (interacting coefficients). The significance of all terms of the polynomial function was statistically evaluated using the F-value with a probability (p) of 0.001, 0.01 or 0.05. Regression coefficients were then used to generate contour plots and overlay plots [4,33]. DOE and statistical analysis were performed using Minitab 17.1 software (Minitab Inc., State College, PA, USA).

The response surface methodology (RSM) was used to verify the presence of combined effects between the different variables and to determine a range of optimal conditions to maximize the amount of extracted Chl. Each variable has three levels: −1, 0, and +1. A

total of 32 combinations, including three replicas of the central point, were performed in random order according to the central composite design [34]. The levels of the variables x1, x2, x3, x4, x5 and X1, X2 X3, X4, X5 are shown in Table 1.

Table 1. Design of Experiment (DOE) for the determination of optimal process parameters (temperature (°C), time (h), enzyme dose (enzyme mix, U/g), $ZnCl_2$ dose (Zn, ppm), buffer/substrate ratio (B/S)) on the amount of chlorophyll extracted (Chl, µg/g).

Treatment Trial	Temperature (°C)	Time (h)	Enzyme Mix (U/g)	Zn (ppm)	B/S	Chl (mg/g)
	X1 (x1)	X2 (x2)	X3 (x3)	X4 (x4)	X5 (x5)	
1	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	791
2	25 (−1)	5 (+1)	50 (+1)	300 (+1)	5 (−1)	1299
3	25 (−1)	5 (+1)	10 (−1)	300 (+1)	30 (+1)	877
4	25 (−1)	1 (−1)	10 (−1)	300 (+1)	5 (−1)	783
5	25 (−1)	5 (+1)	50 (+1)	0 (−1)	30 (+1)	626
6	25 (−1)	1 (−1)	10 (−1)	0 (−1)	30 (+1)	597
7	50 (+1)	5 (+1)	50 (+1)	0 (−1)	5 (−1)	350
8	50 (+1)	1 (−1)	50 (+1)	300 (+1)	5 (−1)	1283
9	50 (+1)	5 (+1)	10 (−1)	300 (+1)	5 (−1)	993
10	50 (+1)	1 (−1)	50 (+1)	0 (−1)	30 (+1)	509
11	25 (−1)	5 (+1)	10 (−1)	0 (−1)	5 (−1)	869
12	50 (+1)	5 (+1)	50 (+1)	300 (+1)	30 (+1)	817
13	50 (+1)	5 (+1)	10 (−1)	0 (−1)	30 (+1)	590
14	50 (+1)	1 (−1)	10 (−1)	300 (+1)	30 (+1)	331
15	25 (−1)	1 (−1)	50 (+1)	300 (+1)	30 (+1)	636
16	25 (−1)	1 (−1)	50 (+1)	0 (−1)	5 (−1)	1286
17	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	791
18	50 (+1)	1 (−1)	10 (−1)	0 (−1)	5 (−1)	621
19	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	791
20	50 (+1)	3 (0)	30 (0)	150 (0)	17.5 (0)	718
21	37.5 (0)	5 (+1)	30 (0)	150 (0)	17.5 (0)	752
22	37.5 (0)	3 (0)	50 (+1)	150 (0)	17.5 (0)	716
23	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	714
24	37.5 (0)	3 (0)	30 (0)	300 (+1)	17.5 (0)	970
25	37.5 (0)	3 (0)	30 (0)	150 (0)	5 (−1)	755
26	25 (−1)	3 (0)	30 (0)	150 (0)	17.5 (0)	1130
27	37.5 (0)	3 (0)	30 (0)	150 (0)	30 (+1)	659
28	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	739
29	37.5 (0)	3 (0)	30 (0)	150 (0)	17.5 (0)	742
30	37.5 (0)	3 (0)	30 (0)	0 (−1)	17.5 (0)	895
31	37.5 (0)	1 (−1)	30 (0)	150 (0)	17.5 (0)	999
32	37.5 (0)	3 (0)	10 (−1)	150 (0)	17.5 (0)	750

2.5. Chlorophyll Recovery by Enzyme Assisted Extraction

Chl extraction from unsold spinach was performed as proposed by Özkan et al. [7] with some modifications:

Pretreatment and homogenization of spinach leaves. Whole spinach leaves (25 g) were blanched for 5 s at 100 °C to inactivate the endogenous chlorophyllases which could lead to Chl degradation. Subsequently, the leaves were homogenized with a mixer (Phillip HR 2068 blender) for 1 min at maximum speed, using part of the buffer. The ratio of biomass (spinach) and McIlvaine buffer (pH 5; 0.1 M) was varied according to the experimental plan (Table 1).

EAE treatment. The homogenized spinach was resuspended in 0.1 M McIlvaine buffer (pH 5) and the treatments were carried out following DOE reported in Table 1. In detail, the value ranges for each variable were: T = 25–50 °C, t = 1–5 h, total enzyme dose = 10–50 U/g, Zn = 0–300 ppm, and B/S = 5–30. Each range has been identified considering the conditions reported in the literature for pigment extraction [7,28]. The reaction mixture was kept under

continuous stirring in the dark in a bioreactor (1 L), which was equipped with an external water jacket for temperature control and connected to a thermostat (MPM Instrument, Type M 900-TI, Bernareggio, Italy). After incubation, the homogenate was centrifuged (Heraeus Megafuge 16R Centrifuge, Thermo Scientific, Milan, Italy) at 4500 rpm for 5 min at 10 °C and the completely clear supernatant was discarded.

2.6. Chlorophyll Content and Colorimetric Characterization

An amount of 1 g of pellet was added to 10 mL of absolute ethanol. After stirring, the solution was centrifuged at 4500 rpm for 5 min at 10 °C. The recovered supernatant was used for the spectrophotometric measurement (UV-visible, Shimadzu UV 2450, Milan, Italy), recording the spectra (from λ 230 to 700 nm) using a 1 mm quartz cuvette (Hellma, Milan, Italy). The amount of Chl was determined through the following equation, obtained via modifications of the equations of Özkan et al. [7] and Lichtenthaler [35] (Equation (3)).

$$\text{Chl } (\mu g/g) = (5.24 \times \text{Abs}_{664} + 22.24 \times \text{Abs}_{649}) \times \text{DF} \quad (3)$$

where: Abs_{664} = Absorbance at λ 664 nm, Abs_{649} = Absorbance at λ 649 nm, DF = Dilution factor.

The supernatant was also used for colorimetric characterization. Color measurements were performed using a CR-5 colorimeter (Konica Minolta, Tokyo, Japan) with the CIELa*b* system. The colorimetric analyses were performed in triplicate, with five measurements for each sample unit. Furthermore, extracts were evaluated through two derived color parameters: hue angle (h°) and chroma (C*) [26].

2.7. Application of Chlorophyll-Based Colorant in Real Food

After the EAE, the pellet containing Chl was freeze-dried (bench freeze-dryer, Labconco Corporation, Kansas City, MI, USA) and used for the application in a typical Moroccan dish: "Baghrir". It is a sweet made with semolina (23% *w*/*w*), flour (9.20% *w*/*w*), sugar (1.74% *w*/*w*), salt (0.57% *w*/*w*), dry yeast (0.34% *w*/*w*), chemical yeast (1.84% *w*/*w*), and water (63.31% w/w). Chl-based extract was added to the Baghrir at different concentrations (1%, 2.5%, 5%, 7.5 and 10% *w*/*w* fluor based). An amount of 10 g of dough was cooked on a pan over medium heat until bubbles appeared on one side only (about 5 min). The final product was colorimetrically characterized by CIELa*b*.

2.8. Statistical Analysis

All trials were performed in triplicate, and results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) to find the effect of individual factors and their interaction on colorimetric parameters ($p < 0.01$). Tukey's post-hoc test (HSD) was also performed ($p < 0.05$) using EXCEL® extension DSAASTAT for multiple comparison between samples.

3. Results and Discussion

3.1. Plant Material and Enzyme Preparations

Spinach was characterized in terms of moisture, DM, pH, and composition of the cell wall polysaccharides (cellulose, hemicellulose, and pectin). The obtained results (Table 2) are in line with those reported in the literature [36,37]. The main structural polysaccharides were: cellulose 40% (5.20 ± 0.08 g/100 g_{dwb}); hemicellulose 41% (5.35 ± 0.08 g/100 g_{dwb}); and pectin 19% (2.43 ± 0.14 g/100 g_{dwb}). On the basis of the spinach cell wall composition, it was possible to develop a tailored enzyme mix composed of 40% CL, 41% XL, and 19% PG.

Table 2. Chemico-physical characterization of spinach.

Moisture (%)	Dry Matter (%)	pH	Cellulose (g/100 g_{dwb})	Hemicellulose (g/100 g_{dwb})	Pectin (g/100 g_{dwb})
93.6 ± 1.2	6.4 ± 0.9	6.29 ± 0.02	5.20 ± 0.08	5.35 ± 0.08	2.43 ± 0.14

dwb: dry weight basis.

Each enzyme preparation was initially characterized in terms of total protein content (0.024 mg_{BSAeq}/mL for CL and PG, 0.027 mg_{BSAeq}/mL for XL, Table 3). The SDS-PAGE profile (proved that all enzyme preparations were characterized by the presence of a dominant band which was in line with the previous reports [4].

Table 3. Protein content and kinetic parameters (V_{max}, K_M, k_{cat}, K_a) of enzyme preparations (cellulase (CL), xylanase (XL) and polygalacturonase (PG)).

	Protein Content (mg_{BSAeq}/mL)	V_{max} (I.U./mg_{BSAeq})	K_M (mg/mL)	k_{cat} (min^{-1})	Ka (min^{-1}/mg mL^{-1})	R^2
CL	0.024 ± 0.002	195.8 ± 9.94	10.41 ± 1.07	3.24 × 106 ± 9.94	3.11 × 105 ± 3.3 × 103	0.98
XL	0.027 ± 0.003	93.84 ± 2.02	0.0057 ± 0.0007	4.14 × 105 ± 2.02	7.29 × 107 ± 1.1 × 105	0.97
PG	0.024 ± 0.001	16950 ± 620	0.375 ± 0.034	6.13 × 108 ± 620	1.63 × 109 ± 1.3 × 107	0.99

V_{max}: maximum rate at which the enzyme catalyses the reaction. K_M: Michaelis-Menten constant. $k_{cat} = V_{max}/[E]_{tot}$, where $[E]_{tot}$ is the molar concentration of the enzyme. $K_a = k_{cat}/K_M$.

The kinetic curves of the three enzyme preparations all followed the hyperbolic behavior of the Michaelis-Menten equation (Figure 1) and the estimated kinetic parameters were reported in Table 3.

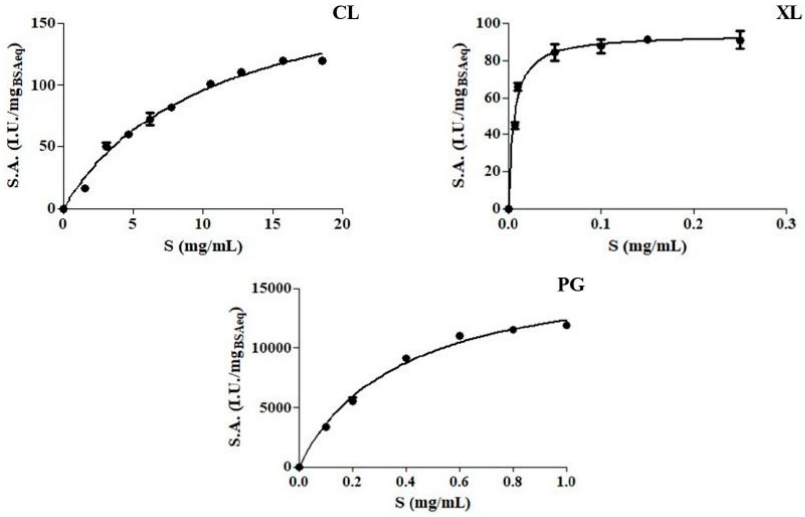

Figure 1. Michaelis–Menten plots of the enzyme preparations (cellulase (CL), xylanase (XL) and polygalacturonase (PG)). The specific activity (S.A., IU/mg_{BSAEq}) were determined at 45 °C in McIlvaine buffer (0.1 M, pH 5), varying the substrate concentration (S, mg/mL).

3.2. Enzyme-Assisted Extraction for Chlorophyll Recovery

To maximize the amount of Chl recovered from spinach through an innovative eco-friendly EAE process, the applied conditions should be optimized since they have a strong influence on the amount of pigment extracted and its stability [38]. The effects of process variables (T, t, enzyme mix, Zn, B/S) on the amount of Chl extracted from spinach leaves

were evaluated by RSM. Table 1 shows the results of the full factorial design, and Table 4 shows the coefficients of the mathematical model and statistical parameters, which display that the RSM developed was adequate (Model *p*-value = 0.003).

Table 4. Regression coefficients, model *p*-value, R2 and adjusted R2 for the different polynomial models (Note: Subscripts: 0 = constant term; 1 = temperature; 2 = time; 3 = Enzyme dose; 4 = Zinc dose, 5 = buffer/substrate).

Regression Coefficient	Chl
b_0	+794.3
b_1	−105.1 **
b_2	+7.0
b_3	+61.7 *
b_4	+91.5 **
b_5	−144.2 ***
b_{11}	+90.5
b_{22}	+41.9
b_{33}	−100.9
b_{44}	+99.0
b_{55}	−126.7
b_{12}	−22.6
b_{13}	−18.5
b_{14}	+71.1 *
b_{15}	+31.4
b_{23}	−101.2 **
b_{24}	+95.6 **
b_{25}	+81.2 **
b_{34}	+59.9
b_{35}	−47.5
b_{45}	−55.8
Model *p*-value	0.003
R^2	0.93
R^2 adj	0.77

* significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

For the response variable considered (Chl concentration), the R^2 value was equal to 0.93, indicating that the regression model was able to effectively explain how the variables considered and their interactions affected the response.

Furthermore, the significance of each equation coefficient was determined using the p-value (Table 4), and the smaller the p-value, the more significant the corresponding coefficient [39]. The analysis of variance was performed to determine the significance of the linear, quadratic, and interaction effects of the independent variable on the dependent variable. All the considered variables, except time, had a statistically significant linear effect (*p*-value < 0.05, Table 4). In detail, T (*p*-value < 0.01), Zn concentration (*p*-value < 0.01) and B/S (*p*-value < 0.001) were the factors that contributed the most (both positively and negatively) to the extraction of Chl when their linear effect was considered. The quadratic effect was not significant (*p*-value > 0.05) for any variable (Table 4). Otherwise, the interactions among the variables that most affected the model (*p*-value < 0.05) were T (°C) × Zn (ppm), time (h) × Enzyme mix (U/g), t (h) × Zn (ppm) and t (h) × B/S. It may be observed that time was significant only in terms of interaction with other variables. The results allowed us to develop a useful equation [40] to predict the amount of Chl extracted by varying T (x_1), t (x_2), enzyme mix (X_3), Zn (X_4) and B/S (X_5) (Equation (4)).

$$\begin{aligned} Chl = {} & +794.3 - 105.1x_1 + 7.0x_2 + 61.7x_3 + 91.5x_4 - 144.2x_5 + 90.5{x_1}^2 + 41.9{x_2}^2 - 100.9{x_3}^2 + 99.0{x_4}^2 - 126.7{x_5}^2 - \\ & 22.6x_1x_2 - 18.5x_1x_3 + 71.1x_1x_4 + 31.4x_1x_5 - 101.2x_2x_3 + 95.6x_2x_4 + 81.2x_2x_5 + 59.9x_3x_4 - 47.5x_3x_5 - 55.8x_4x_5 \end{aligned} \quad (4)$$

The interactions between the variables that most affected the response are depicted through the contour plots (Figure 2). Setting the values for T = 37.5 °C, Zn = 150 ppm and

B/S = 17.5 (Figure 2a), it may be observed that the highest Chl concentration (>800 μg/g) was recovered at the highest enzymatic mix doses (>28 U/g), and also in the shortest extraction time (1 h). An extended extraction time appeared necessary in view of minimizing the enzymatic dose (up to 15 U/g) (Figure 2a), thus lowering the production costs. The effect of the interaction T (°C) × Zn (ppm) on the amount of Chl extracted is shown in Figure 2b (value set t = 3 h, Enzyme mix = 30 U/g and B/S = 17.5). It is interesting to point out that the highest concentration of Chl was reached at temperatures between 25 and 32 °C, independent of the amount of Zn used. By increasing the temperature (>32 °C), the protective action exerted by Zn on the green pigment became more evident. The effect of the interaction t (h) × Zn (ppm) clearly proved the protective action of Zn, notably at a longer extraction time (Figure 2c) (set conditions T = 37.5 °C, Enzyme mix = 30 U/g and B/S = 17.5).

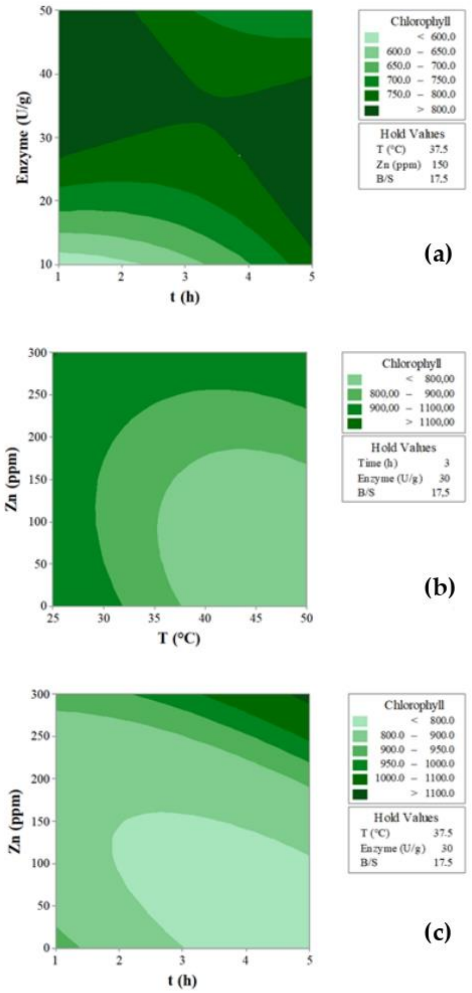

Figure 2. Contour plots showing the interactions between the variables with the greatest influence (p value ≤ 0.05) on the amount of chlorophyll extracted (μg/g): (**a**) time (h) × enzyme dose (U/g), (**b**) temperature (°C) × Zn (ppm), (**c**) time (h) × Zn (ppm).

The literature lacks studies concerning Chl recovery by enzymes. Özkan et al. [7] described the extraction of zinc-stabilized Chl from spinach using a commercial preparation (Pectinex Ultra SP-L enzyme). They identified three parameters that affect the extraction (enzyme preparation concentration, extraction time, and temperature), and in line with our results, observed a significant and positive effect of the enzyme dose and temperature variables. This same relationship between enzyme dose, extraction time, and temperature was also observed on Pandan leaves [28]. The optimal extraction conditions found by Özkan et al. [7] were: (i) enzyme concentration 8%, (ii) temperature 45 °C, (iii) time 120 min, and (iv) Zn dose 300 ppm. Moving beyond the state of the art, our research developed a tailored enzyme mix formulated on the base of polysaccharide composition of the spinach cell wall. The optimized process conditions for maximizing the Chl recovery are as follows: 25 °C, 1 h, 50 U/g of enzyme mix, 300 ppm of Zn and 17.5 B/S ratio. These results prove that Chl extraction could be a green route to coloring foodstuffs, giving much greater value to unsold spinach and developing an eco-friendly and high-performing extraction process.

3.3. Colorimetric Properties of the Extracts

In addition to the quantification of the extracted pigment, other parameters to be considered for a more comprehensive picture of the green color quality are the CIELa*b* parameters (L*, a*, b*) and color indices (C*, h°). L* represents brightness (0 to 100), a* the green/red component (−a* = green, +a* = red), and b* the blue/yellow component (−b* = blue, +b* = yellow). In particular, as reported in the literature [7,28], the assessment of greenness is expressed by the parameter a*.

The values of L*, a*, b*, h° and C* and the corresponding resulting color of the Chl-based extracts from spinach under different conditions are summarized in Table 5.

Table 5. Visual color attributes [a*, b*, L*, hue (h°), chroma value (C*)] of the chlorophyll-based extracts from spinach under different conditions.

Treatment Trial	L*	a*	b*	C*	h°	Color
1	47.14 ^{f}	−20.26 ^{i}	74.5 ^{g}	77.2 ^{hij}	105.2 ^{ij}	
2	28.21 ^{f}	−12.59 ^{f}	47.2 ^{p}	48.9 ^{o}	104.9 ^{ij}	
3	48.05 ^{f}	−34.09 ^{l}	73.4 ^{gh}	80.9 ^{fg}	114.9 ^{l}	
4	65.07 ^{c}	−26.68 ^{j}	33.4 ^{q}	42.7 ^{p}	128.6 ^{a}	
5	55.89 ^{e}	−27.86 ^{j}	84.1 ^{d}	88.6 ^{d}	108.3 ^{gh}	
6	72.4 ^{b}	−39.27 ^{m}	95.5 ^{b}	103.2 ^{a}	112.4 ^{e}	
7	42.75 ^{gh}	−1.71 ^{b}	54.62 ^{o}	54.6 ^{n}	91.8 ^{n}	
8	23.29 ^{j}	−14.11 ^{f}	45.65 ^{p}	47.8 ^{o}	107.2 ^{gh}	
9	30.95 ^{i}	−3.48 ^{bc}	51.92 ^{o}	52.0 ^{n}	93.8 ^{mn}	
10	63.86 ^{cd}	−26.72 ^{j}	92.67 ^{b}	96.4 ^{bc}	106.1 ^{jh}	
11	31.19 ^{i}	−5.56 ^{d}	52.72 ^{o}	53.0 ^{n}	96.0 ^{l}	
12	38.96 ^{g}	−0.18 ^{a}	65.31 ^{l}	65.3 ^{m}	90.2 ^{n}	
13	52.7 ^{f}	−10.97 ^{f}	82.75 ^{d}	83.5 ^{ef}	97.6 ^{l}	
14	77.51 ^{a}	−36.47 ^{l}	98.42 ^{a}	105.0 ^{a}	110.3 ^{fg}	
15	60.62 ^{d}	−44.18 ^{n}	85.24 ^{d}	96.0 ^{b}	117.4 ^{d}	
16	40.74 ^{g}	−43.38 ^{n}	62.93 ^{l}	76.4 ^{ij}	124.6 ^{b}	
17	48.14 ^{f}	−19.26 ^{i}	73.48 ^{gh}	76.0 ^{ij}	104.7 ^{j}	
18	58.51 ^{d}	−27.68 ^{j}	87.73 ^{c}	92.0 ^{c}	107.5 ^{hi}	
19	50.17 ^{f}	−20.98 ^{i}	71.22 ^{ijk}	74.2 ^{j}	106.4 ^{hi}	
20	42.86 ^{i}	−3.32 ^{bc}	70.53 ^{k}	70.6 ^{k}	92.7 ^{mn}	
21	43.86 ^{g}	−5.78 ^{cd}	71.35 ^{jk}	71.6 ^{k}	94.6 ^{m}	
22	49.32 ^{g}	−17.03 ^{h}	77.75 ^{f}	79.6 ^{fjk}	102.4 ^{j}	
23	49.25 ^{f}	−15.8 ^{gh}	77.67 ^{f}	79.3 ^{fjk}	101.5 ^{j}	
24	39.1 ^{f}	−14.25 ^{f}	64.26 ^{l}	65.8 ^{l}	102.5 ^{j}	

Table 5. *Cont.*

Treatment Trial	L*	a*	b*	C*	h°	Color
25	46.74 ^{gh}	−19.72 ^{i}	72.94 ^{hi}	75.6 ^{j}	105.1 ^{j}	
26	35.78 ^{f}	−30.49 ^{k}	57.01 ^{n}	64.7 ^{l}	118.1 ^{d}	
27	53.07 ^{h}	−19.13 ^{i}	82.32 ^{de}	84.5 ^{e}	103.1 ^{j}	
28	48.98 ^{f}	−16.02 ^{gh}	76.99 ^{fg}	78.6 ^{ghi}	101.8 ^{k}	
29	47.22 ^{f}	−14.99 ^{g}	76.78 ^{fg}	78.2 ^{ghi}	101.0 ^{k}	
30	40.36 ^{f}	−11.6 ^{e}	66.47 ^{l}	67.5 ^{l}	99.9 ^{k}	
31	38.77 ^{f}	−37.51 ^{m}	60.88 ^{m}	71.5 ^{k}	121.6 ^{c}	
32	52.76 ^{g}	−31.77 ^{k}	79.15 ^{e}	85.3 ^{de}	111.9 ^{f}	

C* [Chroma = $(a^{*2} + b^{*2})^{0.5}$]; h° [hue angle = 180° + (arctan b*/a*)]. Values with different small letters (a–q) differ significantly (Tukey's test, $p = 0.05$) according to treatment trial.

Regarding the parameter a*, the variables that seem to have the most influence are temperature and time. In detail, we may observe how the lowest values (a* < −30, nuance towards green) are obtained at the lowest temperature (25 °C) and extraction time (1 h). Under such mild conditions, the amount of Zn did not seem to be relevant, whereas its protective action became evident in the extracts recovered at higher temperatures and for longer extraction times (>37.5 °C and >1 h). As expected, the color saturation (C*) and hue (h°) parameters, resulting from the a* value, followed the same trend (Table 5).

3.4. Overlay Plot of Chlorophyll and a Value*

To identify the region where it is possible to maximize both the concentration of Chl (800 < Chl < 1300 μg/g) and the value of a* (−30 < a* < −44), their corresponding contour plots were superimposed in an overlay plot (Figure 3) at fixed T (25 °C) and B/S (17.5).

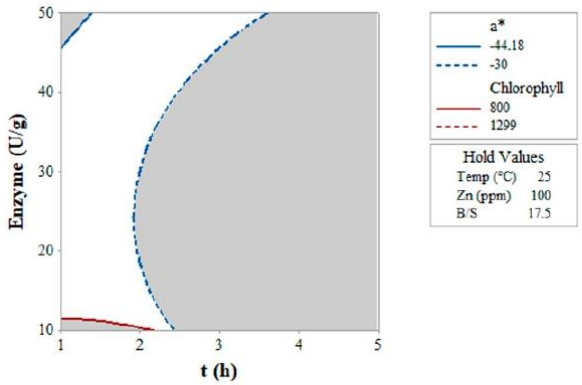

Figure 3. Overlay plot obtained by superimposing the contour plots of chlorophyll extracted (μg/g) and the quality of green (a*) as functions of enzyme dose (U/g) and time (t, h). The white area suggests the optimal region for maximizing both the amount of recovered chlorophyll and the quality of green color.

The white area in Figure 3 delimited the optimal conditions to simultaneously maximize Chl and a* within the imposed ranges, using a Zn concentration of 100 ppm (value set). It corresponded to: time < 2 h and an enzyme mix dose 12–45 U/g.

3.5. Application of Green Colorant in a Food Matrix

The Chl-based extract from spinach, recovered by applying the selected conditions suggested by the overlay plot (T: 25 °C, t: 1 h, Enzyme mix: 30 U/g, Zn: 150 ppm, and B/S: 17.5), was used in the formulation of a real food (Baghrir) at different doses (0, 1, 2.5, 5, 7.5, and 10% *w*/*w* flour basis). The pictures and the coordinates of the tristimulus values

(CIELa*b*) in Table 6 suggest a significant variation of the colorimetric parameters L* and a* between the product without colorant (0% *w*/*w*) and the product at low concentrations (1% *w*/*w*).

Table 6. Visual color attributes (L*, a*, b*) of the real food (Baghrir) prepared by adding different amounts of chlorophyll-based extracts from spinach (0–10% *w*/*w* flour basis).

Colorant (%)	L*	a*	b*	Sample
0%	38.4 ^{a}	2.64 ^{a}	18.16 ^{a}	
1.0%	36.43 ^{b}	−1.53 ^{b}	18.98 ^{a}	
2.5%	23.94 ^{d}	−2.09 ^{c}	15.99 ^{b}	
5.0%	22.01 ^{e}	−3.92 ^{d}	13.83 ^{c}	
7.5%	26.75 ^{c}	−3.98 ^{d}	13.93 ^{c}	
10%	26.15 ^{c}	−4.41 ^{d}	11.37 ^{c}	

Values with different small letters (a–e) differ significantly (Tukey's test, $p = 0.05$) according to colorant (%).

Concerning a*, it varied significantly towards negative values (more intense green nuance) up to a Chl-based extract concentration equal to 5% *w*/*w*. Over this dose, the addition of increased amount of colorant did not affect the colorimetric parameters a* and b* (Table 6).

4. Conclusions

The present work reports a biotechnological tool developed for the green recovery of chlorophyll from spinach, to be used as a natural food colorant. The process was performed using a tailored enzymatic mix, designed considering the polysaccharide composition of spinach cell wall. Response surface methodology allowed us to narrow the area in which it is possible to identify the highest amount of recovered chlorophyll, and concurrently, to have the best quality of green (T: 25 °C, Zn: 150 ppm e B/S: 17.5, t: <2 h, and enzyme mix dose between 12 and 45 U/g). The chlorophyll-based extract was successfully applied to give different green colorations to a real food. The main drawback of this approach is the cost of the biocatalyst and the intrinsic instability of the extracted pigment. Therefore, further studies will be devoted to minimizing the amount of the enzyme mix and to investigating the stability of the novel colorant preparations under different storage conditions (such as temperature and light exposure).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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ORIGINAL ARTICLE

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Stabilized chlorophyll-based food colorants from spinach: Kinetics of a tailored enzymatic extraction

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Abstract: An organic solvent-free method based on limited dosing options (biocatalyst and zinc chloride) for the quick and mild recovery of chlorophyll (Chl) from spinach has been proposed. This tailored, custom-made protocol has been designed to produce stable green natural colorants. The kinetics of pigment extraction turned out to be a very useful tool to identify the proper conditions, in terms of biocatalyst dose (0.10–50 U/g), extraction time (1–48h), and $ZnCl_2$ amount (50–300ppm), both for enhancing the recovery yield and preserving the green color. Considering the extraction kinetics, the recovery yield, and the colorimetric data, the suitable conditions to produce stable green and food-grade colorants are 0.10 U/g of enzyme, 3h, and 150ppm of $ZnCl_2$ at 25°C. The extraction yield of Chl (4863μg/U) was about 51% greater than control, with a higher extraction rate constant (5.43×10^{-4} g/(μg min)). Considering the impact of $ZnCl_2$ amount on Chl, its protective action resulted to be more noticeable toward Chl a: at 150ppm, an increased amount of about 2.5 and 1.5 times was found for Chl a and Chl b, respectively, in comparison to the reference (0ppm $ZnCl_2$).

KEYWORDS
chlorophyll a, chlorophyll b, enzyme-assisted extraction, green pigment, zinc chloride

Practical Application: This research demonstrates how a suitable kinetic approach helps to provide a tailored protocol, customized for the vegetable matrix, to produce stable green natural colorants from spinach. Lowering enzyme dosage and $ZnCl_2$ amount during the extraction of chlorophyll at low temperature is crucial for its potential use as a colorant in food industry, providing high economic values through saving time and environmental protection.

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1 | INTRODUCTION

Chlorophylls (Chls) consist of a hydrophilic head (porphyrin), a lipophilic hydrocarbon tail (phytol group), and a central magnesium ion bonded to the tetrapyrrole ring (Nabi et al., 2023). Due to their lipophilic hydrocarbon chains, Chls are generally considered to be insoluble in polar solvents (Ebrahimi et al., 2023). Stored mainly in chloroplasts, only two types of Chls (a and b) are present in higher plants, whereas others (c–f) are found in algae and bacteria. Chl a and Chl b differ structurally by the presence of a methyl or aldehyde group at the Carbon-7 position, respectively (Paliwal et al., 2016). These distinct structural features result in variations in their color: Chl a has a blue–green nuance, whereas Chl b has a green–yellow color (Carrillo et al., 2022). Chl a and b from plants, and recently authorized algal Chl (Chen et al., 2023), are used as colorants in food and pharmaceutical industries (Nabi et al., 2023), also for their recognized health properties (e.g., antioxidant [Li et al., 2023], anti-inflammatory, anticancer [Derrien et al., 2018], and antimutagenic properties [Ebrahimi et al., 2023]).

Several factors (e.g., heat, low pH, light, or enzymes) may degrade Chl during the extraction process. The replacement of Mg^{2+} with two H^+ ions (pheophytinization) triggers the conversion of blue–green Chl a into gray pheophytin a, whereas green–yellow Chl b turns into a brown pheophytin b (Indrasti et al., 2018). The degradation of Chl a occurs 2.5–10 times faster than that of Chl b (Eckardt, 2009). The loss of Mg^{2+} already starting from the initial stages of the extraction process, and the resulting color change affected Chl applications in food (Ghosh et al., 2022). The use of Cu^{2+} has been suggested as a valid approach to obtain commercial stable green copper Chls (E141(i) and E141(ii)) (Viera et al., 2019), which are more resistant to acids and heat and have improved antioxidant properties (Ebrahimi et al., 2023). However, the toxicity of copper has led to the investigation of other metal bivalent ions, such as zinc (Mazzocchi et al., 2023; Özkan & Bilek, 2015). The usefulness of zinc chloride during the Chl extraction has been documented in other studies (Mazzocchi et al., 2023; Özkan & Bilek, 2015; Senklang & Anprung, 2010). It is conceivable that the early addition of the Zn^{2+} ion, since the beginning of pigment extraction, may contribute to readily re-establish the correct conformation of the molecule, thus preserving its coloring properties.

Actually, all the natural Chl-based food colorants ((E140(i), E140(ii), E141(i), and E141(ii)) permitted in the EU are produced by the conventional solvent extraction (Viera et al., 2019). Despite the fact that solvent extraction is simple and does not require complex/expensive equipment, this non-environmentally friendly methodology shows numerous disadvantages, including the need for a large amount of solvent, a long extraction time, along with the possible pigment degradation, which affects the recovery yield (Mazzocchi et al., 2023). Green processing for the recovery of Chl from plant tissues, such as enzyme-assisted extraction (EAE), is gaining more attention (Benucci et al., 2022) due to the high recovery yield, fast extraction, low energy consumption, and no solvent usage (Saad et al., 2019). The major downside of that strategy is the high cost of biocatalysts, mainly due to the use of unspecific commercial preparations usually applied in unbalanced doses (Özkan & Bilek, 2015) because they are not based on a reasoned approach.

Kinetic studies of Chl extraction from vegetable tissue using solvents (e.g., methanol and ethanol) (Miazek & Ledakowicz, 2013), microwave-assisted extraction (Nguyen et al., 2021), or pulsed electric field (Zhang et al., 2020) turned out to be a very useful tool to identify the proper conditions for enhancing the recovery yield.

In our previous work (Mazzocchi et al., 2023), an EAE protocol for the recovery of Chl from spinach has been developed by using a tailored enzymatic mix, designed considering the polysaccharide composition of spinach cell wall, instead of commercial preparations that are not flexible in relation to the vegetable tissue composition.

This paper offers a deep extension of the precedent work aimed at increase Chl recovered yield per unit of enzyme and, concurrently, the greenness of the extract. The novelty and significant contribution of this research consist of providing useful information to interpret, and possibly foresee, the Chl release trend from a leaf vegetable matrix by the tailored EAE protocol. The kinetic study, along with the resulting modeling parameters, may be additionally helpful for the extraction of other pigments from different vegetable sources. Moreover, this study contributes to provide the most suitable operating conditions for the production of stable green natural colorants.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Biocatalysts (Merck) applied in this research were as follows: polygalacturonase (≥0.3 U/mg) and cellulase (0.8 U/mg) from *Aspergillus niger*; xylanase (≥2500 U/mg) from *Aspergillus oryzae*. All reagents used were purchased from VWR International.

2.2 | Plant material preparation

Unsold spinach (*Spinacia oleracea*) was kindly provided by a local supermarket (Unicoop Tirreno S.C.) and kept under refrigerated conditions until extraction. Spinaches of 25 g

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TABLE 1 Experimental conditions applied for the recovery of chlorophyll (Chl) by enzyme-assisted extraction.

Experimental conditions	
Temperature (°C)	25
Time (h)	1, 3, 6, 16, 24, 36, 48
Total enzyme dose (U/g)	0.10, 0.25, 0.50, 1.0, 3.0, 6.0, 10, 30, 50
$ZnCl_2$ dose (ppm)	0, 50, 100, 150, 200, 300
Buffer/Substrate ratio	17.5
pH	5.0

Note: For each treatment, three experimental replicates were conducted.

were subjected to blanching (5 s at 100°C) to neutralize the chlorophyllases. After that, they were mixed using a mixer (Phillip HR 2068 mixer) for 1 min at maximum power using extraction buffer (McIlvaine 0.1 M, pH 5). According to our previous study, the ratio of biomass (spinach pulp) to buffer was set at 17.5 (Mazzocchi et al., 2023). In order to have a more representative starting vegetable material (with a more representative average concentration of target compounds), three different batches of spinach (1 kg each) were combined before the enzymatic extraction. Experimental trials were replicated three times separately.

2.3 | Effect of the biocatalyst dose

As reported in our previous study (Mazzocchi et al., 2023), using the polysaccharide cell wall composition of the vegetable, a tailor-made enzyme blend containing cellulase (40%), xylanase (41%), and polygalacturonase (19%) was formulated. The homogenized spinach, resuspended in McIlvaine buffer, was kept in a bench-scale stirred bioreactor (1 L, in the dark) by regulating the temperature (25°C) using an external water jacket and connected to a thermoregulator (MPM instrument, type M 900-TI). The treatment was carried out using 300 ppm of $ZnCl_2$, adding different total doses of biocatalyst: 0.10, 0.25, 0.50, 1.0, 3.0, 6.0, 10, 30, and 50 U/g at various extraction times (1, 3, 6, 16, 24, 36, and 48 h). A control sample (Ctrl) was prepared without the addition of the enzyme mixture. For the experimental plan (Table 1), three batch replicates were conducted for each sample.

2.4 | Effect of zinc chloride

In order to verify the effect of the zinc chloride during the EAE of Chl from spinach, different $ZnCl_2$ concentrations were tested (50, 100, 150, 200, and 300 ppm), according to the study of Özkan and Bilek (2015). The procedure was done at 25°C for 3 h by applying the lowest tested enzyme dose (0.10 U/g) and an intermediate biocatalyst dose (1.0 U/g). A reference sample (Ref = 0 ppm $ZnCl_2$) was made without adding $ZnCl_2$. Three experimental replicates were conducted.

2.5 | Chlorophyll content and colorimetric characterization

After enzymatic extractions, the obtained pulp was centrifuged (Heraeus Megafuge 16R centrifuge, Thermo Scientific) for 5 min at 4500 rpm (10°C), and the clear supernatant was removed. Afterward, 10 mL of absolute ethanol was used to solubilize 1 g of pellet, and the obtained solution was centrifuged (under the same conditions previously used). Absorbance analysis (λ: 230–700 nm, UV–visible, Shimadzu UV 2450) was carried out on the recovered supernatant using a 1 mm quartz cuvette (Hellma). The amount of Chl a, Chl b, and total Chl was determined through the following equation with some modifications (Lichtenthaler, 1987; Özkan & Bilek, 2015):

$$\text{Chl a}\ (\mu g/g) = (13.36 \times A_{664} - 5.19 \times A_{649}) \times \text{DF} \quad (1)$$

$$\text{Chl b}\ (\mu g/g) = (27.43 \times A_{649} - 8.12 \times A_{664}) \times \text{DF} \quad (2)$$

$$\text{Tot Chl}\ (\mu g/g) = \text{Chl a} + \text{Chl b} \quad (3)$$

where A_{664} is absorbance at λ 664 nm; A_{649} is absorbance at λ 649 nm; DF is dilution factor.

Extraction yield was estimated as the ratio between total Chl (μg/g) and biocatalyst dose (U/g):

$$\text{Yield}\ (\mu g/U) = \text{Tot Chl}\ (\mu g/g)\ /\text{biocatalyst dose}\ (U/g) \quad (4)$$

The green extract was calorimetrically characterized using a CR-5 colorimeter (Konica Minolta) with the CIELa^*b^* system (illuminant D65 and visual angle of 10°). Three analytical replicates were conducted for each sample (five measurements for each sample unit). Moreover, two derived color parameters were determined: hue angle (h°) and chroma (C^*) (Lombardelli et al., 2021; Mazzocchi et al., 2023):

$$h° = 180° + (\arctan\ b*/a*) \quad (5)$$

$$C^* = \left(a^{*2} + b^{*2}\right)^{0.5} \quad (6)$$

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2.6 | Mathematical model for chlorophyll extraction process

As reported by Nguyen et al. (2021), the second-order model has been proven to be appropriate for describing the extraction process of active compounds from plants. It has also been successfully applied for pigment recovery by microwaves (Nguyen et al., 2021) and pulsed electric fields (Zhang et al., 2020). The second-order equation applied for Chl recovery by EAE is

$$C = \frac{C_\infty^2\, kt}{1 + C_\infty\, kt} \tag{7}$$

where C_∞ is the saturated concentration (μg/g), and k is the second-order extraction rate constant (g/(μg min)) calculated by linear regression.

2.7 | Statistical analysis

Data were analyzed using (*i*) two-way analysis of variance (ANOVA) to verify the effect of the enzyme dose and treatment time and their interaction on the colorimetric parameters ($p < 0.01$); and (ii) one-way ANOVA to test for significant differences between enzyme dose and process time. When significance was reached, Tukey's honestly significant difference (HSD) test ($p < 0.05$) was applied (EXCEL® Add-in macro DSAASTAT). A Pearson correlation analysis ($p < 0.01$) was performed between the k values (second-order kinetic constant) and the different enzyme doses (0.1–50 U/g) computed with GraphPad Prism 5.0 (GraphPad Software, Inc.).

3 | RESULTS AND DISCUSSION

3.1 | Chlorophyll kinetics during enzyme-assisted extraction: effect of the biocatalyst dose

The changes in Chl a, Chl b, and total Chl concentration (μg/g) during the EAE from spinach leaves under different conditions (e.g., enzymatic dose and extraction time) are shown in Figure 1. In all trials, the content of recovered Chl was dose-dependent and increased remarkably as the amount of biocatalyst increased (Figure 1, Table S1). Irrespective of the biocatalyst dose, the concentration of Chl recovered by EAE was always greater as compared to that without enzyme (Ctrl). Özkan and Bilek (2015) observed a 39% increase in Zn–Chl yield by applying EAE with respect to solvent extraction alone. It is interesting to highlight that the Chl a/b ratio was approximately 2.7, in line with those reported in the literature (Indrasti et al., 2018), and remained constant as the enzyme dose changed.

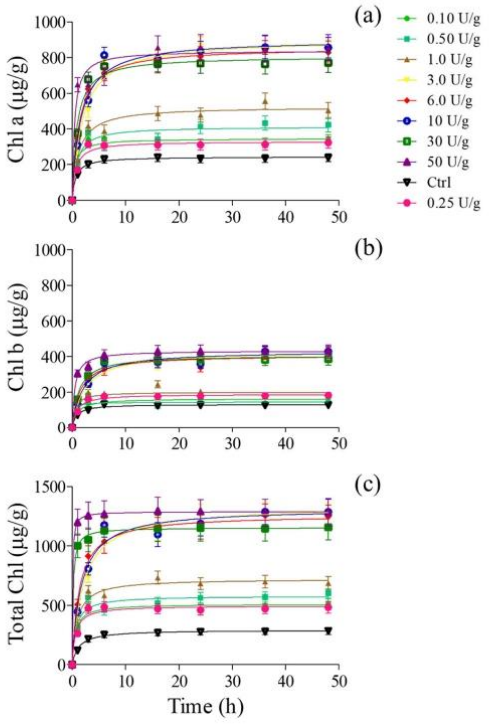

FIGURE 1 Extraction kinetics of (a) chlorophyll (Chl) a, (b) Chl b, and (c) total Chl (Chl a + Chl b), during enzyme-assisted extraction (EAE) from spinach, at different enzyme doses (0.1–50 U/g) and extraction times (1–48 h). Recovery of Chl (μg/g) was performed at 25°C and 300 ppm of $ZnCl_2$. Ctrl is the control sample (treatment without enzyme). Reported values are mean ± 95% confidence interval of triplicate measurements.

The results in Figure 1a, b (Table S1) suggest the Chl (a and b) extraction curves followed a hyperbolic behavior and were consistent with the ideal two-phase extraction kinetic of plant pigment. Indeed, it is widely recognized that the extraction process involves two phases (Nguyen et al., 2021). The first phase (faster) is characterized by the hydrolytic degradation of cellular structures thanks to the action of enzymes, subsequently followed by the dissolution of the soluble constituents in the solvent. The second stage consists in the external diffusion of the soluble components through the porous structures and, therefore, in their solubilization (Goula et al., 2017). As depicted in Figure 1a,b, the greater part of Chl a and Chl b was quickly recovered during the first hour of extraction only at the highest enzyme dose (30 and 50 U/g). A longer time (3 h)

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TABLE 2 Second-order kinetic constant (k, g/(μg min)) and R^2 values obtained for chlorophyll (Chl) extracted at different enzyme doses (0.1–50 U/g) and times (1–48 h).

Dose (U/g)	k (g/(μg min))	R^2
0.10	5.43×10^{-4a}	0.97
0.25	5.51×10^{-4a}	0.98
0.50	4.70×10^{-4b}	0.97
1.0	3.88×10^{-4c}	0.99
3.0	2.08×10^{-4c}	0.98
6.0	2.04×10^{-4c}	0.98
10	1.62×10^{-4d}	0.97
30	1.68×10^{-4d}	0.98
50	1.63×10^{-4d}	0.98

Note: Statistical analysis of data was performed by one-way analysis of variance (ANOVA). Values with different small letters (a–d) differ significantly (Tukey's test, $p = 0.05$) according to enzyme dose.

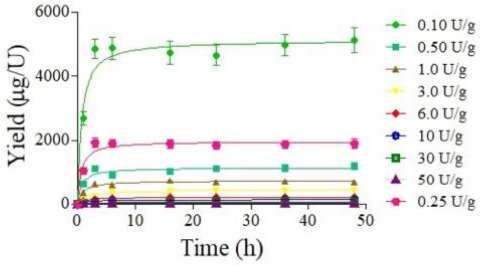

FIGURE 2 Extraction yield (μg/U) of total chlorophyll (Chl). Treatments were carried out using different enzymatic doses (0.1–50 U/g) for various process times (1–48 h) at 25°C and 300 ppm of $ZnCl_2$. Reported values are mean ± 95% confidence interval of triplicate measurements.

was required to achieve the maximum Chl amount when a lower dose of biocatalyst was used (0.10–1.0 U/g) (Figure 1). In all trials, no remarkable increase in pigment content was revealed over extended times (>3 h), suggesting the solid–liquid system reached the saturation state (Table S1).

Similarly, the concentration of total Chl followed a comparable behavior (Figure 1c), and the greatest value (about 1200 μg/g) was recovered after 1 h of extraction at the dose of 50 U/g. At the lowest dose (0.10 U/g), 490 μg/g of Chl were extracted after 3 h of treatment (Figure 1c, Table S1). In any case, the suggested biocatalytic approach, based on a tailored enzymatic mix, turned out to be more effective in the recovery of pigment than commercial standard preparation (50.747 mg_{TCC}/100 $g_{spinach\ pulp}$, at 45°C, Özkan & Bilek, 2015), also considering the low process temperature applied (25°C).

Similar results have been reported for Chl solvent extraction (methanol: 51 $mg/100g_{fresh\ spinach}$, acetone: 90 $mg/100g_{fresh\ spinach}$) (Khalyfa et al., 1992).

To develop a kinetic model for predicting the pigment extraction behavior, the rate of Chl recovery was expressed by a second-order extraction rate constant (k). The determination coefficients ($R^2 = 0.97$–0.99) in Table 2 suggest the model was adequate for fitting the experimental data. Indeed, the second-order model has been proven to be appropriate for describing the extraction process of active compounds from various plants (Nguyen et al., 2021; Zhang et al., 2020). Significantly, greater k values (5.43×10^{-4} and 5.51×10^{-4} g/(μg min)) were obtained for Chl recovered by EAE from spinach leaves at 0.10 and 0.25 U/g (Table 2). At higher enzyme doses (10–50 U/g), k decreased by approximately three times (1.68×10^{-4} and 1.62×10^{-4} g/(mg min)), proving the loss of process efficiency. As a confirmation of this, a correlational analysis between the k values and the different enzyme doses (0.1–50 U/g) was conducted. The value of the Pearson correlation coefficient ($r = -0.618$, $p < 0.01$) suggests a moderate negative correlation.

Frequently, in order to guarantee an efficient process, a high extraction yield is essential, even if this does not necessarily guarantee a high concentration of molecules with high added value (Aspé & Fernández, 2011). Therefore, in this study, attention has been given to the enzymatic extraction yield, calculated as the ratio between the pigment concentration (μg/g) and the enzymatic dose (U/g). Even though this trend followed a hyperbolic behavior (Figure 2) resembling that previously described, the highest extraction yield (about 4860 μg/U) was revealed using the lowest enzyme dose (0.10 U/g) after 3 h of extraction. At 50 U/g, the extraction yield significantly diminished (about 30 μg/U) after 1 h, reaching a plateau (Table S2). The amount of Chl recovered by the tailored enzymatic mix at low temperature (25°C) was in line with the results found by Ahmadi et al. (2022) and Mehdipoor Damiri et al. (2021), who applied a commercial pectolytic preparation (PectineX Ultra SP L) for the recovery of Chl from alfalfa and clover under different extraction conditions (40°C, in the absence of bivalent ions able to preserve the pigment structure). In addition to determine the concentration and yield of pigment, it is essential to take into account the quality of the green color obtained through the CIELa^*b^* parameters (L^*, a^*, b^*) and the color indices (C^*, $h°$) (Mazzocchi et al., 2023). As is well documented, the evaluation of greenness is mainly expressed by the parameter a^* (Özkan & Bilek, 2015; Senklang & Anprung, 2010). The values of L^*, a^*, b^*, $h°$, and C^* and the corresponding resulting color of Chl-based extracts are summarized in Table 3. All samples obtained by EAE were characterized by a significantly more intense greenness than Ctrl.

Irrespective of the enzyme dose, the lowest a^* values (<−35, nuance toward green) were obtained at the

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TABLE 3 Visual color attributes [a^*, b^*, L^*, hue (h°), chroma value (C^*)] of the chlorophyll (Chl)-based extracts.

Dose (U/g)	Time (h)	L^*	a^*	b^*	C^*	h°	Simulated color
0.10 U/g	**1**	$71.83^{b,A}$	$-39.94^{ab,B}$	$96.56^{ab,A}$	$104.5^{a,A}$	$112.5^{ef,AB}$	
	3	$49.13^{e,D}$	$-44.77^{a,A}$	$84.55^{ab,AB}$	$93.0^{ab,BC}$	$114.6^{bc,A}$	
	6	$63.72^{d,B}$	$-33.19^{cd,C}$	$89.88^{abc,AB}$	$95.8^{abc,B}$	$110.3^{bc,B}$	
	16	$58.15^{b,C}$	$-16.51^{de,F}$	$86.67^{ab,AB}$	$88.2^{ab,C}$	$100.8^{f,D}$	
	24	$50.76^{d,D}$	$-13.56^{d,F}$	$79.65^{ab,B}$	$80.8^{b,D}$	$99.7^{bc,D}$	
	36	$63.15^{c,B}$	$-20.91^{b,E}$	$84.01^{a,AB}$	$86.6^{ab,C}$	$104.0^{b,C}$	
	48	$33.91^{c,E}$	$-25.05^{a,D}$	$54.66^{de,C}$	$60.1^{d,E}$	$114.6^{a,A}$	
0.25 U/g	**1**	$80.32^{a,A}$	$-34.64^{cd,B}$	$98.67^{a,A}$	$104.6^{a,A}$	$109.3^{f,BC}$	
	3	$69.41^{b,B}$	$-41.68^{b,A}$	$93.58^{a,AB}$	$102.4^{a,AB}$	$114.0^{cd,A}$	
	6	$75.53^{a,AB}$	$-35.64^{c,B}$	$95.51^{ab,A}$	$101.9^{a,AB}$	$110.5^{bc,B}$	
	16	$77.45^{a,A}$	$-28.45^{ab,C}$	$87.16^{ab,ABC}$	$91.7^{a,BC}$	$108^{abc,BC}$	
	24	$79.88^{a,A}$	$-23.89^{b,D}$	$80.37^{ab,BC}$	$83.8^{b,CD}$	$106.6^{a,CD}$	
	36	$70.11^{b,B}$	$-20.48^{b,E}$	$77.89^{ab,C}$	$80.5^{bc,CD}$	$104.7^{ab,D}$	
	48	$70.85^{a,B}$	$-20.01^{b,E}$	$75.95^{ab,C}$	$78.5^{ab,D}$	$104.8^{c,D}$	
0.50 U/g	**1**	$65.51^{cd,A}$	$-41.27^{a,A}$	$89.65^{ab,A}$	$98.7^{b,A}$	$114.7^{de,A}$	
	3	$67.92^{b,A}$	$-35.09^{cd,B}$	$92.43^{a,A}$	$98.9^{ab,A}$	$110.8^{de,B}$	
	6	$69.83^{bc,A}$	$-39.47^{b,A}$	$91.42^{abc,A}$	$99.6^{ab,A}$	$113.4^{ab,AB}$	
	16	$67.86^{b,A}$	$-21.21^{c,C}$	$86.81^{ab,AB}$	$89.4^{ab,B}$	$103.7^{ef,C}$	
	24	$52.9^{d,B}$	$-16.29^{c,D}$	$80.01^{ab,B}$	$81.7^{b,C}$	$101.5^{b,C}$	
	36	$43.93^{e,C}$	$-5.87^{f,E}$	$70.49^{bc,C}$	$70.7^{cd,D}$	$94.8^{ef,D}$	
	48	$44.09^{b,C}$	$-23.69^{a,C}$	$59.85^{cd,D}$	$64.4^{cd,E}$	$111.6^{b,B}$	
1.0 U/g	**1**	$61.38^{def,A}$	$-37.43^{abc,A}$	$86.32^{ab,A}$	$94.1^{bc,A}$	$113.4^{de,A}$	
	3	$55.75^{cd,B}$	$-31.7^{de,B}$	$83.31^{ab,B}$	$89.1^{b,B}$	$110.8^{de,B}$	
	6	$52.98^{e,BC}$	$-25.5^{fg,C}$	$80.45^{cd,C}$	$84.4^{cd,C}$	$107.6^{c,C}$	
	16	$49.20^{f,CD}$	$-11.46^{f,D}$	$77.03^{b,CD}$	$77.9^{b,D}$	$98.5^{f,D}$	
	24	$49.86^{de,CD}$	$-2.66^{h,F}$	$74.25^{abc,D}$	$74.3^{c,D}$	$92.1^{d,F}$	
	36	$43.65^{e,E}$	$-1.96^{g,F}$	$60.94^{cd,F}$	$61.0^{de,F}$	$91.8^{f,F}$	
	48	$46.07^{b,DE}$	$-6.82^{ef,E}$	$69.81^{bc,E}$	$70.1^{bc,E}$	$95.6^{de,E}$	
3.0 U/g	**1**	$60.6^{def,B}$	$-40.62^{ab,B}$	85.93^{ab}	97.8^{b}	$118.5^{bc,A}$	
	3	$60.4^{c,B}$	$-44.74^{a,A}$	85.59^{ab}	96.6^{ab}	$117.6^{ab,A}$	
	6	$63.78^{d,AB}$	$-44.13^{a,AB}$	88.6^{abc}	99.0^{ab}	$116.5^{a,A}$	
	16	$69.02^{b,A}$	$-30.26^{a,C}$	87.5^{ab}	92.6^{a}	$109.1^{a,B}$	
	24	$68.45^{b,A}$	$-25.98^{a,D}$	86.4^{a}	90.2^{a}	$106.7^{a,B}$	
	36	$66.55^{bc,A}$	$-26.25^{a,D}$	85.9^{a}	89.8^{ab}	$107.0^{a,B}$	
	48	$67.98^{a,A}$	$-20.15^{b,E}$	85.1^{a}	87.5^{a}	$103.3^{c,C}$	
6.0 U/g	**1**	$42.18^{g,B}$	$-40.32^{ab,A}$	$65.4^{c,B}$	$80.1^{c,B}$	$125.3^{a,A}$	
	3	$50.85^{de,A}$	$-41.53^{ab,A}$	$76.06^{c,A}$	$86.7^{b,A}$	$118.6^{a,B}$	
	6	$41.21^{fg,B}$	$-24.33^{fg,B}$	$65.95^{de,B}$	$70.3^{e,C}$	$110.2^{bc,C}$	
	16	$34.96^{g,C}$	$-18.78^{cd,C}$	$56.61^{d,BC}$	$59.6^{c,D}$	$108.4^{a,C}$	
	24	$31.7^{f,D}$	$-11.27^{e,D}$	$53.23^{d,CD}$	$54.4^{de,E}$	$102.0^{ab,D}$	
	36	$28.81^{g,DE}$	$-3.47^{g,E}$	$48.02^{e,CD}$	$48.1^{f,F}$	$94.1^{f,E}$	
	48	$28.08^{d,E}$	$-3.55^{g,E}$	$46.22^{e,D}$	$46.4^{e,F}$	$94.4^{e,E}$	
10 U/g	**1**	$56.42^{f,A}$	$-40.93^{ab,A}$	$82.2^{b,A}$	$94.2^{bc,A}$	$119.2^{b,A}$	
	3	$56.95^{c,A}$	$-38.99^{bc,A}$	$82.78^{ab,A}$	$91.5^{ab,A}$	$115.2^{bc,B}$	
	6	$36.94^{g,B}$	$-22.19^{g,B}$	$60.14^{e,B}$	$64.1^{f,B}$	$110.3^{bc,C}$	
	16	$34.21^{g,B}$	$-15.09^{e,C}$	$56.29^{d,BC}$	$58.3^{c,C}$	$105.0^{b,D}$	
	24	$30.69^{f,C}$	$-9.02^{f,D}$	$52.34^{d,BCD}$	$53.1^{de,D}$	$99.8^{b,E}$	

(Continues)

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TABLE 3 (Continued)

Dose (U/g)	Time (h)	L^*	a^*	b^*	C^*	h°	Simulated color
	36	$28.56^{g,D}$	$-6.38^{ef,E}$	$47.9^{e,CD}$	$48.3^{f,E}$	$97.6^{de,E}$	
	48	$28.21^{d,D}$	$-6.02^{f,E}$	$45.52^{e,D}$	$45.9^{e,E}$	$97.5^{de,E}$	
30 U/g	**1**	$62.61^{de,BC}$	$-41.74^{a,A}$	$86.39^{ab,A}$	$95.9^{b,A}$	$115.8^{cd,A}$	
	3	$58.63^{c,C}$	$-38.93^{bc,A}$	$85.47^{ab,A}$	$93.9^{ab,A}$	$114.5^{bc,A}$	
	6	$67.71^{cd,A}$	$-32.06^{cd,B}$	$81.71^{bcd,AB}$	$87.8^{bc,B}$	$111.4^{b,B}$	
	16	$65.84^{bc,AB}$	$-20.25^{c,C}$	$76.87^{bc,BC}$	$79.5^{b,C}$	$104.8^{b,C}$	
	24	$52.03^{d,D}$	$-14.67^{cd,D}$	$72.32^{bc,BCD}$	$73.8^{c,D}$	$101.5^{b,DE}$	
	36	$49.25^{d,DE}$	$-12.19^{c,D}$	$67.88^{bcd,CD}$	$69.0^{d,E}$	$100.2^{cd,E}$	
	48	$45.60^{b,E}$	$-15.04^{c,D}$	$62.44^{cd,D}$	$64.2^{cd,F}$	$103.5^{c,CD}$	
50 U/g	**1**	$59.36^{ef,A}$	$-36.78^{bc,A}$	$85.01^{ab,A}$	$92.6^{bc,A}$	$113.4^{de,A}$	
	3	$56.9^{c,B}$	$-34.81^{cd,A}$	$84.27^{ab,A}$	$91.2^{ab,A}$	$112.4^{cd,A}$	
	6	$44.9^{f,C}$	$-27.54^{ef,B}$	$68.01^{de,B}$	$73.4^{de,B}$	$112.0^{b,A}$	
	16	$52.47^{ef,B}$	$-17.17^{de,C}$	$63.37^{cd,C}$	$65.7^{c,C}$	$105.2^{b,B}$	
	24	$45.77^{e,C}$	$-11.32^{e,DE}$	$66.2^{c,B}$	$67.2^{d,C}$	$99.7^{bc,C}$	
	36	$34.52^{f,D}$	$-9.37^{d,E}$	$57.61^{de,D}$	$58.4^{ef,D}$	$99.2^{de,C}$	
	48	$33.75^{c,D}$	$-12.89^{d,D}$	$54.93^{de,E}$	$56.4^{d,D}$	$103.2^{c,B}$	
Ctrl	**1**	$70.16^{bc,C}$	$-30.61^{d,A}$	$94.78^{ab,A}$	$98.6^{b,A}$	$101.1^{g,C}$	
	3	$80.01^{a,A}$	$-30.21^{e,A}$	$90.28^{a,B}$	$95.2^{ab,A}$	$108.5^{e,A}$	
	6	$73.04^{ab,BC}$	$-30.1^{de,A}$	$96.71^{a,A}$	$99.3^{ab,A}$	$107.3^{c,A}$	
	16	$61.23^{cd,D}$	$-26.61^{b,B}$	$94.78^{a,A}$	$98.4^{a,A}$	$105.7^{b,AB}$	
	24	$62.58^{c,D}$	$-6.72^{g,C}$	64.78^{cd}	$65.1^{e,D}$	$95.9^{d,D}$	
	36	$78.25^{a,A}$	$-7.68^{e,C}$	$89.85^{a,B}$	$92.2^{a,B}$	$103.0^{bc,BC}$	
	48	$70.23^{a,C}$	$-8.52^{e,C}$	$69.85^{bc,C}$	$70.4^{bc,C}$	$97.0^{de,D}$	

Note: Statistical analysis of data was performed by two-way analysis of variance (ANOVA). Treatments were carried out using different enzymatic doses (0.10–50 U/g) for various process times (1–48 h) at 25°C and 300 ppm of $ZnCl_2$. Values with different capital letters (A–F) differ significantly (Tukey's test, $p = 0.05$) according to process time. Values with different small letters (a–f) differ significantly (Tukey's test, $p = 0.05$) according to enzyme dose.

shortest extraction times (1 or 3 h). As regards the colorimetric parameters C (color saturation) and h° (hue), they showed the same trend as the parameter a^*, given that they are mainly determined by its value (Table 3).

3.2 | Chlorophyll kinetics during enzyme-assisted extraction: effect of zinc chloride

The data available in the literature report that using a Zn^{2+} concentration of 300 ppm, it is possible to obtain zinc–Chl complexes from spinach pulp for the production of stable food coloring (Özkan & Bilek, 2015). In this study, we investigated the possibility of preserving the color stability of Chl-based extracts by employing a lower amount of $ZnCl_2$ during EAE.

Figure 3a,b illustrates the effect of $ZnCl_2$ addition on the formation of Zn–Chl derivatives at the lowest tested enzyme dose (0.10 U/g) and at an intermediate dose (1.0 U/g). The total amount of recovered Chl increased as Zn^{2+} concentrations increased, reaching the maximum level at 150 ppm of $ZnCl_2$, both at 0.10 and 1.0 U/g. No significant variations were observed at higher levels (150–300 ppm, Figure 3a). A similar trend was observed in terms of extraction yield (Figure 3b) (Table S3).

Considering the effect of $ZnCl_2$ concentration on Chl a and Chl b, its protective action was more evident toward Chl a (Figure 4). In detail, at 150 ppm, an increased amount in comparison to the reference sample (Ref = 0 ppm $ZnCl_2$) of about 2.5 and 1.5 times was revealed for Chl a and Chl b, respectively. These results appeared relevant given that Chl a is the least stable form of pigment, as described in literature. Indrasti et al. (2018) reported that Chl a degrades 2.5 times faster than Chl b due to heat, pH, and oxidation phenomena.

Colorimetric data (Table 4) show that the use of a high level of Zn^{2+} significantly improved the greenness ($-a^*$ value) of the Chl-based extracts. The $-a^*$ value reached the maximum level at 150 ppm, and no remarkably color value differences were observed between 150 and 300 ppm when compared to the reference sample (Ref = 0 ppm $ZnCl_2$). The protective effect of Zn^{2+} was more evident at the lowest enzyme dose (0.10 U/g). Accordingly, C^* and

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TABLE 4 Visual color attributes [a^*, b^*, L^*, hue ($h°$), chroma value (C^*)] of the chlorophyll (Chl)-based extracts.

Dose (U/g)	$ZnCl_2$ (ppm)	L^*	a^*	b^*	C^*	$h°$	Simulated color
0.10	0	53.92^{bc}	-33.58^{d}	80.14^{ab}	80.1^{cd}	114.5^{ab}	
	50	59.36^{ab}	-30.31^{e}	86.49^{a}	81.6^{c}	109.3^{b}	
	100	49.59^{c}	-39.66^{b}	74.76^{b}	86.6^{b}	120.3^{a}	
	150	66.08^{a}	-41.21^{a}	71.28^{bc}	91.8^{a}	119.4^{a}	
	200	40.99^{d}	-39.97^{b}	64.34^{c}	75.7^{d}	121.8^{a}	
	300	47.32^{cd}	-34.86^{c}	73.31^{bc}	81.2^{c}	115.4^{ab}	
1.0	0	54.98^{ab}	-34.88^{b}	80.35^{ab}	80.6^{b}	116.3	
	50	54.88^{ab}	-34.97^{b}	81.92^{ab}	89.1^{ab}	113.1	
	100	58.94^{ab}	-40.52^{a}	84.89^{a}	94.1^{a}	115.5	
	150	46.48^{b}	-35.58^{ab}	71.42^{b}	79.8^{b}	116.5	
	200	61.54^{a}	-37.23^{ab}	87.57^{a}	95.2^{a}	113.0	
	300	65.36^{a}	-37.79^{ab}	90.16^{a}	97.8^{a}	112.7	

Note: Statistical analysis of data was performed by one-way analysis of variance (ANOVA). Treatments were carried out using different enzyme doses (0.10 or 1.0 U/g) for 3 h at 25°C. Values with different small letters (a–e) differ significantly (Tukey's test, $p = 0.05$) according to $ZnCl_2$ amount.

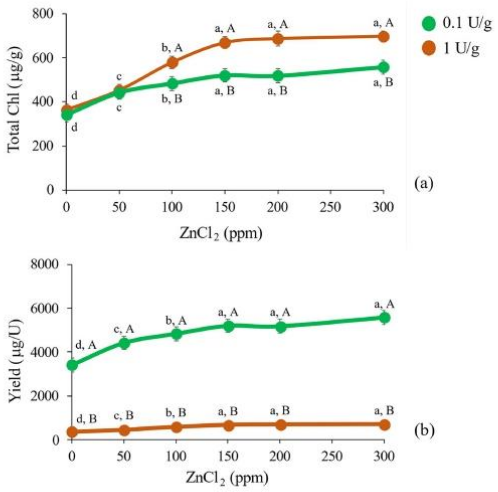

FIGURE 3 Effect of the $ZnCl_2$ concentration (ppm) on (a) total chlorophyll (Chl) (µg/g) and (b) extraction yield (µg/U) during enzyme-assisted extraction (EAE). Treatments were carried out for 3 h using different enzymatic doses (0.10 and 1.0 U/g) at 25°C. Reported values are mean ± 95% confidence interval of triplicate measurements. Statistical analysis of data was performed by two-way analysis of variance (ANOVA). Values with different letters (a–d) differ significantly (Tukey's test, $p = 0.05$) according to $ZnCl_2$ amount (ppm). Values with different capital letters (A–B) differ significantly (Tukey's test, $p = 0.05$) according to enzyme dose.

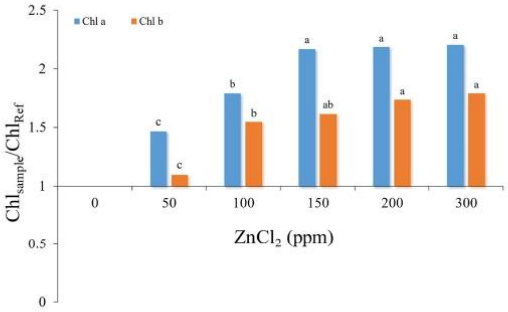

FIGURE 4 Ratio between chlorophyll (Chl) amount (µg/g) in enzyme-assisted extraction (EAE) sample (0.10 U/g) and reference sample (Ref = 0 ppm $ZnCl_2$) in the presence of different $ZnCl_2$ concentrations (ppm). Values with different letters (a–c) differ significantly (Tukey's test, $p = 0.05$) according to $ZnCl_2$ amount (ppm).

$h°$ parameters followed the same trend of the a^* value (Table 4). Overall, our data suggested that 150 ppm $ZnCl_2$ was enough to ensure the formation of Zn–Chl complexes during the EAE, employing low enzyme dose at mild condition (25°C). Considering the extraction kinetics, the recovery yield and the colorimetric data, the most suitable conditions for a tailored enzymatic extraction to produce green Chl-based extracts are as follows: 0.10 U/g of enzyme dose, 3 h, and 150 ppm of $ZnCl_2$ at 25°C.

4 | CONCLUSION

The idea behind the present study was to apply a tailored enzyme mix *ad hoc* formulated considering the vegetable composition of the matrix cell wall, first affected by the vegetative organs (fruits, tubers, or leaves) and, second, by the vegetable species. Our results proved that the

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biocatalyst dose significantly affected the recovered yield of Chl as well as the quality of greenness. At mild conditions (25°C), the highest Chl extraction yield and the best quality of green were reached using the lowest enzyme dose (0.10 U/g) for short processing time (1–3 h). The protective effect of $ZnCl_2$ was more evident toward Chl a, already during the extraction process. An amount of 150 ppm was enough to ensure the formation of green stable Zn–Chl complexes employing low enzyme dose and short extraction time. Our tailored and low-temperature (25°C) approach clearly appeared more effective than those based on the use of unspecific commercial enzymes (45°C), both in terms of recovered pigment and yield per enzyme unit.

The method developed for spinach is clearly applicable to other green leafy vegetables, as long as the polysaccharide composition of the pigment-holding cell wall is determined in order to optimize the *ad hoc* enzymatic formulation. Furthermore, the kinetic study applied in the present study may be helpful for the extraction of other pigments from different vegetal sources.

AUTHOR CONTRIBUTIONS

Claudio Lombardelli and **Caterina Mazzocchi**: Conceptualization; methodology; formal analysis; investigation; resources; data curation; writing—original draft; visualization. **Ilaria Benucci**: Conceptualization; methodology; validation; data curation; writing—review and editing; visualization; supervision; funding acquisition. **Marco Esti**: Conceptualization; validation; data curation; writing—review and editing; supervision; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Metallo-chlorophyll derivatives as food colorant: is it possible to recover intact chloroplasts from spinach leaf by Enzyme-Assisted Extraction?

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Highlights

- Enzyme Assisted Extraction and Cu^{2+}/Zn^{2+} used for recovering intact chloroplasts
- Metal ions preserved the greenness of chlorophyll (Chl) into intact chloroplasts
- Green meringues were prepared using Chl-based colorant

1 **Metallo-chlorophyll derivatives as food colorant: is it possible to recover intact chloroplasts**
2 **from spinach leaf by Enzyme-Assisted Extraction?**

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Abstract

The green color is highly desired in food industry, as well as in the new emerging scientific discipline called “molecular gastronomy”, since it provides an indication of freshness. The commercial green dyes (E140 and E141) consist of chlorophyll derivatives extracted from natural sources by organic solvent. This study proposes a novel, mild, and tailored approach for the recovery of intact chloroplasts from spinach leaves by Enzyme-Assisted Extraction (EAE) in the presence of metal ions (Cu^{2+} and Zn^{2+}) in a synergistic combination.

The extraction process is optimized through response surface methodology, considering variables such as extraction time and metal ions concentration. Results show that EAE, combined with metal ions, produces a higher yield of intact chloroplasts compared to traditional organic solvent extraction. Furthermore, the presence of metal ions significantly improves Chl stability, preserving its greenness during extraction. The stability of the Chl extract is confirmed by colorimetric analysis, which reveals a strong correlation between metal ions concentration and the intensity of the green color. When applied to a food matrix, such as meringues, the Chl-based vegetal extract enhances the green color without adversely affecting the product's organoleptic quality if the concentration is less than 3% w/w.

Keywords: Food Pigment; Green colorant; Copper; Zinc; Meringues

1. Introduction

Chlorophyll (Chl), the vibrant green pigment found in plants and algae, plays a crucial role in photosynthesis and has garnered interest for its potential applications as food colorant and for its health benefits (Hsiao et al. 2020). The commercial Chl-based colorants are usually obtained by organic solvent extraction (Viera et al., 2018).

Chl stability is often compromised by heat, oxygen, light, and acidic conditions, leading to color changes and degradation (Indrasti et al. 2018), due to its structural characteristics. When Chl loses its magnesium ion (Mg^{2+}), undergoes a pheophytinization reaction and it is transformed into pheophytin, a derivative that appears olive-green (Amin et al. 2021). This transformation is heavily influenced by environmental factors such as pH and temperature (Nabi et al., 2023, Lombardelli et al., 2024). In nature, Chl is stored inside chloroplasts, organelles found in plant, that protect and stabilize the pigment (Staehelin, 2003; Indrasti et al. 2018; Li et al., 2021). Indeed, Chl is bound to proteins in a protein-Chl complex, surrounded by a lipidic bilayer, which helps shield it from harmful external factors. This structure allows Chl to perform its function without undergoing rapid degradation (Miazek & Ledakowicz, 2013). When plant cell structures are damaged, the release of Chl leads to an increased susceptibility to degradation (Indrasti et al. 2018). Given the challenges posed by Chl sensitivity, several strategies have been employed to enhance its stability during the extraction phase (Hu et al. 2022): (i) inhibition of enzyme activity, high-temperature blanching can effectively inhibit the activity of Chl-degrading enzymes, although this process may also lead to nutrient loss and accelerate the demagnetization of Chl (Managa et al. 2020); (ii) alkalization, alkaline conditions favor the preservation of Chl, although this effect may diminish over time (Kwartiningsih et al. 2021); (iii) ion replacement, substitution of the Mg^{2+} ion with more stable bivalent cations, such as zinc (Zn^{2+}) or copper (Cu^{2+}), leading to metal-Chl complexes (Amin et al., 2021). These derivatives retain the green color while being more resistant to heat and acidic conditions (Amin et al. 2021). Notably, Cu^{2+} not only stabilizes Chl but also exhibits protective effects against degradation. Research has shown that the addition of Cu^{2+} may enhance the retention of Chl pigments during processing, helping to

maintain color and nutritional quality (Indrasti et al. 2018). Research by Zheng et al. (2014) demonstrated that adding Cu^{2+} to grape puree significantly improved Chl retention compared to Mg^{2+}, Zn^{2+}, and K^{+}. Moreover, considering that the tolerable upper intake limit for Zn^{2+}in adults is 40 mg/day, its addition in the Chl extract may represent a safe option besides enhancing Chl stability (Chasapis et al. 2020). Treatments with Zn^{2+} salts have been shown to form stable green Chl derivatives in various vegetables. Senklang and Anprung (2010) reported the formation of Zn-pheophytin in pandan leaves, enhancing their color and antioxidant properties. Mazzocchi et al. (2023) and Lombardelli et al. (2024) proved that the protective effect of $ZnCl_2$ (150 ppm) was more evident toward Chl-a rather than Chl-b, already during the Enzyme-Assisted Extraction (EAE) process from spinach. Both in the stabilization of pigments and in their use as dietary supplements, Zn^{2+} and Cu^{2+} play a crucial role. These trace elements, when combined, enhance the stability and vibrancy of pigments, as well as support essential physiological functions if taken as dietary supplements, ensuring a balanced intake for overall health. Indeed, daily intakes of Cu (0.07 mg/kg of body weight; EFSA, 2014) and Zn (0.18 mg/ kg of body weight; EFSA, 2023) are required to maintain a steady state because the body has no specialized storage system for these two micro-elements. Several studies have demonstrated that the ideal Zn:Cu ratio is between 8:1 to 15:1 (Osredkar & Sustar, 2011) and 4:1 to 12:1 (Watts, 2010). This balance is crucial for various physiological functions, including immune response, oxidative stress management, and enzyme function. Maintaining an optimal ratio ensures that both minerals can support their respective biological roles effectively, preventing deficiencies and potential health issues related to imbalances (Matuszczak et al., 2024; Escobedo-Monge et al., 2023).

Currently, despite extensive experiments focused on the extraction and quantification of Chl from vegetable sources, no single method that combines broad applicability, ease of reproduction, and high sensitivity has been identified. Bearing in mind several factors during Chl extraction and their interaction, the identification of a single procedure capable of efficiently recovery the pigment, also preserving its greenness, is quite complex (Berhe et al. 2024).

105 In light of these challenges, we propose a novel, mild, and tailored approach for the recovery of intact
106 chloroplasts from spinach leaves by EAE in the presence of metal ions (Zn^2 and Cu^{2+}) in a synergistic
107 combination. By keeping Chl within its natural cellular environment, this method may protect the
108 pigment from oxidation and degradation. Additionally, this extraction process explores the synergistic
109 effects of Cu^{2+} and Zn^{2+} to further enhance the Chl stability, providing a food green colorant to be
110 applied for staining a baked good usually white, such as the meringue.
111
112 **2.Materials and methods**
113 **2.1 Raw material, Enzymes and Chemicals**
114 Spinach (*Spinacia oleracea*) was provided by Unicoop Tirreno S.C. (Viterbo, Lazio, Italy) and kept
115 at 4 °C until use. Enzyme preparations were as follows: cellulase (0.8 U/mg) and polygalacturonase
116 (≥0.3 U/mg) from *Aspergillus niger* and xylanase (≥2500 units/mg) from *Aspergillus oryzae*, all
117 obtained from Merck (Milan, Italy). Additionally, all reagents, including acetone for solvent
118 extraction and chloroplast isolation kit were purchased from Merck (Milan, Italy).
119 **2.2 Extraction protocols**
120 Enzyme-Assisted Extraction (EAE). The EAE was performed following the method developed by
121 Lombardelli et al. (2024). Briefly, 25 g of spinach were blanched for 5 sec, blended and added to 440
122 mL of McIlvaine buffer (0.1 M, pH 5) containing the tailored enzyme mix (total dose 0.1 U/g
123 consisting of: cellulase 40%, xylanase 41% and pectinase 19%). The incubation time (1-3 hours) and
124 the total dose of metal ions (Cu^{2+}: Zn^{2+}, 1:10) were varied according to the experimental plan drawn
125 up by Design of Experiment (DOE, Table 1). The temperature was set at 25°C. At the end of the
126 incubation, the extract was centrifuged (4500 rpm, 10 min at 4°C, Heraeus Megafuge 16R Centrifuge,
127 Thermo Scientific, Milan, Italy) and the pellet subjected to analysis using a chloroplast isolation kit.
128 Solvent Extraction (SE). The SE was performed following the method developed by Ašimović et al.
129 (2016) using 80% (v/v) acetone as a solvent. The extraction was performed maintaining the same

130 solid-liquid ratio and the same operating conditions applied for EAE (Table 1). After centrifugation,
131 the pellet was used for chloroplast isolation kit.

132 **2.3 Optimization of the extraction process conditions**

133 In order to determine (i) the effect of extraction time and total metal ions dose on the Chl-containing
134 chloroplast and (ii) the best conditions to maximize the recovery of intact chloroplast, a DOE based
135 on a central composite design with the quadratic model was applied. Considering that the yield of
136 isolated chloroplasts is expressed on a unit Chl basis (mg of Chl/mL), the measured dependent
137 variable (y) was the concentration of Chl (mg/mL). The variance for each evaluated factor was
138 divided into linear, quadratic, and interactive components, and represented using the second-order
139 polynomial function as follows:

140 $$y = b_0+b_1x_1+b_2x_2+b_{11}x_1^2+b_{22}x_2^2+b_{12}x_1x_2 \quad (1)$$

141 The coefficients of the polynomial were represented by b_0 (constant term), b_1 and b_2 (linear
142 coefficient), b_{11} and b_{22} (quadratic coefficient) and b_{12} (interactive coefficient). The significance of all
143 the terms in the polynomial function was statistically assessed using the F-value at a probability (p)
144 of 0.001, 0.01 or 0.05. The regression coefficients were then used to generate contour plots. The
145 experimental design and statistical analysis were performed using Minitab 17.1 (Minitab Inc.,
146 Pennsylvania, USA). Response surface methodology (RSM) was used to verify the presence of
147 combined effect between the total metal ions dose and extraction time (independent variables). These
148 variables were coded as X1 and X2 respectively and each had three levels: −1, 0, and +1. A total of
149 18 combinations, which included three replicates of the center point, were carried out in random order
150 according to the central composite design. The level of variables for (x1 and x2) and (X1 and X2) are
151 shown in Table 1.

152 **2.4 Isolation of chloroplast**

153 The isolation of intact chloroplast was performed using the specific kit supplied by Merck (Milan,
154 Italy), following the procedure reported in the technical data sheet. Briefly, intact chloroplasts can be
155 separated from broken chloroplasts using the 40% Percoll® method. At the end of the procedure, a

156 suspension containing only intact chloroplasts was obtained. This suspension was used to determine
157 the chloroplast yield, expressed on a unit Chl basis (mg of Chl/mL). This requires the extraction of
158 the Chl from the chloroplast suspension with an organic solvent: 10 µL of the chloroplast suspension
159 was added to 1 mL of an 80% acetone solution and mixed. Then, sample was centrifuged for 2 minutes
160 at 3000 rpm, retaining the supernatant and reading the absorbance at 652 nm (A_{652}) (UV-visible,
161 Shimadzu UV 2450, Milan, Italy), using 80% acetone solution as reference blank. To obtain the mg
162 of Chl per mL of chloroplast suspension the following equation was applied:
163 $$mg_{chlorophyll}/mL = (A_{652} \times 100)/36 \quad (2)$$
164
165 **2.5 Meringue preparation**
166 The optimal extract, characterized by the highest Chl content and the most suitable colorimetric
167 parameters, was used in the preparation of a real food. Meringue, a sweet baked good typically white,
168 has been selected in order to better appreciate the coloring impact of the recovered green pigment.
169 Meringues were prepared by mixing 3 egg whites (100 mL) with 220 g of powdered sugar (added in
170 two steps) using electric whisks (Phillip, Milan, Italy) at speed 3 (600 rpm) for 10 min to obtain
171 soft/stiff peak batter structure. The prepared meringue batter was divided into 6 equivalent portions
172 and the appropriate dosage of freeze-dried colorant was added (0, 1, 2, 3, 5 and 7% w/w sugar basis)
173 and squeezed into waxed paper trays before baking. The baking temperature was adjusted to 75 °C
174 for 3 hours using an oven (Venticell, MMM Medcenter Einrichtungen GmbH, Planegg/München,
175 Germany). The cooked meringues (n. 150) were subjected to colorimetric and sensory analysis.
176
177 **2.6 Colorimetric determinations**
178 Color measurement of the extract and meringue was carry out using a CR-5 colorimeter (Konica
179 Minolta, Tokyo, Japan) by a D65 illuminant on the basis of L*, a*, and b* values, where L* value
180 indicates the lightness and its value ranges from 0 to 100, a* value gives the degree of the red-green
181 color, with a lower negative a* value suggesting more green (-a), and the b* value indicates the degree

of the yellow-blue color, with a higher positive b value suggesting more yellow (+b). The colorimeter was calibrated using a standard white and black plate. The ΔE (color difference) between samples was calculated by equation reported in Lombardelli et al. (2021).

2.7 Sensory Analysis

Meringue samples were analyzed for their organoleptic characteristics by a panel of 25 trained members (26 - 47 years of age, included 14 female and 11 male panelists) using a 5-point hedonic scale. The panelists scored different attributes, described each one to facilitate scoring (Table 2). The meringues were evaluated for their brightness, color homogeneity, green color intensity, smell (herbaceous), crumb color, internal color, texture, hardness, taste, herbaceous flavor and overall acceptability. The samples were placed on white plates and identified with random three-digit numbers. The panelists evaluated all samples in a testing area with good light condition and instructed to rinse their mouth with water between samples to minimize any residual effects.

2.8 Statistical Analysis

All trials were performed in triplicate, and results were expressed as the mean ± standard deviation. The mean comparisons were carried out using an analysis of variance (ANOVA) to find the effect of individual factors and their interaction on colorimetric parameters ($p < 0.01$). Tukey's post-hoc test (HSD) was also performed ($p < 0.05$) using EXCEL® extension DSAAS-TAT for multiple comparison between samples.

3. Results and Discussion

3.1 Chloroplast recovery by Enzyme-Assisted- and solvent extraction

The recovery yield of intact chloroplasts by EAE and SE, varying the total metal dose (0 - 165 ppm) and the extraction time (1-3 h), was investigated. From a first visual analysis, the difference between the two extraction methods was already evident. In fact, in Fig. 1 it is possible to distinguish broken

208 (top) and intact (bottom) chloroplasts; the latter seems to be more numerous in Fig. 1a, suggesting
209 EAE allowed the recovery of a greater amount of intact chloroplasts compared to SE (Fig. 1b).
210
211 **3.1.1 Extraction process optimization**
212 The effect of process variables (time and total metal dose) on the amount of Chl in intact chloroplasts
213 was evaluated by RSM. Table 1 shows the results of the full factorial design, and Table 3 displays the
214 coefficients of the mathematical model and statistical parameters, proving that the RSM developed
215 was adequate (Models p-value = 0.000). For the response variable considered (Chl concentration),
216 the R^2 values were equal to 0.96 for both extraction methods (EAE and SE), indicating that the
217 regression models were able to effectively explain how the variables, and their interactions affected
218 the response. Furthermore, the significance of each equation coefficient was determined using the p-
219 value (Table 3) (Mazzocchi et al., 2023). The analysis of variance was performed to determine the
220 significance of the linear, quadratic, and interaction effects of the independent variables (extraction
221 time and total metals dose) on the dependent variable (Chl concentration). Considering the EAE
222 process, both the extraction time and metal dose had a statistically significant linear effect (p-value <
223 0.001, Table 3), contributing to the recovery of intact chloroplasts. The quadratic effect was
224 significant (p-value <0.05) only for metal dose (Table 3). Otherwise, no interactions among the
225 variables were revealed (p-value > 0.05). The results allowed us to develop a useful equation to predict
226 the amount of intact chloroplasts recovered varying metal dose (X1) and extraction time (X2)
227 (Equation 3):

228 Chl = 0,2243 - 0,000342 Metals + 0,0656 Time + 0,000007 Metals*Metals + 0,0076 Time*Time
229 + 0,000125 Metals*Time (3)

230 Considering SE, only the linear and the quadratic terms of time were significant (p-value < 0.001),
231 and the equation has changed as follows:

232 Chl = 0,07911 + 0,01964 Time - 0,000009 Metals - 0,00831 Time*Time + 0,000000 Metals*Metals
233 - 0,000017 Time*Metals (4)

234 The interactions between the variables are depicted through the contour plots (Fig. 2). It is interesting
235 to note that for EAE, the greatest amount of recovered pigment and, therefore, the highest yield of
236 intact chloroplasts was obtained as the extraction time and metal dose increased (Fig. 2a). For the SE,
237 however, the yield of intact chloroplasts was greater as the extraction time decreased, regardless of
238 the metal dose (Fig. 2b), proving the detrimental effect of solvent on the organelles integrity.
239 Considering data reported in Fig. 3a, it was confirmed that the amount of Chl in intact chloroplasts
240 depends both on the extraction time and on the presence of metals. Indeed, the greatest concentration
241 was obtained in the samples recovered using the maximum dose of metals (165 ppm) for 2h (trial 14)
242 with no remarkable difference extending the extraction time up to 3h (trials 3 and 9). Irrespective of
243 the process time, a notable difference in the amount of intact chloroplasts was observed between the
244 sample with the maximum dose of metals (trials 2, 6, 14, 3 and 9) and the sample without metals
245 (trials 1, 7, 16, 8 and 10). Trials with halved metal dose were characterized by an intermediate Chl
246 content (Fig. 3a). For the SE, data reported in Fig. 3b confirmed that the amount of intact chloroplasts
247 depended on the extraction time, whereas the presence of metals was not relevant. Indeed, with the
248 same extraction time and different metal dose, the concentration of chloroplasts was similar (trial 1
249 and 7 *vs* trials 2 and 6 *vs* trial 12). Unlike the enzymatically extracted samples, the greater content of
250 intact chloroplasts was identified in the tests with a shorter extraction time (1h), probably because a
251 prolonged contact with organic solvent destroyed the organelles.
252
253 **3.1.2 Colorimetric properties of green extract**
254 In addition to the pigment quantification, CIELa*b* parameters have been considered for
255 characterizing the extracts (Table 4 and 5). In particular, the greenness is expressed by the a* value;
256 the more negative it is, the greener the sample is. For EAE trials the total color difference (ΔE) was
257 also evaluated in order to better highlight the color changes. In detail, a total color difference ranging

from 0 to 2 is considered unrecognizable, a $\Delta E \geq 5$ is rated significant and recognizable, whereas a remarkable variation is found when $5 \leq \Delta E < 12$ and a different color if $\Delta E \geq 12$.

The lowest a* value for EAE samples (Table 4) was observed by applying the highest metal dose at 2h (trial 14) and 3h (trials 3 and 9), while preserving the greenness. By decreasing the metal dose, the color shifted towards duller nuances until obtaining the worst green color (higher a* value) for the tests without metal ions (trials 1,7 and 16), as also highlighted by the ΔE value [calculated as the difference with trial 3 (reference sample with the lowest a*)]. Considering the colorimetric data of the SE samples (Table 5), the presence of metal ions did not preserve the color which tended to turn on duller and less brilliant nuances, tending to greyish hue when the extraction time extends. These results fit with what has been reported in the literature. It is well known that the use of organic solvents may easily lead to a partial degradation of the extracted molecules and, consequently, to a qualitative color deterioration (Taghavi et al., 2023).

Considering the obtained results, it is possible to hypothesize that the synergistic use of a tailored enzymatic mix and a suitable combination of metal ions (Zn^{2+} and Cu^{2+}) may be an efficient method for the recovery of Chl within intact chloroplasts.

3.2 Application of the green extract in a food matrix: the case study of meringues

Meringues are a baked mixture of egg white and sugar and the quality is mainly linked to the processing methods, the ingredients of the product recipes (Yüceer & Caner, 2021), as well as to the typical pure white. The freeze-dried Chl-based sample, containing intact chloroplast (trial 14), was used in the formulation of meringues at different doses (0, 1, 2, 3, 5, and 7 % w/w sugar based). The pictures and the coordinates of the tristimulus values (CIELa*b*) in Table 6 suggested a significant variation of the colorimetric parameters L*, a* and b* between the meringue without colorant (0% w/w) and the product at lowest concentration (1% w/w). Concerning a*, it significantly varied towards negative values (more intense green nuance) up to a Chl-based extract concentration equal

283 to 3% w/w. Over this dose, the addition of increased amount of colorant did not affect the colorimetric
284 parameters a* and b* (Table 6).
285 The effect of powder colorant incorporation on the organoleptic characteristics of meringues is
286 presented in Fig. 4. The sensory evaluation suggested that, irrespective of the dosage, the colorant
287 addition had a significant effect on meringues quality ($p < 0.05$) except for internal color (Fig. 4).
288 Excluding the attributes related to the intensity of the green color and the overall acceptability, it may
289 be note that, considering the dose of colorant added, no significant differences were observed among
290 the control (0%) and the 1 and 2% samples for taste, smell (herbaceous), texture, hardness and
291 herbaceous flavor. The sample containing 3% pigment slightly differed from the 0-2% samples, with
292 the only exception of brightness (Fig. 4). The meringues with the highest dose of colorant (5 and 7%)
293 were similar, but completely different from the other samples with the exception of crumb color and
294 color homogeneity. Regarding the intensity of the green color, as expected, the 3%, 5% and 7%
295 samples obtained the highest score (Fig. 4). The overall acceptability on a five-point hedonic scale
296 was higher than 4.6 for meringues at 1-3% and did not differ from the control. However, for 5 and
297 7%, the overall acceptability was rated as poor (3.2 and 2.6 respectively), due to the strong taste and
298 aroma of these samples. The sensory analysis data suggested that the most suitable dose of colorant
299 that can be used in an original and innovative green meringue preparation is 3% (w/w), without
300 significantly affecting the sensory characteristics.
301
302 **4. Conclusions**
303 This study demonstrates that chlorophyll (Chl) may be effectively recovered inside their natural
304 envelope (chloroplast) by a tailored approach, which combines the simultaneous use of enzyme-
305 assisted extraction (EAE) in the presence of metal ions (zinc and copper), applied in ideal ratio for
306 health benefits. This method exploits the mild and selective hydrolytic action of enzymes, preserving
307 the structural integrity of chloroplast, and concurrently it stabilizes the pigment thanks to the
308 protective action of the two bivalent ions.

309 The combined use of enzymes and metal ions allowed for more efficient extraction and better
310 preservation of Chl, maintaining its vibrant green color and minimizing pigment degradation,
311 compared to traditional organic solvent extraction.
312 Colorimetric analysis revealed that the stability of the color is strongly correlated with the
313 concentration of metal ions and the dose of 165 ppm helped to better preserve an intense green color
314 even at longer extraction times.
315 The application of the extracted Chl, as natural colorant in a typical pure white food matrix, such as
316 meringues, confirmed that at lower concentrations (1-3% w/w) the pigment not only gives the desired
317 color but also did not compromise the organoleptic characteristics of the product, including taste,
318 aroma, and texture. In contrast, higher concentrations (5-7% w/w) led to an overpowering herbal
319 flavor, reducing the overall acceptability of the product.
320 Overall, the proposed methodology offers a more efficient process for extracting Chl, avoiding the
321 use of organic solvents, but also enhances the quality and stability of natural colorants for industrial
322 applications.
323
324 **CRediT authorship contribution statement**
325 **Caterina Mazzocchi**: Writing – original draft, Methodology, Investigation, Formal analysis **Claudio**
326 **Lombardelli**: Writing – review & editing, Methodology, Conceptualization, Formal analysis. **Ilaria**
327 **Benucci**: Writing – review & editing, Conceptualization, Visualization, Validation, Data curation,
328 Resources, Supervision. **Marco Esti**: Writing – review & editing, Conceptualization, Funding
329 acquisition, Project administration.
330
331 **Declaration of competing interest**
332 The authors declare no conflicts of interest.
333 **Data availability**
334 Data will be made available on request.

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Figure caption

Fig. 1. Percoll gradient allowing the separation of broken (top) and intact (bottom) chloroplast extracted by (**a**) Enzyme Assisted Extraction and (**b**) Solvent Extraction.

Fig. 2. Contour plots showing the interactions between the variables on the amount of chlorophyll into intact chloroplast (mg/mL): (**a**) time (h) x metals dose (ppm) for Enzyme Assisted Extraction, (**b**) time (h) x metals dose (ppm) for Solvent Extraction.

Fig. 3. Chlorophyll concentration (mg/mL) into intact chloroplast obtained by (**a**) Enzyme Assisted Extraction and (**b**) Solvent Extraction.

Fig. 4. Spider plot representing the mean sensory analysis scores for meringues at different concentration of green chlorophyll-based colorant (0 – 7 % w/w sugar based).

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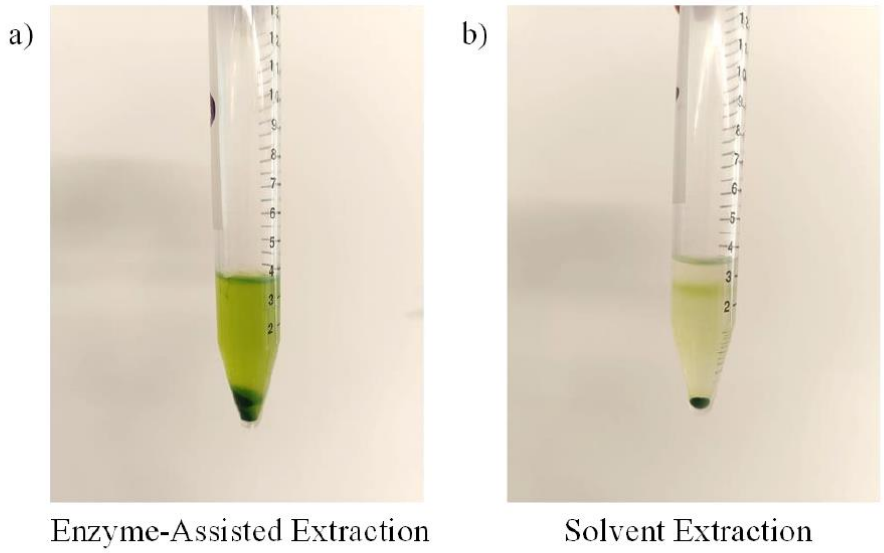

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14 **Fig. 1**

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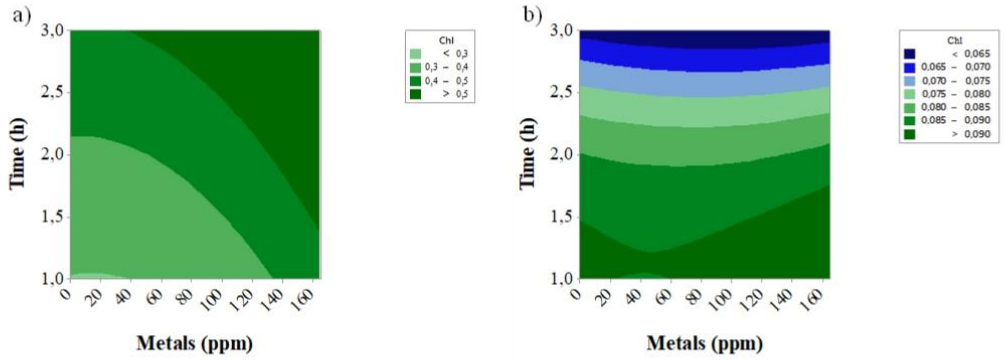

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18 **Fig. 2**

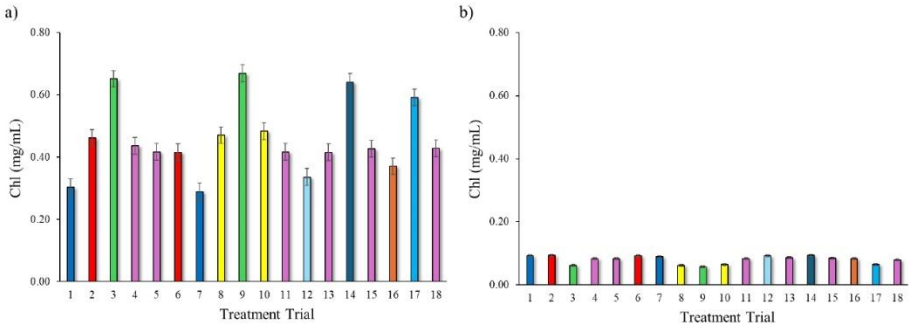
a)
b)
Chl (mg/mL)
0.80
0.60
0.40
0.20
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Treatment Trial

Fig. 3

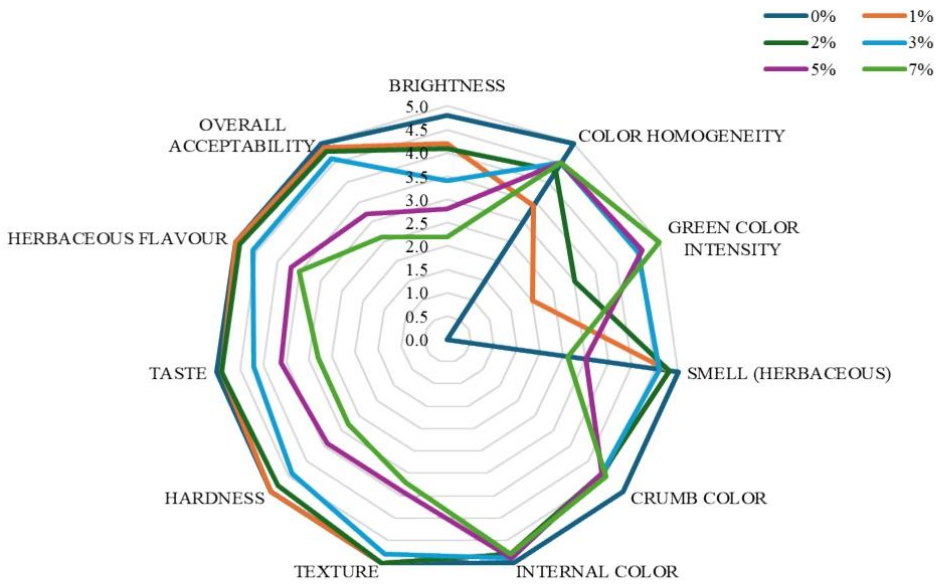
0%
1%
2%
3%
5%
7%
BRIGHTNESS
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
COLOR HOMOGENEITY
GREEN COLOR INTENSITY
SMELL (HERBACEOUS)
CRUMB COLOR
INTERNAL COLOR
TEXTURE
HARDNESS
TASTE
HERBACEOUS FLAVOUR
OVERALL ACCEPTABILITY

Fig. 4

Table (Editable version) Click here to access/download;Table (Editable version);Table.docx

Table 1. Design of Experiment (DOE) for the determination of optimal process parameters (total metal ions, ppm and extraction time, h) on the amount of chlorophyll (Chl, mg/mL) into intact chloroplast.

Treatment trial	Total metal ions dose (ppm)	Extraction time (h)	Chl (mg/mL)	
	X1 (x1)	X2 (x2)	EAE	SE
1	0 (-1)	1 (-1)	0.30	0.091
2	165 (+1)	1 (-1)	0.46	0.093
3	165 (+1)	3 (+1)	0.65	0.061
4	82.5 (0)	2 (0)	0.44	0.083
5	82.5 (0)	2 (0)	0.42	0.083
6	165 (+1)	1 (-1)	0.41	0.092
7	0 (-1)	1 (-1)	0.29	0.089
8	0 (-1)	3 (+1)	0.47	0.061
9	165 (+1)	3 (+1)	0.67	0.057
10	0 (-1)	3 (+1)	0.48	0.064
11	82.5 (0)	2 (0)	0.41	0.083
12	82.5 (0)	1 (-1)	0.34	0.092
13	82.5 (0)	2 (0)	0.41	0.086
14	165 (+1)	2 (0)	0.64	0.093
15	82.5 (0)	2 (0)	0.43	0.085
16	0 (-1)	2 (0)	0.37	0.083
17	82.5 (0)	3 (+1)	0.59	0.064
18	82.5 (0)	2 (0)	0.43	0.079

Table 2. List of attributes and their descriptor scale for sensory analysis

ATTRIBUTES	DESCRIPTOR SCALE
Brightness	1 (opaque) --> 5 (bright)
Color homogeneity	1 (inhomogeneous) --> 5 (homogeneous)
Green color intensity	1 (pale) --> 5 (intense)
Smell (herbaceous)	1 (very herbaceous) --> 5 (herbaceous absent)
Exterior color	1 (uninviting) --> 5 (very inviting)
Internal color	1 (not very uniform) --> 5 (uniform)
Texture	1 (granulous) --> 5 (homogeneous)
Hardness	1 (soft/too hard) --> 5 (correct)
Taste	1 (unpleasant) --> 5 (pleasant)
Herbaceous flavour	1 (excessive) --> 5 (absent)
Overall acceptability	1 (negative) --> 5 (positive)

Table 3 Regression coefficients, model p-value, R^2 and adjusted R^2 for the different polynomial models (Note: Subscripts: 0 = constant term; 1 = metals dose; 2 = time)

Regression Coefficient	EAE Chl	Solvent Chl
b_0	+0.2243	+0.07911
b_1	-0.000342***	-0.000009
b_2	+0.0656***	+0.001964***
b_{11}	+0.000007**	+0.0000001
b_{22}	+0.0076	-0.00831***
b_{12}	+0.000125	-0.000017
Model p-value	0.000	0.000
R^2	0.96	0.96
R^2 adj	0.93	0.94

* significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

Table 4. Visual color attributes (L*, a*, b*) and total color difference (ΔE) of chlorophyll into intact chloroplast recovered by Enzyme Assisted Extraction at different conditions.

Treatment Trial	L*	a*	b*	ΔE
1	76.14	-27.79	99.8	**17.1**
2	75.89	-30.63	97.07	**13.8**
3	74.45	-44.35	95.96	**0.0**
4	77.38	-34.5	97.82	**10.4**
5	79.71	-32.51	98.64	**13.2**
6	75.78	-29.27	98.77	**15.4**
7	76.78	-28.01	99.5	**16.9**
8	75.96	-29.57	98.1	**15.0**
9	75.05	-43.55	96	**1.0**
10	76.1	-30.02	98.2	**14.6**
11	78.25	-33.99	97.87	**11.2**
12	76.56	-28.99	99.01	**15.8**
13	78.15	-34.65	97.33	**10.5**
14	75.23	-42.99	98.24	**1.8**
15	76.01	-33.99	99.21	**11.0**
16	76.85	-30.12	97.25	**14.5**
17	75.03	-34.52	98.75	**10.2**
18	77.25	-34.02	98.41	**11.0**

Table 5. Visual color attributes (L*, a*, b*) of chlorophyll into intact chloroplast recovered by Solvent Extraction at different conditions.

Treatment Trial	L*	a*	b*	Simulated color
1	80.99	-20.97	32.85	
2	82.25	-21.32	34.22	
3	95.24	-10.23	24.44	
4	88.79	-19.88	20.54	
5	90	-18.96	20.54	
6	79.47	-20.13	31.98	
7	79.12	-19.98	30.54	
8	96.47	-9.37	24.47	
9	96.24	-11.43	24.87	
10	95.22	-11.04	24.95	
11	87.25	-18.35	19.55	
12	97.01	-10.1	23.89	
13	88.52	-18.25	21.01	
14	79.35	-22.01	32.54	
15	89.57	-19.58	20.96	
16	91.24	-17.87	21.02	
17	97.01	-10.11	23.89	
18	88.78	-17.99	20.56	

67 **Table 6.** Visual color attributes (a*, b*, L*) of meringues prepared by adding different amount of green
68 chlorophyll-based colorant recovered by Enzyme Assisted Extraction (0 – 7 % w/w sugar based)

Colorant (%)	L*	a*	b*	Sample
0%	95.29^{e}	-0.30^{d}	9.48^{a}	
1.0%	86.69^{d}	-6.51^{c}	23.2^{b}	
2.0%	81.91^{c}	-8.29^{b}	29.15^{c}	
3.0%	75.72^{b}	-9.35^{a}	33.16^{d}	
5.0%	68.89^{a}	-9.38^{a}	34.11^{d}	
7.0%	64.45^{a}	-9.52^{a}	32.86^{d}	

Values with different letters (a-e) differ significantly (Tukey's test, p = 0.05) according to color.

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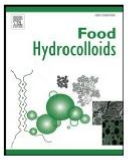

Enhancing the water solubility and stability of zinc modulated chlorophyll by alginate-whey protein isolate complexes at different pH values

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ABSTRACT

Chlorophyll (Chl), a natural pigment, is widely used in the food, pharmaceutical, and cosmetic industries for its vibrant green color. However, its application is limited by its poor solubility and stability in aqueous solutions, especially under acidic conditions. This study investigates a novel approach to enhance the solubility and stability of zinc modulated chlorophyll (Chl-Zn^{2+}) by complexation with whey protein isolate (WPI) and alginate (ALG). Encapsulation of Chl-Zn^{2+} with WPI prevented its precipitation between pH 3 and pH 7, except at the isoelectric point (pI) of WPI (around pH 4.5). The visual appearance, colorimetric measurements, and absorbance spectra indicated that Chl-Zn^{2+} formed stable complexes with WPI at various pH. Additionally, the inclusion of ALG prevented isoelectric precipitation at pH 4.5, likely due to electrostatic interactions between WPI and ALG, leading to the formation of stable Chl-WPI-ALG complexes. These complexes significantly improved the color stability and solubility of Chl-Zn^{2+} during storage under both neutral and acidic conditions. Fluorescence spectroscopy and particle size measurements confirmed the formation of these complexes and their enhanced stability. This study suggests that the use of WPI and ALG to encapsulate Chl-Zn^{2+} could be an effective method to expand its application in acidic food products, providing a stable and vibrant natural colorant.

1. Introduction

Chlorophyll is a very widespread pigment in nature and if extracted from natural sources may be used as an alternative to synthetic dyes due to its bright green color. In addition to this, it has been suggested that chlorophyll have biological activities such as antioxidant and antibacterial properties (Halim et al., 2010). Its molecular structure consists of a porphyrin macrocycle esterified with a phytol group, which is mainly responsible for the liposolubility of the pigment (Galaffu et al., 2015).

Although chlorophyll is a natural food colouring agent, there are disadvantages associated with its use. Chlorophyll easily undergoes degradation during processing and storage due to environmental factors such as the extent of exposure to oxygen/light and changes in pH value, presence of enzymes and high temperature, resulting in a color change from bright green to dark yellow. This instability and its insolubility in water severely limit its application, especially in the food industry (Kang et al., 2019; Zhang et al., 2019). To overcome these limitations previous studies have shown that exchanging the unstable Mg^{2+} with a more stable Zn^{2+} may enhance the stability of the chlorophyll (Mazzocchi et al., 2023; Özkan & Ersus Bilek, 2015). Also, the encapsulation of chlorophyll could be a solution. Agarry, Wang, Cai, Wu, et al. (2022) have encapsulated chlorophyll using different carrier agents such as zein, casein, and whey protein isolate (WPI). These carrier agents are effective in protecting chlorophyll from acidic medium (pH 2 to 6, chlorophyll retention; 39.3–97.8%) and from light exposure (chlorophyll retention; 41.6–65.5%) (Agarry et el., 2022a). Kang et al. (2019) used different blends of gum arabic and maltodextrin for encapsulation of chlorophyll by spray drying, suggesting that encapsulation with maltodextrin alone showed the highest storage stability (94.7%–97.5%). In a recent study, soy protein isolate (SPI) and chitosan were used as carriers for its encapsulation. The authors demonstrated that the predicted and experimental encapsulation efficiency was 98% and 96% (using a 1:7 ratio of chitosan:SPI, a 1:14.8 ratio of chlorophyll: SPI, respectively). Furthermore, using the complex coacervation and vibrating nozzle technology, green nanoparticles with satisfactory

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encapsulation efficiency (94.9–96.1%, w/w) and good loading capacity (3.32–4.22%, w/w) were obtained (Agarry, Wang, Cai, Kan, & Chen, 2022). Zhang et al. (2019) used WPI to improve the solubility and stability of chlorophyll by producing microcapsules by spray drying. The results show an increase in the encapsulation efficiency and solubility of chlorophyll by 3.8% and 7.8%, respectively. Different encapsulation methods could affect the final appearance of colorant. Indeed, spray drying often results in larger particles, typically in the micron range and these larger particles may shatter light affecting the coloring properties of the chlorophyll. On the other hand, complex coacervation may produce much smaller particles, often in the nanometer range, which do not scatter light and therefore do not affect the coloring properties.

In addition to having a high nutritional value, WPI is also characterized by its essential functional properties, such as emulsification, gelling and film formation. Due to these characteristics and the relatively low cost, WPI may be used as a carrier for the encapsulation of hydrophobic components, such as chlorophyll (Agarry, Wang, Cai, Wu, et al., 2022; Zhang et al., 2019). Even though WPI showed much better binding and coating properties than other materials (e.g. gelatin, starch and maltoxtrin), its isoelectric point (pI) and in particular that of β-Lactoglobulin (BLG, a major component of WPI) at pH 5.2 tends to make it aggregate and precipitate. For this reason, the use of alginate, a hydrocolloid from brown algae, could be used in the formulation of the encapsulation carrier, as it forms a stable electrostatic complex with BLG, especially around pH 5.2 (Hosseini et al., 2015; Møller et al., 2020.)

The aim of this study was to investigate the encapsulation chlorophyll in WPI alone or combined with Alginate (ALG), as an effective and cost-saving method to improve stability, solubility, pH-response and color properties of the green pigment, recovered by tailored enzyme-assisted extraction from spinach including Zn^{2+} modulation (Chl-Zn^{2+}) (Mazzocchi et al., 2023). Firstly, we hypothesised that WPI could be used as a carrier for chlorophyll in order to increase its solubility and stability in acidic environment. However, its properties may be influenced by pH. The second hypothesis was that ALG could prevent precipitation of WPI via electrostatic stabilization, when the pH is close to the pI of WPI.

2. Materials and methods

2.1. Materials

BiPro whey protein isolate (WPI) (92.6%) was kindly provided from Agropur (Le Sueur, Minnesota, USA). It contains about 85% of BLG (Van der Meeren, El-Bakry, Neirynck, & Noppe, 2005). Alginic acid sodium salt (ALG) was acquired from Sigma-Aldrich (Steinheim, Germany). Deionized (18Ω) and filtered water from a MilliQ water system (Millipore Corporation, MA, USA) was used for all preparations.

2.2. Methods

2.2.1. Extraction of Chl-Zn^{2+} based colorant and solution preparation

Chl-Zn^{2+} was extracted from spinach according to Mazzocchi et al. (2023) as follows: blanched spinach was resuspended in 0.1 M McIlvaine buffer (pH 5.0) in a lab-scale bioreactor and the treatments were carried at 25 °C, 150 ppm of $ZnCl_2$, ratio buffer/spinach equal to 17.5 and enzyme mix dose of 30 U/g (composed of 40% cellulase, 41% hemicellulase and 19% pectinase). The Chl-Zn^{2+} based colorant obtained was freeze-dried (bench freeze-dryer, Labconco Corporation, Kansas City, MI, USA). The powder was dissolved in ethanol and then centrifuged at 3500 g for 10 min (SL 40R centrifuge, ThermoFisher Scientific, Waltham, MA, USA). The obtained green supernatant was quantified in terms of chlorophyll and used as a stock solution for encapsulation.

Solution of 0.1% (w/w) WPI was prepared in 50 mM phosphate buffer containing 0.02% (w/w) sodium azide. WPI was mixed using a magnetic stirrer for 15 min before the addition of ALG (half the dose of WPI based on weight). The solutions were mixed for 4 h at room temperature and stored at 4 °C for maximum 2 weeks. WPI and WPI + ALG solutions were obtained and were later used to dissolve Chl-Zn^{2+}, please see the optimization section in 2.2.3.

2.2.2. Preparation of Chl-Zn^{2+} complexes

Chl-Zn^{2+} (30 μM) was dissolved in 50 mM phosphate buffer (0% WPI, Control) or in the WPI solution or in WPI + ALG solution. The samples were mixed for 15 min and the pH was adjusted to values of 3.0, 4.5 and 7.0 using HCl (0.1M) (pH range typical for food application). The visual appearance of the samples was evaluated 1 h after preparation and after 1 week (stored in the dark at 20 °C).

2.2.3. Determination of WPI concentration and behaviour at different pH

For the determination of the most suitable concentration of WPI for Chl-Zn^{2+} encapsulation, different concentrations of protein were tested (0, 0.005, 0.025, 0.050 and 0.1% w/v) at pH 3.0. 30 μM of Chl-Zn^{2+} were dissolved in different concentrations of WPI and stirred for 45 min. The solutions were centrifuged at 3500 *g* for 10 min and then the absorbance spectrum was measured (230–700 nm, Cary 60 UV–vis spectrophotometer (Agilent Technologies, CA, USA). The content of Chl in solution was used to determine the WPI concentration needed. Once the amount of WPI was optimized, the selected concentration was used alone or combined to ALG (0.025% w/v) to evaluate the behaviour of Chl-Zn^{2+} at different pH.

Therefore, 30 μM Chl-Zn^{2+} were dissolved in 0% WPI, WPI or WPI + ALG solutions. The samples were mixed for 15 min before adjusting the pH with HCl until reaching values of 3.0, 4.5 and 7.0. Finally, the samples were centrifuged at 3500 g for 10 min and the supernatant obtained used for spectrophotometric analysis. Based on the absorbance, Chl-Zn^{2+} concentration was determined through the following equation (Lichtenthaler, 1987; Mazzocchi et al., 2023; Özkan & Ersus Bilek, 2015):

$$\text{Chl } (\mu g/mL) = (5.24 \times Abs_{664} + 22.24 \times Abs_{649}) \times DF \quad (1)$$

where: Abs_{664} = Absorbance at λ 664 nm, Abs_{649} = Absorbance at λ 649 nm and DF = Dilution factor.

The absorbances at 664 nm and 669 nm were used in the chlorophyll calculation equation because they represent the wavelengths of maximum absorption for chlorophyll *a* and chlorophyll *b*, respectively. This allows the concentration of these chlorophylls in a sample to be accurately quantified. Using these specific wavelengths, the concentration of each type of chlorophyll can be calculated separately and then added together to obtain the total chlorophyll concentration.

2.2.4. Colorimetric analyses

Two mL of Chl-Zn^{2+} samples dissolved in 0% WPI, WPI, or WPI + ALG were analysed using a Nunc 12-well multiplate and a CR-400 portable colorimeter (Konica-Minolta, Tokyo, Japan). Tristimulus values (L*, a*, b*) were acquired. The color was measured in triplicate. The color difference (ΔE*) was determined using the following equation (Lombardelli et al., 2021):

$$\Delta E^* = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (2)$$

Where L_0^*, a_0^*, b_0^* are the values of the reference and L*, a*, b* are the values of the sample.

2.2.5. Fluorescence spectroscopy

Fluorescence spectroscopy was used to investigate the interaction between WPI and Chl-Zn^{2+}. WPI (0.050%) was mixed with different concentrations of Chl-Zn^{2+} (0–30 μM) at either pH 3.0 or pH 7.0. The samples were mixed for 30 min before measuring the fluorescence on a FL 6500 fluorescence spectrophotometer from PerkinElmer (Shelton, CT, USA). The emission spectra were recorded in the range 305–450 nm using an excitation wavelength of 280 nm, exciting the aromatic

residues in WPI. Since Chl absorbs light at both excitation and emission wavelengths, this may result in an inner filter effect. For this reason, absorbance was recorded for each Chl concentration and used for corresponding corrections during calculation of the binding parameters. The inner filter effect was accounted by using the following formula (Wiese et al., 2009) (3):

$$F = F_u \cdot 10^{(\varepsilon\,\lambda ex\, +\varepsilon\,\lambda em)\cdot l/2\cdot L0} \quad (3)$$

Where Fu is the experimentally measured uncorrected fluorescence and F is the corresponding corrected value for the fluorescence; ε λex or ε λem represents the molar extinction factor at the excitation or emission wavelength and l is the path length (in this case l/2 = 0.5 cm) in the measuring cell and L_0 is the concentration of ligand (in this case chlorophyll). In order to determine the fluorescence quenching changes during interactions between Chl-Zn^{2+} and WPI, the Stern-Volmer equation (4) was used (Cao et al., 2021; Khalifa et al., 2018).

$$F_0 / F = 1 + K_{sv} \times [Q] = 1 + K_q \times \tau_0 \times [Q] \quad (4)$$

Where F_0 and F are WPI fluorescence in the absence or presence of Chl (quencher), K_{sv} is the Stern-Volmer quenching constant, K_q is the bimolecular quenching rate constant, τ_0 is average lifetime of unquenched fluorophore (WPI), equals 10^{-9} s (Dufour et al., 1994) and [Q] is the concentration of Chl.

2.2.6. Particle size measurements

Chl-Zn^{2+} (30 μM) was dissolved in 0% WPI, WPI (0.050%) or WPI (0.050%) + ALG (0.025%) solutions. Control samples without Chl-Zn^{2+} were also prepared. The samples were mixed for 15 min before the pH was adjusted to pH 3.0, 4.5 and 7.0. Subsequently, the samples were centrifuged at 3500 *g* for 10 min and the particle size was determined by dynamic light scattering on a Zetasizer (Malvern Panalytical, Worchestershire, UK). The intensity-weighted distribution was used to find the average particle size, which was not measured in samples with clear visual precipitation. Samples with some precipitation (such as samples with WPI at pH 4.5) were measured.

2.2.7. Solubility of freeze dried Chl complexes

Solutions of 0% WPI, WPI (0.050%) or WPI (0.050%) + ALG (0.025%) at pH 7.0 containing 30 μM Chl-Zn^{2+} were prepared and stirred for 15 min before being frozen overnight, and then freeze-dried (ScanVac Coolsafe 110 - 4 pro, LaboGene, Allerød, Denmark). The obtained powder was resolubilized in MilliQ water and 50 mM phosphate buffer (pH 3.0 and 4.5) to achieve the same initial concentration. The supernatant after centrifugation (3500 *g* for 10 min) was measured using spectrophotometer in the range 230–700 nm. The solubility yield (SA) of Chl-Zn^{2+} complexes was calculated according to the following equation (Zhang et al., 2020).

$$SA(\%) = \left(\frac{Total\ Chl - Solubility\ Chl}{Total\ Chl}\right) x\ 100 \quad (5)$$

where $Total_{Chl}$ is the Chl concentration before freeze drying, and $Solubility_{Chl}$ is the water-soluble Chl.

2.2.8. Stability test

Chl-Zn^{2+} (30 μM) was dissolved in 0% WPI, WPI and WPI + ALG solutions. The samples were mixed for 15 min before adjusting the pH at 3.0, 4.5 and 7.0 using 0.1 M HCl. The absorbance spectra (230–700 nm) were measured 1 h after sample preparation and at different time intervals for a total of 168 h, during dark storage at room temperature (20 °C). The stability was expressed using residual concentration (%) normalized to the starting concentrations of different samples, as a function of the incubation time.

2.2.9. Statistical analysis

All analyses were performed in triplicates and results were expressed as mean ± standard deviation. The normality of data was verified by Shapiro-Wilk's test ($p > 0.05$). Results were analysed for statistical significance using: (i) two-way analysis of variance (ANOVA) for testing the effect of the single factors (type of encapsulation and pH); and ii) one-way analysis of variance (ANOVA) test to test for significant differences between samples ($p < 0.01$). Tukey's post-hoc test (HSD) was also performed ($p < 0.05$) using the EXCEL® DSAASTAT extension for multiple comparison between samples.

3. Results and discussion

3.1. Design and characterization of Chl-Zn^{2+} complexes by absorbance spectrophotometry

Firstly, different concentrations of WPI were analysed at pH 3.0 to identify the most suitable WPI concentration for Chl-Zn^{2+} complexation. Chl-Zn^{2+} concentration obtained from spectrophotometric analysis showed an increase as the WPI concentration increased (Fig. 1). This behaviour was observed up to 0.050% WPI, whereas no differences were observed at higher WPI concentration, suggesting that a WPI concentration of 0.050% was sufficient for the encapsulation of Chl-Zn^{2+}. This WPI concentration corresponds to about 30 mM, reaching a final inclusion ratio of Chl:WPI of 1:1. Given these results, the concentration of 0.050% WPI was used for the subsequent analyses. The effect of changing pH on Chl-Zn^{2+} absorption was investigated by measuring the absorbance at pH 3.0, 4.5 and 7.0 with or without WPI and ALG (0% WPI, 0.050% WPI or 0.050% WPI + 0.025% ALG) (Fig. 2). The obtained absorbance spectra were in line with previous results of Chl in solution (Kang et al., 2018). At pH 3.0, no peaks characteristic for chlorophyll were observed in the visible region for the non-encapsulated samples (0% WPI). The characteristic peaks of chlorophyll (around 649 and 664 nm, Mazzocchi et al., 2023; Özkan & Ersus Bilek, 2015) were recorded for the encapsulated samples at pH 3 (WPI), demonstrating a successful encapsulation of Chl. The inclusion of ALG (WPI + ALG) at pH 3.0, gave no well-defined absorption peak due to precipitation of the sample as a consequence of a strong electrostatic interactions between the negatively charged polysaccharide and the positively charged protein (Harnsilawat et al., 2006). As expected, at pH 4.5 no peaks were detected neither in the 0% WPI nor in the WPI sample, indicating no Chl to in solution. It has previously been shown that the presence of WPI at

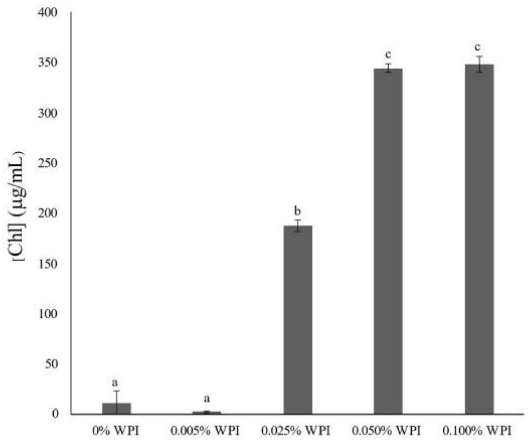

Fig. 1. Behaviour of chlorophyll (30 μM) dissolved in different concentrations of WPI (0–0.100%) at pH 3.0. Different letters (a–c) show significant difference according to Tukey's test ($p < 0.05$). Reported values are mean ± 95% confidence interval of triplicate measurements.

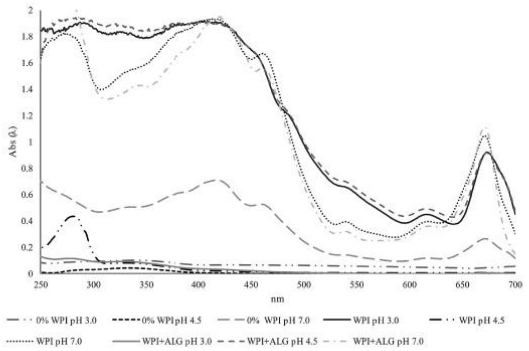

Fig. 2. Absorbance spectra from 250 to 700 nm of 30 μM chlorophyll samples dissolved in 0% WPI, WPI (0.050%) or WPI + ALG (0.050% + 0.025%) at pH 3.0, 4.5 and 7.0.

pH 4.5 leads to precipitation of protein-pigment complex as the pH fell within the isoelectric point (pI) of the primary whey protein (Bromley et al., 2005; Das & Kinsella, 1989; Møller et al., 2020; Sawyer & Kontopidis, 2000). However, the precipitation of WPI was prevented by the addition of ALG and the characteristic Chl peaks were observed in the sample containing WPI and ALG, which has previously been seen for other WPI/ALG formulation with another colorant norbixin (Møller et al., 2020). Finally, even though some Chl was in solution at pH 7.0 without WPI and ALG, the absorption peaks of Chl were remarkably higher in the encapsulated samples compared to the free pigment (0% WPI), while no differences resulted between WPI and WPI + ALG peaks. Moreover, a batochromic effect (red shift) of Chl-a was observed when reducing the pH (from 7.0 to 3.0) and it could be ascribed to the formation of Chl-a aggregates at pH 3.0. Indeed, an acidic environment may favour the formation of Chl-a aggregates and in particular H-type and J-type (Qu et al., 2020). Furthermore, lowering the pH resulted in a decrease of the absorption peaks between 600 and 700 nm, with a diminished Chl detection both in absence or in presence of WPI or WPI + ALG. Furthermore, the sample complexed with WPI at pH 7.0 showed an absorption peak around 280 nm, which represents the characteristic absorbance of its structural hydrophobic aromatic amino acids (tryptophan, tyrosine) and to lower extent cysteine (Dodero & Messina, 2013; Siddique et al., 2017).

Through the absorption spectra, it was possible to calculate the concentration of Chl-Zn^{2+} present in each sample (Fig. 3). The protective action of WPI was evident at pH 3.0, where the encapsulated sample

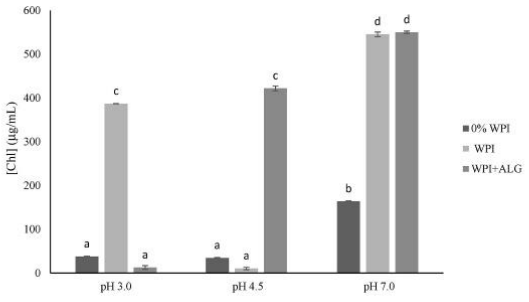

Fig. 3. Final concentration of 30 μM chlorophyll dissolved in 0% WPI, WPI or WPI + ALG at pH 3.0, 4.5 and 7.0. Different letters (a–d) show significant difference according to Tukey's test ($p < 0.05$) Reported values are mean ± 95% confidence interval of triplicate measurements.

showed a higher amount of Chl-Zn^{2+} compared to the 0% WPI and WPI + ALG. As expected at pH 4.5 (close to the protein pI) the precipitation of WPI occurred, although the addition of ALG allowed us to overcome this issue. Even at pH 7.0, the encapsulated Chl-Zn^{2+}, both with WPI and WPI + ALG, showed a clear improvement compared to the control (Fig. 3). The study of absorbance allowed us to observe the behaviour of the protein-pigment complexes at different pH and optimize the encapsulation conditions of the pigment in relation to the pH. Two-way ANOVA proved that both pH and type of encapsulation had a significant effect on the Chl-Zn^{2+} concentration and that there was an interaction between the two factors.

3.2. Visual and colorimetric properties of chlorophyll complexes

The visual and color aspect of the green extract (containing 30 μM Chl-Zn^{2+}) encapsulated (WPI and WPI + ALG) or not (0% WPI), was assessed at the three different pH (3.0, 4.5 and 7.0) just after preparation (1h) and after 1 week of storage (20 °C in the dark) (Fig. 4). One hour after preparation, non-encapsulated Chl-Zn^{2+} samples appeared brownish yellow at lower pH and pale green at pH 7.0. The WPI and WPI + ALG samples were greener at all the pH, although at the lower values (pH 3.0 and 4.5), the color tended towards slightly more yellow shades. Irrespective of the pH, after 1 week of storage the green pigment in non-encapsulated samples precipitated, making the solution completely bleached, likely because of Chl-Zn^{2+} not being soluble in aqueous media. Otherwise, in the WPI sample precipitation occurred at pH 4.5, probably due to the pH being close to pI of the protein (Kristensen et al., 2021; O' Mahony & Fox, 2013). As previously addressed the addition of ALG allowed a stable electrostatic complex formation (Harnsilawat, Pongsawatmanit, & McClements, 2006; Møller et al., 2020). However, the acidic condition (pH 3.0) gave rise to a prompt precipitation of WPI + ALG samples, while at pH 4.5 and 7.0 they maintained a homogeneous up to 1 week of storage. A similar behaviour was found for the carotenoid norbixin when complexed with WPI and ALG (Møller et al., 2020).

Colorimetric measurements were performed to compare the effect of changing pH on color (Fig. 5). The most predominant variations were observed for the a* value (green for negative value) and b* (yellow for positive value). In particular, the values of a* in the non-encapsulated samples were almost equal to 0. The pH lowering resulted in a decrease in green quality (higher a* value), although in WPI and WPI + ALG samples the phenomenon was less evident. This is probably due to the normal degradation of the pigment at acidic pH due to loss of the metal ion, despite the presence of the zinc ion. In the WPI samples, the ΔE value between pH 3.0 and pH 7.0 was equal to 2.6, suggesting a color difference. Indeed, when ΔE is between 1.5 and 3.0 the color appears as a just noticeable difference by the eye, while ΔE values higher than 3.0 give a remarkable color difference (Young & Whittle, 1985). However, in our case with Chl-Zn^{2+} differences in visual appearance were very evident with a ΔE value of 2.6. Concerning the WPI + ALG complex, the ΔE between pH 4.5 and 7.0 was 2.3, indicating a small difference changing the pH value. Comparing WPI and WPI + ALG at pH 7.0, the low ΔE value (0.6) proved no color difference between the samples. When the encapsulated samples were compared with non-encapsulated ones, the former was considerably more colored with ΔE values greater than 14, thus proving the successful effect of pigment encapsulation.

3.3. Particle size of Chl-Zn^{2+} complexes

Since the absorbance measurements suggested the formation of Chl-Zn^{2+} complexes, the average particle size was measured in the absence and/or presence of WPI and ALG (Table 1). In many studies the interactions between proteins and small molecules have been investigated through particle size (Bromley et al., 2005; Li et al., 2019). The low scattering detection in samples only containing Chl-Zn^{2+} suggested a scarce aggregation of non-encapsulated Chl-Zn^{2+} at all the pH tested or that the Chl-Zn^{2+} concentration was too low for the aggregates to be

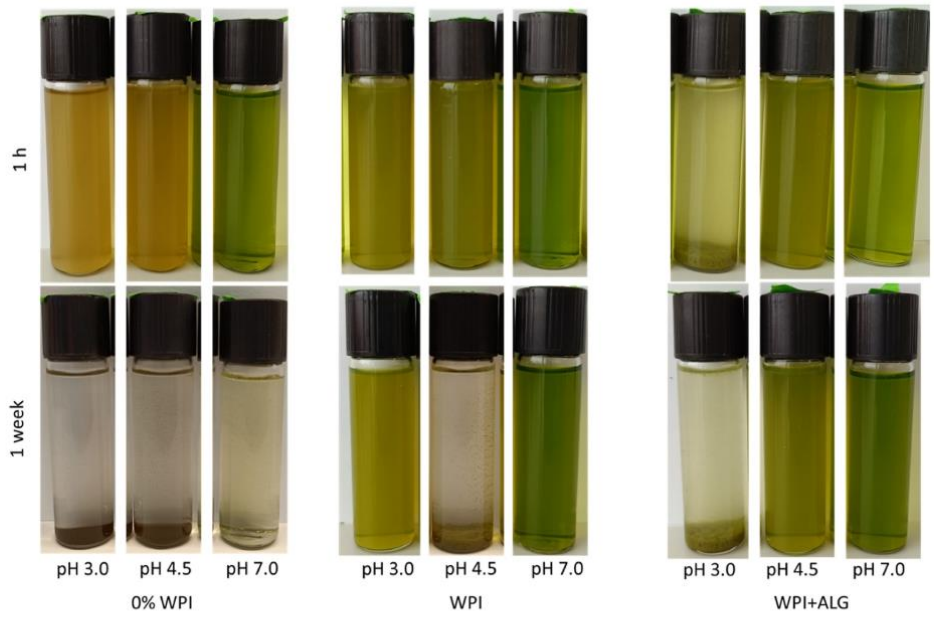

Fig. 4. Appearance of 30 μM chlorophyll dissolved in 0% WPI, WPI or WPI + ALG at pH 3.0, 4.5 and 7.0 just after preparation (1 h) and after 1 week storage in the dark at 20 °C.

detected. Similar behaviour has been observed for norbixin (Møller et al., 2020). The solution containing only WPI [WPI (no Chl)] did not allow us to record any particle size presence, except at pH 4.5 where the protein began to aggregate due to isoelectric aggregation and precipitation of the WPI. In combination with Chl, the presence of WPI, showed an average particle size around 150/200 nm at pH 3.0 and 7.0, where no precipitation occurred, whereas at pH 4.5, the particle size significantly increased due to protein aggregation. When ALG was added to samples (WPI + ALG), the particle size was not detected at pH 3.0 due to the immediate precipitation, which also occurred in samples without Chl-Zn^{2+} [WPI + ALG (no Chl)]. At pH 4.5, in samples with WPI + ALG the detected particle sizes were much smaller than the samples with only WPI. This phenomenon could be explained by the formation of a stable complex through electrostatic interactions that avoids protein isoelectric precipitation. Indeed, ALG may prevent the aggregation of WPI (and in particular of BLG), forming a stable electrostatic complex, due to electrostatic attraction between the oppositely charged molecules at pI of WPI (Harnsilawat et al., 2006). The decrease in particle size at pH 4.5 in samples with ALG addition is consistent with findings by Bromley et al. (2005) and Li et al. (2019), who also observed increased stability of molecules associated with reduced complex size. Since the size of WPI is reported in the literature to be around 10 nm (Fan et al., 2021) (therefore significantly smaller than the values found in our results) and excluding the possibility that the inclusion of a Chl-Zn^{2+} molecule increases the size of the final particle in this way, it could be that the interaction between Chl-Zn^{2+} and WPI leads to the formation of larger complexes containing more than one protein and perhaps even more than one Chl-Zn^{2+} molecule. However, our results agreed with those reported by Møller et al. (2020) with norbixin as colorant. Although the values reported have different scales of magnitude (probably due to the different nature of the pigments), a similar trend is observed. In fact, at pH 3.0, both for norbixin and for Chl-Zn^{2+} a similar size of the pigment + WPI complex was recorded. In samples at pH close to the pI of BLG, an increase in size of aggregates with WPI both in presence and absence of pigment was observed, and a reduction in size when ALG is added for both pigments.

3.4. Solubility test

One of the major drawbacks of Chl application in aqueous food is its very low solubility in water. Recently, Chl encapsulation made it possible to overcome this limitation (Zhang et al., 2020). The solubility of the freeze-dried complex between Chl-Zn^{2+}, WPI and ALG, assessed in water and in buffer at pH 3.0 and 4.5, is shown in Table 2. From a first visual examination of the samples dissolved in water, it was possible to observe how the encapsulated chlorophyll was soluble, while particles floated in the solution of the non-encapsulated one. Samples were then centrifuged, and the supernatant was spectrophotometrically analysed. Both the sample encapsulated with WPI and in that with WPI + ALG the solubility was found to be around 85%, which is in line with what is reported by the literature (Zhang et al., 2020). On the other hand, the solution containing non-encapsulated Chl-Zn^{2+} was completely clear. Regarding Chl-Zn^{2+} dissolved in buffer at pH 3.0, the WPI sample showed good solubility (>70%), while the WPI + ALG sample, despite the tendency to precipitate at this pH, still showed increased solubility compared to free Chl-Zn^{2+}. At pH 4.5, the WPI + ALG sample was characterized by excellent solubility and despite its lower solubility (27%) also the complex with WPI showed promising aqueous soluble properties compared to free Chl-Zn^{2+}.

3.5. Analysis of Chl-Zn^{2+} and WPI interaction by fluorescence spectroscopy

The interaction between Chl-Zn^{2+} and WPI was investigated by fluorescence spectroscopy at both pH 3.0 and pH 7.0. Fig. 6(A and B) shows the fluorescence spectra of WPI with the increasing concentration of Chl-Zn^{2+} (0–30 μM). The emission spectrum of WPI displayed a strong peak emission at ~325 nm at both pH, similar to previous reports on

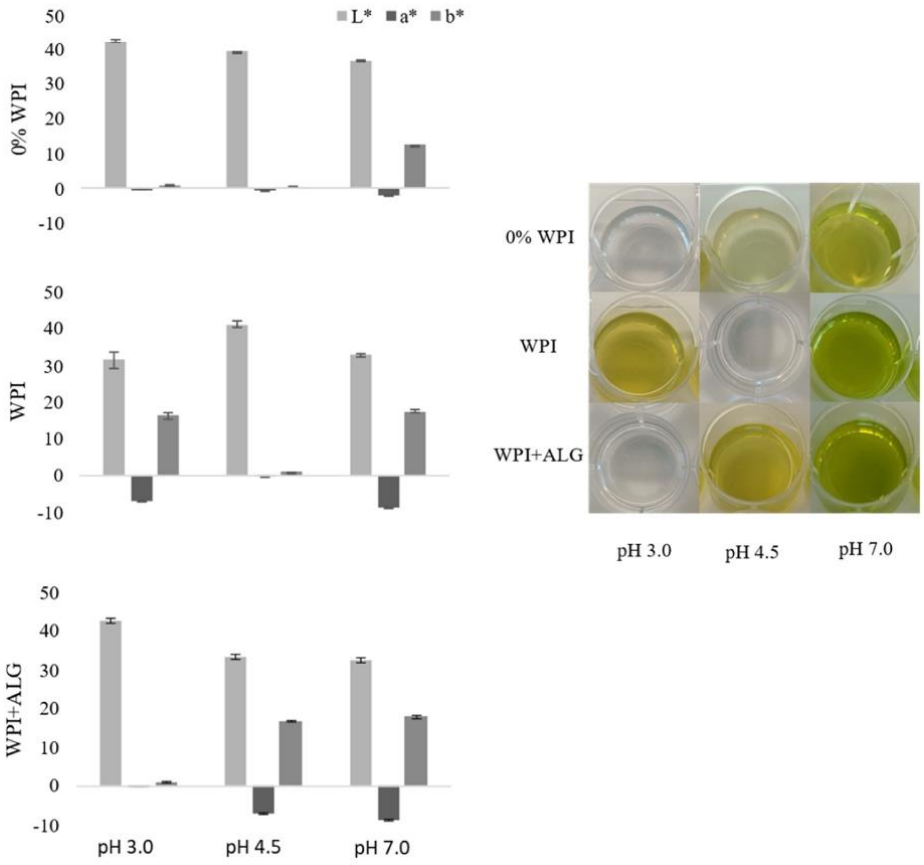

Fig. 5. Tristimulus color measurements of 30 μM chlorophyll dissolved in 0% WPI, WPI or WPI + ALG at pH 3.0 **(A)**, 4.5 **(B)** and 7.0 **(C)** and visual appearance of samples **(D)** after removal of insoluble parts by centrifugation. The color was measured 1 h after preparation of the samples. Reported values are mean ± 95% confidence interval of triplicate measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Intensity-weighted average particle diameter of 30 μM chlorophyll dissolved in WPI or WPI + ALG and solutions of WPI or WPI + ALG at pH 3.0, 4.5 and 7.0. Particle size was determined 1h after sample mixing.

	Mean (±SD)		
	pH 3.0	pH 4.5	pH 7.0
WPI	196.2 ± 7.02	435.6 ± 4.74	149.6 ± 2.02
WPI + ALG	Precipitate	266.3 ± 5.82	154.4 ± 2.45
WPI (No Chl)	ND	468.9 ± 24.25	ND
WPI + ALG (No Chl)	Precipitate	245.5 ± 8.75	ND

ND: not detected due to too low scattering intensity of the dispersions.

Table 2
Solubility of 30 μM chlorophyll dissolved in 0% WPI, WPI (0.050%) or WPI + ALG (0.050 + 0.025%) resuspended in water (pH 6) or in 50 mM phosphate buffer at pH 3.0 and 4.5.

Sample	Water	pH 3.0	pH 4.5
0% WPI	2%	1%	2%
WPI	85%	71%	27%
WPI + ALG	85%	24%	86%

WPI fluorescence (Cui et al., 2021; Møller et al., 2020). The addition of Chl-Zn^{2+} induced a decrease of fluorescence intensity of WPI. Similar behaviour was observed using WPI and norbixin (Møller et al., 2020) and when Chl was encapsulated in soybean protein isolate (SPI) (Cao et al., 2021), thus assuming an interaction between WPI and Chl-Zn^{2+} is established. To further understand the type of quenching, the fluorescence data were analysed using the Stern-Volmer equation (Eq. (4)). As depicted in Fig. 6(C and D), the Stern-Volmer curves of samples at pH 3.0 and 7.0, were linear with $R^2 = 0.96–0.97$, respectively (Table 3). This may represent a single quenching mechanism, either static or dynamic, and K_{sv} values were recognized as the slopes: $K_{sv} = 1.04 \times 10^5$ (pH 3.0) and 9.5×10^4 (pH 7.0) L/mol. The fluorescence lifetime of tryptophan in BLG in the absence of quencher is around $\tau_q = 10^{-9}$ s, and by using these, k_q values were obtained. As shown in Table 3, the K_q values were three orders of magnitude higher than diffusion limited dynamic quenching (approximately 1×10^{10} L/mol·s), suggesting the fluorescence quenching mainly arose from the static quenching by complex

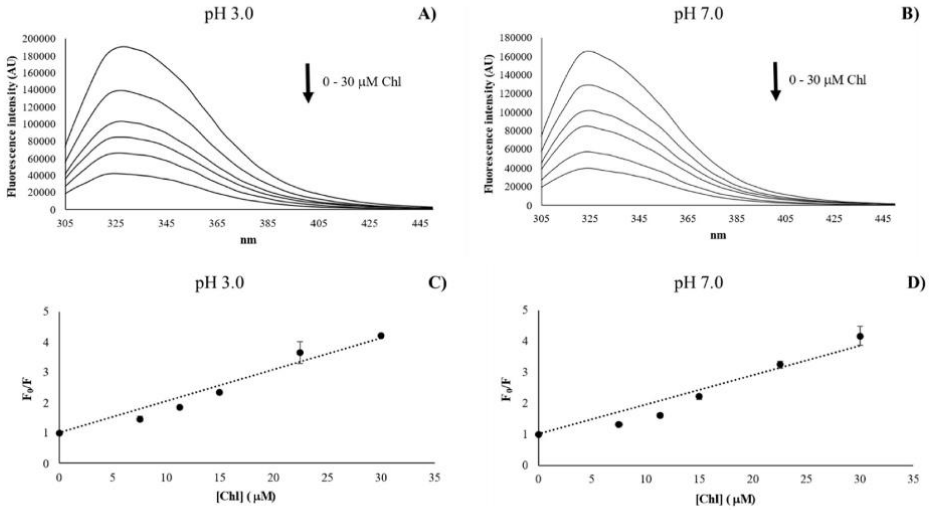

Fig. 6. Effect of chlorophyll on the fluorescence intensity of a WPI solution in 50 mM phosphate buffer at **A)** pH 3.0 and **B)** pH 7.0 using an excitation wavelength of 280 nm. Chlorophyll was added in concentrations from 0 to 30 µM. The fluorescence intensity shown is an average of duplicate measurements. The WPI fluorescence quenching at 325 nm was used to produce Stern-Volmer plots at **C)** pH 3.0 or **D)** pH 7.0.

Table 3
The quenching constant (K_{sv}) and bimolecular quenching rate constant (K_q) for WPI (0.050%) samples at pH 3.0 and 7.0.

pH	K_{sv} (L/mol)	K_q (L/mol·s)	R^2
3.0	1.04×10^5	1.04×10^{14}	0.97
7.0	9.50×10^4	9.48×10^{13}	0.96

formation. This indicates that Chl-Zn^{2+} binds to the aromatic residues in WPI. These results are in line with what was reported by Cao et al. (2021) who studied the mechanism of interaction between SPI and Chl at different temperatures (although in their work they do not consider the inner filter effect of chlorophyll) and with those of Møller et al. (2020) who investigated the complexation between WPI and norbixin at the same pH. Therefore, considering the K_{sv} and k_q values reported in literature, in both cases they appeared to be of the same order of magnitude, confirming the interaction between WPI and Chl-Zn^{2+}.

3.6. Stability of Chl-Zn^{2+} complexes

The stability of Chl-Zn^{2+} was tested for the samples 0% WPI pH 3.0 and 7.0, WPI pH 3.0 and 7.0 and WPI + ALG pH 4.5. The stability test (168 h) was carried out by evaluating total Chl (UV–visible spectrophotometer analysis) during storage in the dark, at room temperature (20 °C). Fig. 7 shows the normalized residual concentration (%) of Chl as a function of the storage time. The encapsulated Chl-Zn^{2+}showed an excellent behaviour in terms of stability, regardless of the encapsulation matrix (WPI or WPI + ALG) and pH (3.0, 4.5 and 7.0), retaining about 98–99% of the initial concentration at the end of the exposure time (Fig. 7). A very different trend was observed for the non-encapsulated Chl-Zn^{2+}samples (0% WPI) at pH 7.0 and 3.0, which decreased of 60 and 70%, respectively, after 168 h of storage. This decrease could be due either to the degradation of the pigment or due to its partial precipitation. The degradation rate constant (k) of the latter two samples was calculated in order to develop a kinetic model to predict the stability behaviour of Chl-Zn^{2+}. The logarithm of residual concentration *vs* time was plotted for the different pH and a linear relationship was obtained,

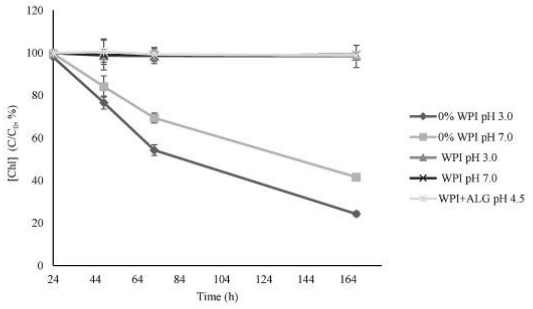

Fig. 7. Normalized residual concentration (%) of free chlorophyll (0% WPI) and encapsulated chlorophyll (WPI, WPI + ALG) stored in the dark at 20 °C. Reported values are mean ± 95% confidence interval of triplicate measurements. C: residual chlorophyll concentration C_0: initial chlorophyll concentration.

demonstrating that Chl-Zn^{2+}degradation followed a first-order kinetic model ($R^2 = 0.99$ for pH 3.0 and 0.98 for pH 7.0), as also found by Koca et al. (2007) and Rudra et al. (2008). Indeed, it is possible to observe that the k values increased as the pH decreased (0.0086 h^{-1} for pH 3.0 and 0.0052 h^{-1} for pH 7.0) resulting in half-life values ($t_{1/2}$) of 80.6 h and 133.3 h for pH 3.0 and 7.0, respectively (as also observed by Koca et al., 2007) As mentioned above, the encapsulated Chl-Zn^{2+} was retained for during the storage time investigated making it impossible to calculate half-lives for the encapsulated Chl-Zn^{2+}, but they can be predicted to be much higher than for the non-encapsulated Chl-Zn^{2+}. The present findings suggested that under these storage conditions, the complexation of Chl-Zn^{2+} with WPI and WPI + ALG occurred successfully, giving rise to a highly protective environment, limiting the pigment degradation at all the tested pH.

4. Conclusion

A method for enhancing the stability and water solubility of Chl-Zn^{2+} by using whey protein isolate (WPI) and alginate (ALG) has been explored. This study demonstrates that the encapsulation of Chl-Zn^{2+} with WPI and ALG may significantly prevent Chl-Zn^{2+} degradation across various pH, making it a viable option for a broader range of food applications, including aqueous foods at low pH. Addition of WPI to Chl-Zn^{2+} may prevent colorant precipitation in acidic solution except around the isoelectric point of WPI. This precipitation was avoided by addition of ALG. These results suggest that the complexation of Chl-Zn^{2+} with WPI and ALG is an effective and simple method for incorporating Chl into aqueous foods, particularly at acidic pH levels such as those found in juices and soft drinks. This approach provides a promising alternative to synthetic dyes, leveraging Chl's natural bright green color and biological activities. This study lays the groundwork for further research into optimizing encapsulation techniques for natural pigments and expanding their application across various industries.

CRediT authorship contribution statement

Caterina Mazzocchi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Anders Hauer Møller:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization. **Ilaria Benucci:** Writing – review & editing, Supervision, Resources. **Marco Esti:** Writing – review & editing, Project administration, Funding acquisition. **Trine Kastrup Dalsgaard:** Writing – review & editing, Supervision, Resources, Data curation, Conceptualization.

Data availability statement

The data presented in this study are available on request from the corresponding authors.

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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3. Conclusions and Future Perspectives

Within the framework of the Circular Economy, the food industry must implement measures to enhance vegetable waste as a valuable source of natural pigments. In the modern food and biotechnology sectors, the application of enzymes for the recovery of natural food colorants is highlighted as a beneficial alternative to conventional techniques due to its mild, eco-friendly, and economically efficient nature. The major challenge for the future is to exploit natural and health-benefiting pigments from food waste, minimizing environmental impact and supporting the concepts of the Circular Economy while also contributing to industry profitability. The results of this PhD thesis prove that the recovery of chlorophyll-containing chloroplast from unsold spinach may be enhanced using a tailored enzymatic mix designed considering the polysaccharide composition of spinach cell wall. Response surface methodology allowed us to narrow the area in which it is possible to identify the highest amount of recovered chlorophyll, and concurrently, to have the best quality of green (T: 25 °C, Zn: 150 ppm e B/S: 17.5, t: <2 h, and enzyme mix dose between 12 and 45 U/g). The chlorophyll-based extract was successfully applied to give different green colorations to a real food. Such a process cannot be carried out without the study of extraction kinetic and recovery yield. Biocatalyst dose significantly affected the recovered yield of Chl, as well as the quality of greenness. At mild conditions (25 °C), the highest Chl extraction yield and the best quality of green were reached using the lowest enzyme dose (0.10 U/g) for short processing time (1–3 h). The protective effect of $ZnCl_2$ was more evident toward Chl a, already during the extraction process. An amount of 150 ppm was enough to ensure the formation of green stable Zn–Chl complexes employing/at low enzyme dose and short extraction time.

Moreover, an in-depth look at the possibility of using an innovative and sustainable method, based on EAE and the simultaneous use two metal ions (zinc and copper) combined at a 10:1 ratio, improved the pigment stability keeping it inside intact chloroplasts, compared to traditional solvent extraction methods. The synergy between enzymes and metal ions allowed an efficient extraction and preserved Chl (inside intact chloroplast), maintaining its vibrant green hue and minimizing degradation caused by temperature, pH, and extraction time. Application of the extracted Chl as a

natural colorant in meringues demonstrated that low concentrations (1-3% w/w) maintained the desired color without affecting taste, aroma, and texture.

Finally, a method for enhancing the stability and water solubility of Chl-Zn^{2+} by using whey protein isolate (WPI) and alginate (ALG) has been explored. This study demonstrates that the encapsulation of Chl-Zn^{2+} with WPI and ALG may significantly prevent Chl-Zn^{2+} degradation across various pH, making it a viable option for a broader range of food applications, including aqueous foods at low pH. Addition of WPI to Chl-Zn^{2+} may prevent colorant precipitation in acidic solution except around the isoelectric point of WPI. This precipitation was avoided by addition of ALG. These results suggest that the complexation of Chl-Zn^{2+} with WPI and ALG is an effective and simple method for incorporating Chl into aqueous foods, particularly at acidic pH levels such as those found in juices and soft drinks.

The method developed in this PhD research for spinach is clearly applicable to other green leafy vegetables, as long as the polysaccharide composition of the pigment-holding cell wall is determined in order to optimize the ad hoc enzymatic formulation. Furthermore, the kinetic study applied may be helpful for the extraction of other pigments from different vegetal sources. This approach provides a promising alternative to synthetic dyes, leveraging Chl natural bright green color and biological activities. Moreover, this research lays the groundwork for further investigations into optimizing encapsulation techniques for natural pigments and expanding their application across various industries.

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List of Papers

Draft: **Mazzocchi, C**., Lombardelli, C., Benucci, I., Esti, M.. Metallo-chlorophyll derivatives as food colorant: is it possible to recover intact chloroplasts from spinach leaf by Enzyme-Assisted Extraction?

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