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# From Citrus Waste to a Promising Biopesticide: Hesperidin Extraction, Nanoformulation, and Bioactivity Evaluation

MASTER DISSERTATION

**Verónica Raquel Sousa Pereira**
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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ORIENTATION
Paula Cristina Machado Ferreira Castilho

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Esta dissertação foi desenvolvida no grupo de Produtos Naturais do Centro de Química da Madeira (CQM), sob a orientação da Professora Doutora Paula Cristina Machado Ferreira Castilho. Foi apresentada à Universidade da Madeira, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Aplicada.

Verónica Pereira

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2023

Funchal, Madeira - Portugal

“What is the bravest thing you've ever said? asked the boy.

'Help,' said the horse. 'Asking for help isn't giving up, it’s refusing to give up.”

— **Charlie Mackesy, The Boy, the Mole, the Fox and the Horse**

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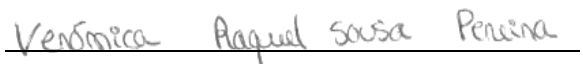
September 2023

Verónica Raquel Sousa Pereira



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## Abstract

Botanical pesticides have gained attention as potential biopesticides and complements to traditional pesticides. Flavonoids have been extensively studied for this purpose because of their involvement in plant defense responses. In particular, hesperidin (HES) is a glycosylated flavanone abundant in citrus waste, whose bactericidal and insecticidal activities have been reported. Despite its potential as a biopesticide, its extraction is usually time-consuming and nonselective, coupled with the use of organic solvents. Moreover, botanical pesticides have some limitations such as rapid biodegradability and low efficiency. The present master's dissertation addresses these issues by proposing an innovative consecutive extraction scheme for pectin and HES from citrus albedo waste. This process describes a new HES extraction method using a basified hydroethanolic mixture, as well as a less time-consuming precipitation method. The efficiency, selectivity, and environmental impact of this method were compared with those of the conventional methanolic HES extraction. The proof of concept for assessing HES purity with quantitative nuclear magnetic resonance technique (qNMR), using benzoic acid as an internal standard, was addressed, along with the attempted HES encapsulation in polysaccharides. The developed consecutive extraction method allowed the extraction and isolation of two highly valued bioactive compounds from citrus waste with high reproducibility and efficiency, with HES extraction yields and purity reaching approximately 0.68% and 84% (determined by HPLC-PDA), respectively, under the best conditions tested (30 minutes, 70 °C, and 1:10 (w/v)). qNMR showed great potential for use in determining HES purity, but further optimization of the method is needed. Pectin-hesperidin nanoparticles formulated with a HES concentration of 0.5 mg/mL (HES-PecNPs 0.5) revealed satisfactory physicochemical properties (452.8 ± 22.1 nm and -16.9 ± 0.7 mV), an encapsulation efficiency of 85.0 ± 1.5%, and a controlled release of HES over 26h.


**Keywords:** biopesticides, hesperidin, citrus waste, extraction, qNMR, nanoparticles

## Resumo

Os pesticidas botânicos têm despertado interesse como potenciais biopesticidas e complementos aos pesticidas tradicionais. Os flavonoides têm sido extensivamente estudados para este fim devido ao papel que desempenham nas respostas de defesa das plantas. Um caso em particular é a hesperidina (HES), uma flavanona glicosilada abundante em resíduos cítricos cujas atividades bactericidas e inseticidas já foram reportadas. Apesar do seu potencial como biopesticida, a sua extração é geralmente demorada e não seletiva, aliada à utilização de solventes orgânicos. Além disso, os pesticidas botânicos têm algumas limitações, como a rápida biodegradabilidade e baixa eficiência. A presente dissertação de mestrado aborda estas questões propondo um esquema inovador de extração consecutiva de pectina e HES a partir de resíduos de albedo cítrico. Este processo descreve um novo método de extração de HES utilizando uma mistura hidroetanólica alcalina, bem como um método de precipitação menos demorado. A eficiência, seletividade e impacto ambiental deste método foram comparados com os da extração metanólica convencional de HES. Foi ainda abordada, sob a forma de prova de conceito, a avaliação da pureza da HES pela técnica de quantificação por ressonância magnética nuclear (qRMN), utilizando ácido benzoico como padrão interno, assim como, a tentativa de encapsulamento da HES em polissacarídeos. O método de extração consecutiva desenvolvido permitiu a extração e o isolamento de dois compostos bioativos altamente valorizados de resíduos cítricos com alta reprodutibilidade e eficiência, com rendimentos de extração e pureza de HES atingindo aproximadamente 0,68% e 84% (determinado por HPLC-PDA), respectivamente, nas melhores condições testadas (30 minutos, 70 °C e 1:10 (p/v)). A técnica de qRMN mostrou grande potencial para uso na determinação da pureza da HES, no entanto, o método carece de otimização. As nanopartículas de pectina-hesperidina formuladas com a concentração de HES de 0,5 mg/mL (HES-PecNPs 0,5) revelaram propriedades físico-químicas satisfatórias (452,8 ± 22,1 nm e -16,9 ± 0,7 mV), uma eficiência de encapsulamento de 85,0 ± 1,5%, e uma liberação controlada de HES durante 26h.

**Palavras-chave:** biopesticidas, hesperidina, resíduo cítrico, extração, qRMN, nanopartículas

# List of Communications

**List of Oral Communications**

Figueira O**, Pereira V**, **Castilho PC**. Sequential extraction of pectin and hesperidin from orange peel wastes: A green chemistry approach. 9th CQM Annual Meeting; 2022 Sep 28-30; Funchal, Portugal. ISBN: 978-989-54090-6-8

**Pereira V**, Figueira O, **Castilho PC**. Hesperidin as a potential biopesticide in environmental applications: extraction, purity determination, and formulation. 10th CQM Annual Meeting; 2023 Jun 1-2; Funchal, Portugal. ISBN: 978-989-54090-7-5

Figueira O, **Pereira V**, Spínola V, Tavares M, **Castilho PC**. Valorization of Orange Peel Waste by Green Consecutive Extraction of Pectin and Hesperidin. PSE Trends in Natural Products: Young Scientists' Meeting; 2023 Jun 28-30; Paris, France

**List of Posters**

Figueira O, **Pereira V**, **Castilho PC**. Hesperidin and Pectin Sequential Extraction for Orange Waste Valorization. Poster session presented at: XXXI International Conference on Polyphenols; 2023 Jul 3-6; Nantes, France

**Pereira V**, Figueira O, **Castilho PC**. Hesperidin-loaded nanoparticles as a promising sustainable release system for environmental applications. Poster session presented at: XXXI International Conference on Polyphenols; 2023 Jul 3-6; Nantes, France

# List of Publications

Figueira O, **Pereira V**, **Castilho PC**. A Two-Step Approach to Orange Peel Waste Valorization: Consecutive Extraction of Pectin and Hesperidin. Foods 2023, 12, 3834.

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## Abbreviations

| | |
|---|---|
| **$ABTS^{+\bullet}$** | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid Radical Cation |
| **AIS** | Alcohol Insoluble Solids |
| **AUA** | Anhydrouronic Acid Content |
| **$Ca^{2+}$** | Calcium Ion |
| **ChNPs** | Chitosan Nanoparticles |
| **CRM** | Certified Reference Material |
| **$d_1$** | Relaxation Delay |
| **DE** | Degree of Esterification |
| **DES** | Deep Eutectic Solvents |
| **DLC** | Drug-Loading Content |
| **DLS** | Dynamic Light Scattering |
| **DMSO** | Dimethyl Sulfoxide |
| **DMSO-d6** | Deuterated Dimethyl Sulfoxide |
| **$DPPH^{\bullet}$** | 2,2-Diphenyl-1-picrylhydrazyl Radical |
| **EE** | Encapsulation Efficiency |
| **EPA** | Environment Protection Agency |
| **EW** | Equivalent Weigh |
| **FTIR-UATR** | Fourier transform infrared spectroscopy coupled with a universal attenuated total reflection |
| **GRAS** | Generally Recognized as Safe Solvents |
| **HCl** | Hydrochloric Acid |
| **HES** | Hesperidin |
| **HES-ChNPs** | Hesperidin-chitosan Nanoparticles |
| **HES-PecNPs** | Hesperidin-pectin Nanoparticles |
| **HES-PecNPs 0.1** | HES-PecNPs formulated with a HES concentration of 0.1 mg/mL |
| **HES-PecNPs 0.3** | HES-PecNPs formulated with a HES concentration of 0.3 mg/mL |
| **HES-PecNPs 0.5** | HES-PecNPs formulated with a HES concentration of 0.5 mg/mL |
| **HPLC-PDA** | High-performance Liquid Chromatography coupled with a diode array detector |

| | |
|---|---|
| **IS** | Internal Standard |
| **$Mg^{2+}$** | Magnesium Ion |
| **MeO** | Methoxyl Group Content |
| **MIC** | Minimum Inhibitory Concentration |
| **NaOH** | Sodium Hydroxide |
| **NMR** | Nuclear Magnetic Resonance |
| **PecNPs** | Pectin Nanoparticles |
| **PIP** | Plant-incorporated-protectant |
| **qNMR** | Quantitative Nuclear Magnetic Resonance |
| **rpm** | Rotation Per Minute |
| **$T_1$** | Longitudinal Relaxation Time |
| **TPP** | Sodium Tripolyphosphate |
| **UPW** | Ultrapure Water |
| **USA** | United States of America |
| **WAC** | Water Absorption Capacity |
| **YP** | Yield of Production |

# I. Introduction

## 1. Biopesticides in Sustainable Agriculture

The Green Revolution of the mid-1900s changed global agriculture by introducing innovative approaches to enhance crop productivity. This historical agricultural phase was characterized by high-yielding varieties, mineral fertilizers, and chemical crop protectants such as pesticides [1]. Together with the mechanization of cultivation and irrigation systems, these changes promoted a significant increase in crop production. This outcome was crucial in the agri-food sector's response to the rising demand for food and its by-products from a continuously growing human population. Although these measures were successful and contributed to decreasing the malnourished population in developing countries, the intensification of these agricultural methods strongly promoted environmental problems, such as soil microbiota impoverishment and loss of water quality [2]. These issues raised concerns about the sustainability of agricultural systems. In light of this situation, researchers and farmers have worked together to adopt new agricultural strategies that do not compromise environmental health, economic profitability, and social equity. As a result of this investigation, promising approaches have emerged, and nowadays, some sustainable practices include rotating crops, adopting agroforestry practices, and applying integrated pest management programs. This last measure focuses on minimizing the use and concentration of applied pesticides while maximizing production and maintaining sustainable agriculture [3]. This encourages the utilization of natural products and biological organisms as pest-controlling agents, known as biopesticides. With the controlled and proper use of biopesticides and new technologies, it will be possible to transition from agriculture characterized by the over-use of agrochemicals and pest resistance, to a more sustainable and eco-friendly one (Figure 1) [4].

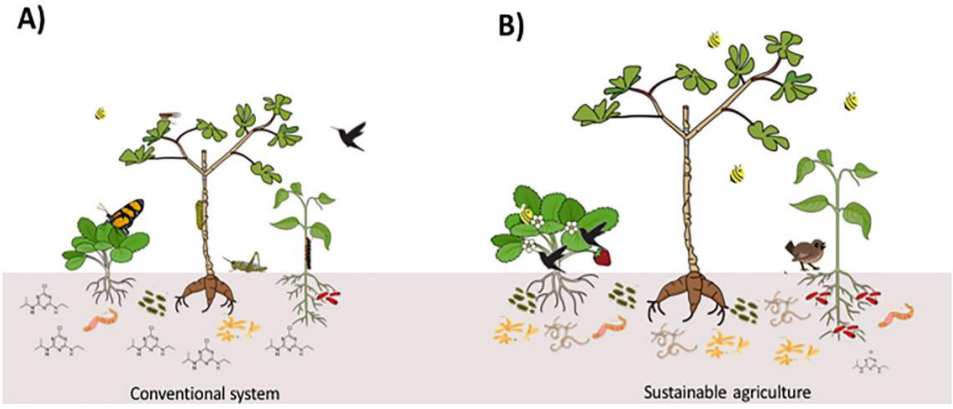


Figure 1 - Illustration of conventional (A) and sustainable (B) agricultural systems [4].

Pests, including animals, weeds, and microbes, infest approximately 40% of the world's crops, resulting in substantial financial damage to farmers owing to harvest losses [3,5]. Pesticides are synthetic chemical products developed to control pests in various agricultural activities. There are several ways to classify them, from the type of target, categorized into insecticides, herbicides, and fungicides, to their mode of action, grouped into killing, mitigating, or repelling agents. They are also classified according to

their chemical structures as organochlorines, organophosphates, carbamates, and pyrethroids [6]. Nowadays, farmers depend heavily on these agrochemicals to have an economically viable production. Despite their successful inhibition of pest activity, they present harmful effects on human health and the environment. These substances can be carcinogenic and teratogenic agents, increasing the risk of developing neurodegenerative diseases, such as Parkinson's [5,7,8]. In nature, pesticides affect soil quality because of their ability to degrade organic matter and damage microbial biodiversity by disrupting the interactions between microorganisms and plant roots or by affecting the nitrogen cycle [4,6]. Water-soluble pesticides reach groundwater by leaching downward into soil layers. Those insoluble compounds bind to soil particles and are susceptible to water run-off and soil erosion, thereby contaminating lakes and rivers [9]. Therefore, biopesticides have received increasing attention as complements to conventional pesticides because they are environmentally and human-friendlier. According to Verified Market Research, in 2021, the biopesticides market was evaluated at USD 4.57 billion and is expected to reach USD 14.63 billion by 2030, expanding at a Compound Annual Growth Rate of 14% between 2022 and 2030 [10].

Defining the term biopesticide is challenging. Different designations have appeared over the years, some generic and others more restricted. One of the broadest definitions is that reported by the United States of America (USA) Environment Protection Agency (EPA), which defines them as “certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals” [11]. In contrast to the USA, the European Union (EU) has not yet defined them. Although the USA has classified biopesticides in a unique regulatory category, in the EU, they fall under the same legislation as all plant protection products (EC 1107/2009), which include pesticides [3,12]. EPA classifies biopesticides into biochemical, microbial, and plant-incorporated-protectant (PIP) pesticides. These natural pesticides and microorganisms may be considered suitable complements to traditional pesticides because studies have shown that they are biodegradable and decompose rapidly in nature because of their contact with air, moisture, high temperatures, and sunlight. These characteristics make them ideal for agricultural applications, because they do not accumulate in soil and water reservoirs, thereby avoiding pollution problems. Biopesticides can be safe to use near the harvest period due to their low toxicity. However, the toxicity of by-products originating from their degradation must be evaluated, despite their natural origin. In addition, they can be effective at low concentrations, resulting in lower operator exposure, are mostly host-specific, and the risk of developing pest resistance is lower for those with more than one mode of action [3,7]. Although these substances offer significant advantages over conventional pesticides, they are associated with some limitations that make them difficult to adopt in pest and disease management. Owing to their biodegradability and performance variation under different climatic conditions, they have low efficiency, are slow to kill, and have a limited shelf life compared to conventional pesticides. Due to these reasons, it is sometimes necessary to reapply them to achieve the desired effect. Their encapsulation in biodegradable polymers, such as polysaccharides, may help overcome this problem by providing a

sustainable release system [13]. Moreover, the manufacturing cost can be high because it involves screening, formulation development, and regulatory clearance [3,14,15].

### 1.1. Microbial Pesticides

Microbial pesticides are microorganisms that are capable of controlling pests directly or indirectly by producing toxins and virulence factors [11,14]. These organisms include bacteria, fungi, viruses, protozoa, and nematodes [15]. Nonetheless, bacteria are the most commonly studied microorganisms due to their low manufacturing costs. Subspecies and strains of *Bacillus thuringiensis* are among the most successful and widely used microbial pesticides. These bacteria produce endotoxins capable of damaging insect gut cells upon ingestion and are, therefore, commonly used against the larval stage of insects, such as mosquitoes, flies, and moths [5,7]. Their high specificity to target pests can be considered a limitation, because multiple microbial species may be necessary to achieve the desired effect. Moreover, they have variable efficiency because their performance is affected by environmental conditions, such as high temperatures [16].

### 1.2. PIP's

EPA defines PIP's as substances with pesticide activity that are produced by genetically modified plants [11]. Over the past decades, transgenic plants have improved agricultural practices by increasing crop yields and enriching the nutritional value of the final product. For instance, plants have been genetically engineered to produce Bt toxins [5]. Although effective, PIP's may not be host specific and can still lead to the development of pest resistance.

### 1.3. Biochemical Pesticides: Botanical Pesticides

Biochemical pesticides are natural compounds that control pests via non-toxic mechanisms. This group includes insect sexual pheromones that interfere with mating and reproduction, plant extracts that attract insects to traps, and essential oils that function as insecticides and repellents [11]. During their evolution, plants have developed the capacity to produce a wide diversity of secondary metabolites, which they utilize in their responses and adaptation mechanisms to biotic (such as a pathogenic infection) and abiotic factors (like drought and nutrient scarcity). Consequently, these phytochemicals, known as botanical pesticides, have attracted the attention as potential natural pesticides. Their insecticide or microbicidal activity depends on their mechanism of action. Botanical insecticides act especially on the nervous system of insects by affecting neuronal channels (such as sodium and γ-aminobutyric acid gated chloride channels), receptors (nicotinic acetylcholine, octopamine, and tyramine receptors) or even enzymes (like acetylcholinesterase) [4]. They can also inhibit respiratory enzymes, affect the digestive system, and bind to specific muscular receptors. Botanical microbicides can inhibit conidial germination, increase membrane permeability, and lead to abnormal bacterial metabolism by interacting with sulfur

compounds [5]. Botanical pesticides may not be host-selective because of their broad range of antimicrobial effects, which may impact the soil microbiota population [4].

Independent of their mechanisms of action, these pesticides can be used in different forms, such as essential oils and plant-based extracts. Of all the currently studied botanical pesticides, essential oils have been the most researched due to their promising activities as insecticides, repellents, and feeding deterrents. Monoterpenes, which are responsible for the biopesticide activity of essential oils, are volatile and lipophilic compounds that can kill insects by interfering with crucial physiological processes [3,17]. The bioactivity of these compounds may be attributed to the inhibition of acetylcholinesterase enzymatic activity. López and Pascual-Villalobos [18] found that seven of the eight monoterpenes studied inhibited this enzyme, where fenchone, S-carvone, and linalool exhibited the highest inhibition values. Nowadays, commercialized essential oils with insecticide or repellent applications include oils from oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), orange (*Citrus sinensis*), garlic (*Allium sativum* L.) and neem (*Azadirachta indica*) [4]. Since ancient times, plant-based extracts have been preferred as crop protectants because of their simple preparation and affordability compared to conventional pesticides and pure isolated natural compounds. Special attention should be given to the chosen extraction solvent because it can affect its performance and quality. The solvent should have low toxicity and boiling point, be able to dissolve as many bioactive components as possible, and maintain their biological properties [15]. Aqueous and ethanolic extracts prepared by maceration are the most commonly studied as bioinsecticides [19]. In addition, their use in sustainable practices is justified by the wide variety of bioactive compounds that they can contain, which are responsible for this extract's bioactive properties, such as antimicrobial and antioxidant properties. In plant-based extracts, alkaloids, phenolic acids, flavonoids, saponins, sterols, tannins, and other compounds can be identified.

Flavonoids are the most abundant non-nitrogenous phytochemicals found in vascular plants, comprising 10,000 known secondary metabolites in the Plant Kingdom [20,21]. They are synthesized via a mixed pathway and accumulate in the cell vacuoles of plant-specific organs such as leaves and fruits, where they have a wide diversity of physiological functions. For example, flavonoids regulate plant development and pigmentation, protect plants against ultraviolet radiation, insects, and pathogens, function as signaling molecules during nodulation, and regulate auxin transport and male fertility [22–24]. These polyphenols comprise the largest group of non-enzymatic antioxidants produced by plants under stress conditions, regardless of whether they are induced by biotic or abiotic factors. Their abundance in these situations is mainly because these molecules are reducing agents [22]. Due to this property, flavonoids have undergone significant research as human health promoters, being recognized as anti-inflammatory, antiviral, antimicrobial, and anticancer agents [13]. Structurally, the flavonoid core is usually referred to as C6-C3-C6 because it consists of two phenyl rings (Figure 2, A and B) connected through a heterocyclic pyran ring (C). Each subclass differs from the others in terms of the level of oxidation, unsaturation, and pattern of

substitution of the C ring, whereas those belonging to the same subclass differ in the substitution of rings A and B. In plants, they can be found in their free form, designated as aglycone, glycosylated, or methylated [20].

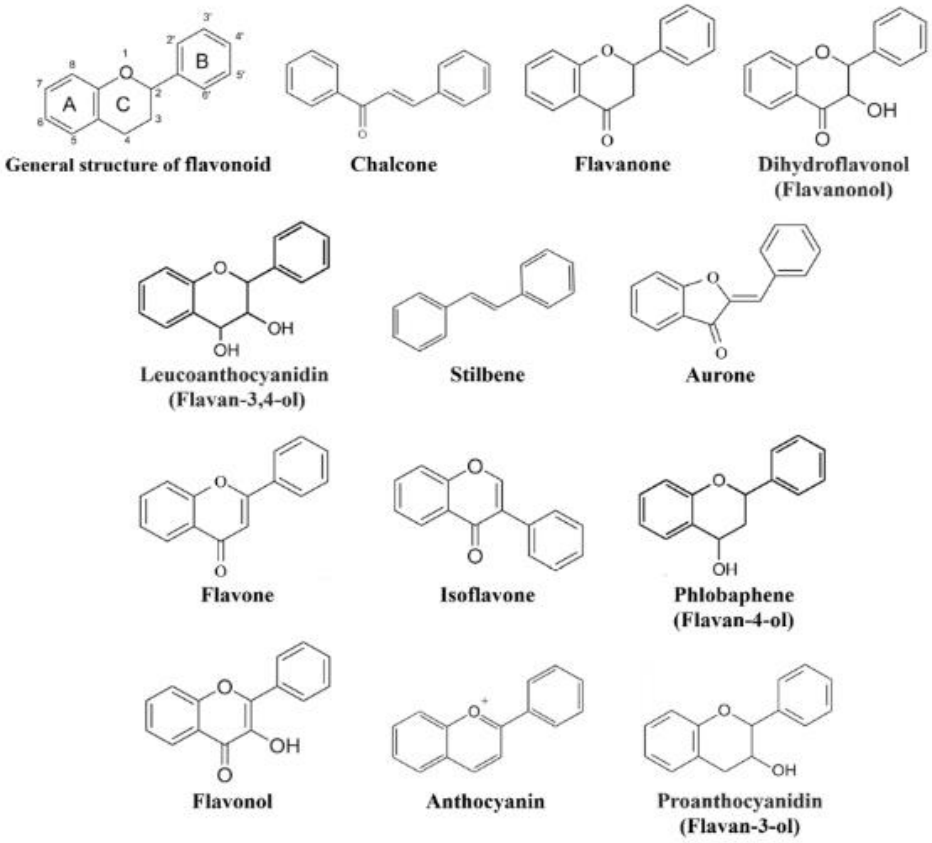


Figure 2 - General chemical structure of flavonoids and different subclasses [21].

Flavonoids have drawn increased attention for pest and disease management owing to their protective effects against insects and herbivores. Table 1 summarizes studies that have investigated the biopesticide potential of different flavonoid subclasses against various global pests. Owing to their low water solubility, flavonoids have been studied in their natural form and as coordination complexes with metals with antimicrobial properties, such as copper and ruthenium. Flavonoid encapsulation in natural biodegradable matrices has also been studied to increase its bioavailability and efficiency as a possible controlled release system in agriculture. Mainly studied as insecticides, flavonoids impact insects' development, behavior, and growth by interfering with their food uptake and reproductive system, as shown in Table 1. Therefore, flavonoids are promising substances that could be helpful in pest and disease management strategies for widely affected crops.

Table 1 - Broad description of some flavonoids researched as biopesticides for specific target organisms and the main results.

| Subclass | Flavonoid | Application | Target Organism | Host | Main Results | Reference |
|---|---|---|---|---|---|---|
| **Flavanone** | Hesperidin (HES) | Bactericide | *Xylella fastidiosa* | Sweet Oranges | In vitro, cis-[Mg(HES)$_2$(phen)]OAc complex was more active (minimum inhibitory concentration (MIC) of 1.4 µmol L$^{-1}$) than HES (3.3 µmol L$^{-1}$). In vivo, HES reduced *Xylella fastidiosa* cells to 16.99% of initial abundance, showing moderate activity | [25] |
| | | Insecticide | *Spodoptera frugiperda* | Corn | [Cu(phen)(HES)] complex increased larval mortality by 96.66% in comparison with the control | [26] |
| | Naringenin | Bactericide | *Xylella fastidiosa* | Sweet Oranges | In vitro, [Ru(narin)(phen)$_2$]PF$_6$ and cis-[Mg(narin)(phen)$_2$]OAc were more active complexes (MIC 0.19 and 34 µmol L$^{-1}$, respectively) than naringenin (7.3 µmol L$^{-1}$). In vivo, naringenin was less active (15.63%) than the respective ruthenium and magnesium complexes (0.12 and 0.65% of initial abundance) | [25] |
| | | Insecticide | *Acryrthosiphon pisum* | Pea | Increasing concentrations increased the pre-reproductive period, decreased fecundity, and increased mortality of adult apterae | [27] |

| Subclass | Flavonoid | Application | Target Organism | Host | Main Results | Reference |
|---|---|---|---|---|---|---|
| | | | *Spodoptera frugiperda* | Corn | All tested complexes were less toxic to *Spodoptera frugiperda* than [Cu(phen)(HES)] complex | [26] |
| **Flavone** | Luteolin | Insecticide | *Acryrthosiphon pisum* | Pea | Passive ingestion was completely blocked at 100 µg $cm^{-3}$ | [28] |
| | Tangeretin, Quercetogetin and 3,5,6,7,8,3',4-heptahydroxyflavone | Fungicide | *Aspergillus parasiticus* | Grains | High mycelium growth inhibition for *Geotrichum candidum.* Significant activity at 50 µg/mL to *Penicillium italicum*, *Colletotrichum gloesporioides*, and *Aspergillus parasiticus* | [29] |
| | | | *Penicillium italicum* | Citrus | | |
| | | | *Geotrichum candidum* | Citrus, Tomatoes, and Carrots | | |
| | | | *Colletotrichum gloesporioides* | Herbaceous and Fruits | | |
| | | | *Fusarium culmorum* | Cereals | | |
| **Flavonol** | Kaempferol | Fungicide | *Fusarium oxysporum* | Banana and Tomato | No inhibition of pure Kaempferol, with high inhibition when encapsulated in lecithin/chitosan nanoparticles | [30] |
| | Quercetin | Insecticide | *Bactrocera cucurbitae* | Melon | Reduced the egg hatching, larval period of second and third instar, larval and pupal weight of the second instar, percentage pupation and emergence of all instars, and food assimilation | [31] |
| | | | *Spodoptera litura* | Tobacco and Cotton | Negatively affected larvae growth, loss of pupae weight, and dose-dependent mortality effect | [32] |

| Subclass | Flavonoid | Application | Target Organism | Host | Main Results | Reference |
|---|---|---|---|---|---|---|
| | Rutin | | *Acryrthosiphon pisum* | Pea | Increasing concentrations increased the pre-reproductive period, decreased fecundity, and increased mortality of adult apterae | [27] |
| | | | *Helicoverpa armigera* | Pigeon pea | High concentrations inhibited larvae owing to the cessation of feeding | [33] |
| | | | *Spodoptera litura* | Tobacco and Cotton | Rutin had a significant effect on larval development, pupal mortality, and malformed adults | |
| **Isoflavone** | Genistein | Insecticide | *Acryrthosiphon pisum* | Pea | Passive ingestion was completely blocked at 1000 µg $cm^{-3}$ | [28] |
| | | | *Oedaleus asiaticus* | Grassland | Negatively regulates insulin-signaling pathway by inhibiting protein tyrosine kinase, resulting in suppressed growth and development | [34] |

## 2. Food Waste as a Source of Botanical Biopesticides: the particular case of Citrus

Food waste includes losses from agricultural fields (infestation and postharvest), the processing chain (avoidable and unavoidable losses during handling and distribution), and consumers. Annually, this waste accounts for 45% of fruit and vegetable production losses due to the lack of resource management and food security, resulting in environmental, social, and economic consequences [35,36]. Younger generations are becoming more aware of this problem owing to the growing knowledge of the environmental issues caused by food waste. For instance, the COVID-19 pandemic showed that among younger people, grocery shopping did not lead to higher consumption and waste because younger generations are better adapted to avoid and reduce food residues [37]. Despite this positive observation, approximately 1.3 billion tons of food waste are produced globally per year, resulting in an estimated economic loss of 1 trillion dollars [38]. This number is predicted to climb even further, probably reaching 2.1 billion tons of food wasted annually by 2030 [39]. This forecast is primarily the result of a continuously growing population that demands more food and its by-products. Traditional disposal methods are currently considered insufficient, expensive, and harmful to the environment owing to the mass production of waste. For example, landfilling causes the release of methane and carbon oxide, which are well-known greenhouse gases, during degradation by microorganisms. Additionally, it is associated with other environmental problems because illegal waste discharge can lead to soil contamination and water pollution. In addition to these issues, waste is an ideal substrate for developing potentially pathogenic organisms [40–42].

In light of this situation, food waste valorization in a circular bioeconomic manner has been a target of interest. Circular bioeconomy stands for the reduction of waste generation, with its recycling and reutilization, by employing several mechanisms that lead to economic growth [36]. Most approaches emphasize food residue reuse for extracting value-added products such as biomaterials and biofuels. Several studies have focused on the characterization of these residues using modern analytical techniques (especially chromatography and mass spectroscopy) to shed light on their potential nutritional and economic value and the extraction of these high-value bioactive compounds using green approaches. These studies include, for example, the valorization of spent-coffee grounds [43], grape stalks and pomace [44], avocado seeds [45], onion skin peels [46], and citrus peels.

Citruses are the most popular fruit group worldwide, owing to their attractive organoleptic properties (color, flavor, and aroma) and nutritional value. They belong to the Rutaceae family, particularly to the genus *Citrus*, which accounts for 1600 species. Only eight of these species are of economic interest, namely sweet oranges (*Citrus sinensis*), sour oranges (*Citrus aurantium* L.), lemon (*Citrus lemon* L.), mandarin or tangerine (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* L.), lime (*Citrus aurantifolia* L.), and citron (*Citrus medica* L.) [47]. Of these species, sweet oranges represent 55 to 70% of the world's total

citrus consumption, being the most cultivated and processed citrus fruit globally [48,49]. Morphologically, citrus fruits can be elongated or more spherical in shape but have a similar anatomical structure, as shown in Figure 3. They are an excellent source of nutrients, including carbohydrates (glucose, sucrose, pectin, etc.), proteins (polygalacturonases, pectin esterases, etc.), lipids (oleic and linoleic acids are the most abundant), vitamins (such as vitamins C and B), minerals (potassium, calcium, etc.), and dietary fibers (pectin, cellulose, hemicellulose, etc.). Additionally, citruses are abundant in highly valued bioactive compounds with health-promoting properties, such as flavonoids (HES, naringenin, tangeretin, etc.), terpenoids (mainly limonene), organic acids (such as citric, maleic, and oxalic acids), carotenoids (zeaxanthin), and synephrine alkaloids [50,51].

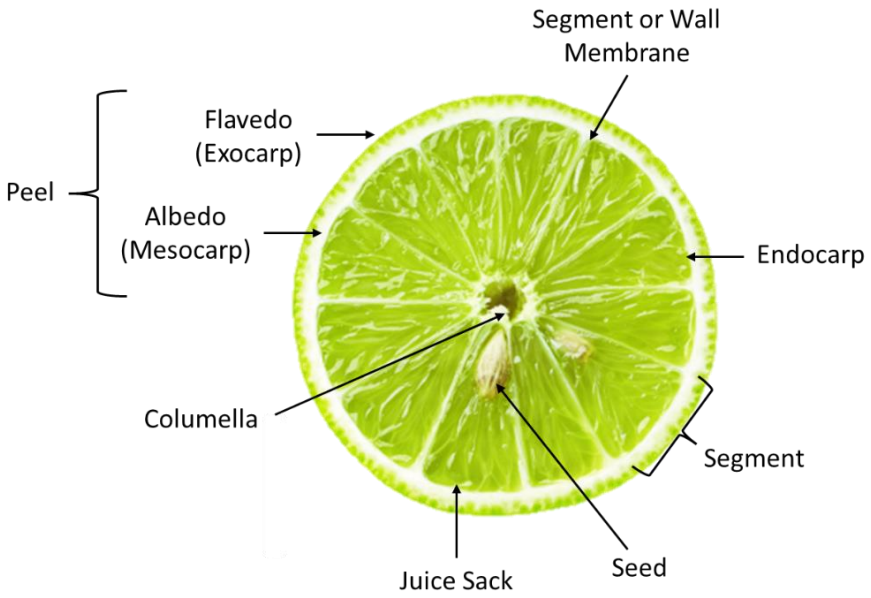


Figure 3 - Anatomical structure of citrus fruit.

Fruit processing industries strongly contribute to global warming due to the high energy requirements and associated problems with waste disposal. In the citrus processing industry, fruits are transported, graded, and after the removal of debris, they are subjected to postharvest cleaning treatments, such as water washing. Following juice extraction and treatment, this product is labeled and commercialized [51]. Only one-third of cultivated citruses are processed, producing around 50 to 60% organic waste, which makes this industry one of the highest contributors towards food waste. Peels, segment membranes, and seeds comprise this residue, accounting for about 50 to 70% of the total fruit mass [52]. Citrus waste disposal is challenging since the fruit moisture content ranges between 70 and 80%, making it a suitable substrate for the proliferation of microorganisms, including mycotoxin-producing fungi and insects [53]. Bearing this in mind, citrus waste, especially peel, has been explored for its potential application to reduce the amount of residue produced and disposal costs. After industrial processing, peel residue can be used directly in agriculture or incorporated into biorefinery systems (Figure 4). Citrus peel

(dry, ensiled, or fresh) can be utilized in animal diets as a partial substitute for cereals because of its nutritional value. Yet, the large-scale application of raw waste is an obstacle because of its high transportation expenses [42]. As an organic soil conditioner and compost, it can increase soil fertility and water retention capacity, and reduce water runoff and soil erosion. However, soil microbial biodiversity may be affected since peels, especially flavedo, are abundant in essential oils with antimicrobial properties. Biorefinery approaches offer significant advantages, as they maximize the value of citrus peel waste by obtaining a large variety of products, such as biofuels and value-added compounds. Currently, citric waste is used for food purposes as an additive or a prebiotic, but also for non-food applications such as biodegradable packaging materials and cosmetic products with anti-aging properties [53].

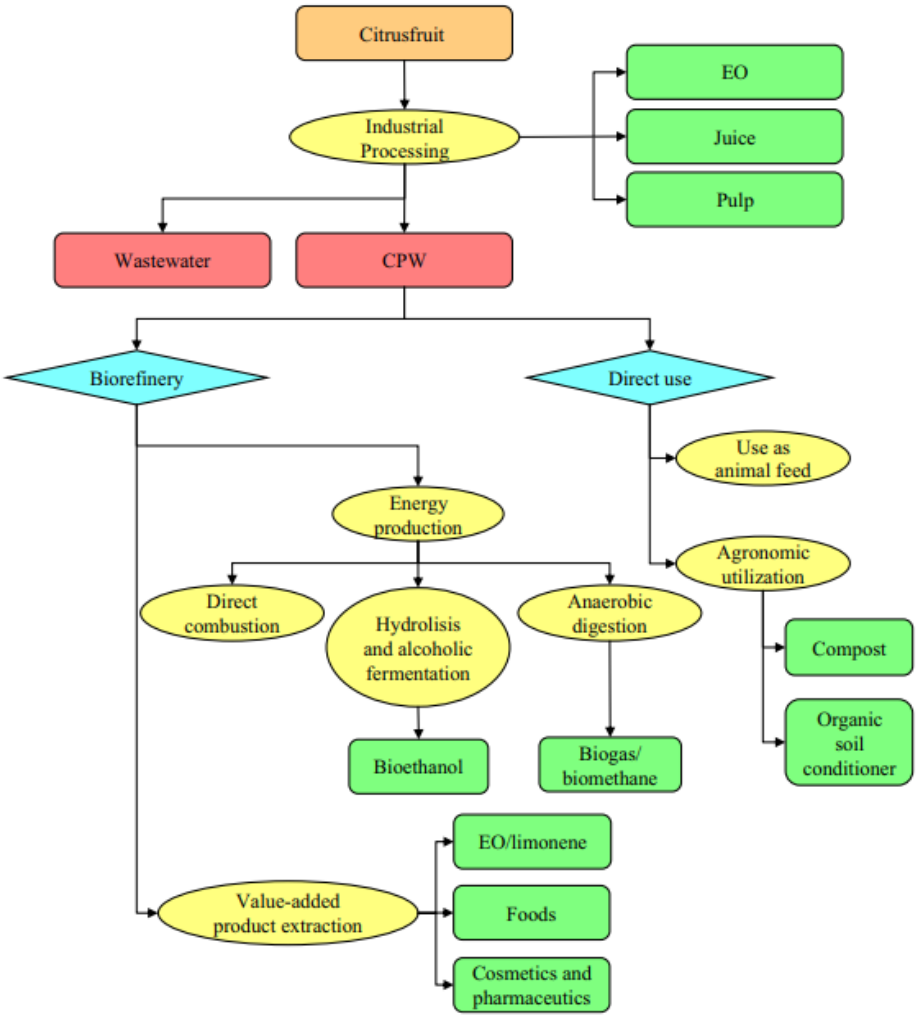


Figure 4 - Citrus peel waste valorization scheme in agriculture and biorefineries [42].

## 2.1. Citrus Flavonoids

The flavonoid biosynthesis pathway in citrus leads to the formation of different subclasses and intermediates (Figure 5). Flavonoid synthesis begins with the condensation of three malonyl-CoA molecules derived from the acetate-malonate pathway, with one molecule of p-coumaroyl-CoA, produced via the phenylpropanoid pathway. This reaction originates from the first important intermediate in this

pathway, naringenin chalcone, through the intervention of chalcone synthetase. Subsequently, chalcone isomerase isomerizes this metabolite to form the flavanone naringenin. Through the intervention of different enzymes, this key intermediate generates other flavanones, flavones, and dihydroflavonols. These groups give rise to flavonols, leucoanthocyanidins, anthocyanidins, and polymethoxylated flavones [54,55].

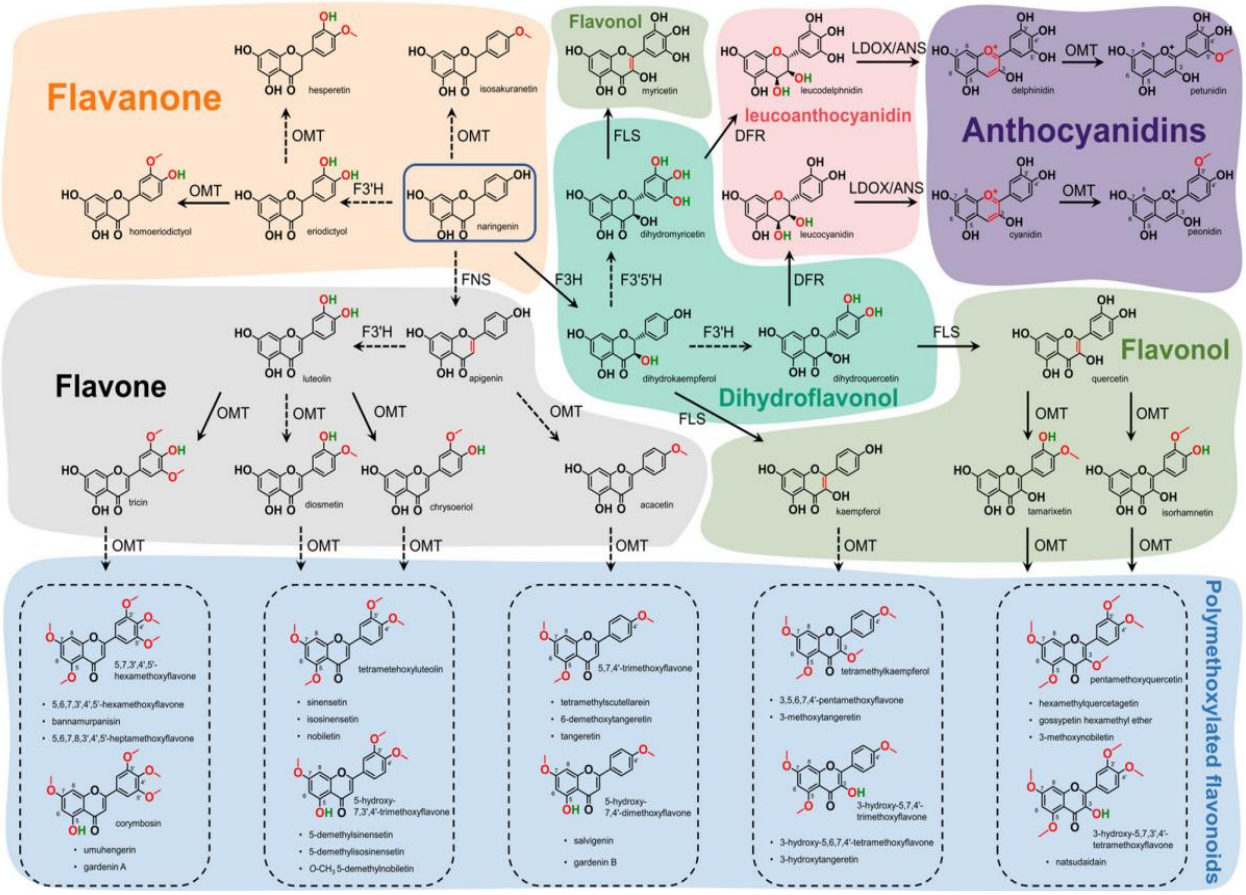


Figure 5 - Flavonoid biosynthesis pathway in citrus fruits [55].

Flavonoids are found in all citrus plant parts but are more abundant in the fruit peel. Flavanones are the predominant subclass, followed by flavones and flavonols. Nonetheless, citrus species greatly influence the flavonoid profile and content, in a similar way to other bioactive substances and nutrients. Although flavanones are the most abundant subclass in citruses, the most prevalent one changes between species. For example, HES is predominant in sweet oranges and mandarins, whereas naringin is found in sour oranges, grapefruits, and pummelos [50]. These differences rely mostly on variations in the genetic code and its expression. In most *Citrus* species, flavonoid concentration is higher during organ development, especially in the middle stages (60 to 80 days after pollination), decreasing during complete maturation, possibly due to the high expression of chalcone synthase and isomerase [54,56]. Therefore, plant age and the degree of fruit maturation affect flavonoid concentration. Nevertheless, citrus flavonoid content may also be affected by post-harvesting treatments [54].

Nowadays, a wide variety of citrus flavonoid applications are known and rely mostly on their antioxidant nature. The antioxidant activity of citric flavonoids may be attributed to their 3',4'-catechol structure, which acts as an inducer, and to the presence of a hydroxyl group at position C3, which is an enhancer [57]. Their ability to act via different redox mechanisms makes them interesting molecules for use as therapeutic agents for several diseases in the biomedical field, and as potential fungicides and bactericides. These compounds have been extensively studied as anti-inflammatory agents in medical and pharmaceutical fields. Research on the anti-inflammatory properties of citrus flavonoids has opened the door to exploring their application in disorders linked to chronic inflammation. These include neurodegenerative diseases (like multiple sclerosis, Alzheimer and Parkinson's disease), cancer (such as colon cancer), cardiovascular disorders (including angina and myocardial infarction), and obesity. Additionally, citrus flavonoids have been investigated as anti-allergic and anti-microbial agents, especially as antivirals. Owing to their anti-aging properties, they are also used in the cosmetic industry as active ingredients in creams [54,57,58]. On the other hand, citrus flavonoids are commonly used as additives in the food industry. Flavanones, such as naringin, are added to beverages and sweets to give them a bitter flavor. Anthocyanins are used as coloring agents in various food products to make them more appealing to consumers and to adjust or prevent discoloration induced during processing. Citric flavonoids can act as food preservatives by protecting against lipid peroxidation owing to their antioxidant capacity [54]. Due to their unique chemical structures, polymethoxylated and glycosylated flavanones are good markers for detecting adulterated juices [59]. In addition, citrus flavonoids have also been studied as biopesticides in agricultural practices, as previously shown in Table 1. HES stands out from other citric flavonoids given its research in all the aforementioned fields and its high concentrations in citrus waste.

### 2.1.1. Hesperidin

HES ($C_{28}H_{34}O_{15}$; 3',5,7-trihydroxy-4'-methoxy-flavanone-7-*O*-rutinoside; Figure 6A) is a flavanone glycoside with an aglycone and disaccharide named hesperetin and rutinose, respectively. This flavonoid is a pale-yellow crystalline powder that is soluble in solvents such as pyridine, dimethyl sulfoxide, formamide, and highly alkaline aqueous solutions. It has low solubility in methanol and glacial acetic acid, and is insoluble in most organic solvents, including ether, acetone, chloroform, and benzene [60]. In citruses, it is found in higher concentrations in peel and is the most abundant flavanone of several species, such as sweet and red oranges (28.6 and 43.6 mg/100 mL of juice, respectively), clementine (39.9 mg/ 100 mL of juice), and lemon (20.5 mg/100 mL of juice) [61]. Apart from these fruits, this flavonoid was also identified in other plant species, such as *Mentha piperita* L. (504.2 mg/L) and *Stevia rebaudiana* (493.4 mg/L) [62]. Even though HES predominates in nature, hesperetin (Figure 6B) is the main responsible for its bioactivity. Hesperetin can be isolated via acid or enzymatic hydrolysis of hesperidin [63,64].

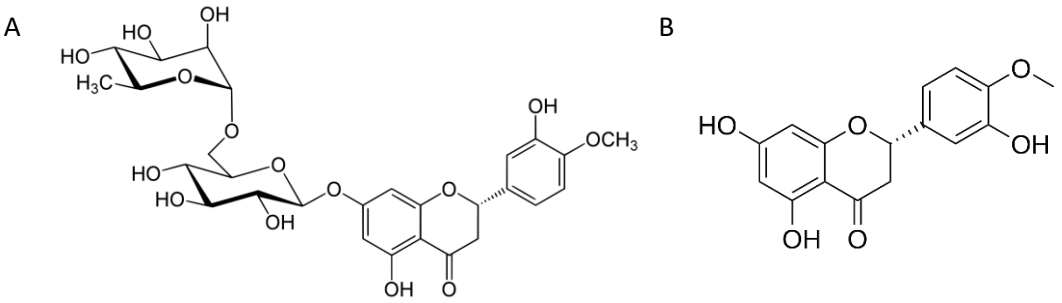


Figure 6 - Chemical structure of HES (A) and hesperetin (B).

HES has been extensively studied for its biological and pharmacological properties, which are briefly summarized in Figure 7. This flavanone has proven antioxidant [65,66] and anti-inflammatory [67,68] activities, which potentiated HES investigation as an anticancer [62,69,70], antiviral [61,71,72], anti-obesity [73], anti-aging [60], cardio- [64] and neuroprotective [74,75] agent. Besides these biomedical applications, HES has also drawn growing interest as a biopesticide, as previously shown in Table 1. HES's insecticidal effect (pure or in coordination complexes) may be attributed to its inhibitory activity against acetylcholinesterase [76,77]. However, the mechanisms underlying its antibacterial properties are not fully understood.

**Antioxidant**

- HES antioxidant activity relies on its radical scavenging activity and antioxidant cellular defenses via the ERK/MAPK and Nrf2 signaling pathways [65,66]

**Anti-inflammatory**

- HES enhanced self-renewal ability and chondrogenesis of mesenchymal stem cells by inhibiting pro-inflammatory cytokines [67]
- Commercialized with diosmin as Daflon® to treat venous circulation disorders [68]

**Neuroprotective Effects**

- Improves neural growth factors and endogenous antioxidant defense functions, decreasing the neuroinflammatory and apoptotic pathways [73]
- Has anxiolytic, sedative, anticonvulsant, antinociceptive, and antidepressant effects [74]

**Anticancer**

- HES anticancer mechanisms relies on apoptosis induction and cell cycle arrest at different checkpoints (G1, G2/M, or S phase) [62,68]
- In vitro and in vivo, HES induced pinX1 protein expression, an important protein in regulating lung cancer progression [69]

**Cardioprotective Effects**

- HES lowered plasma-free fatty acids, plasma and hepatic triglyceride levels, and hepatic fatty acid oxidation. As a result, decreased lipid accumulation in adipocytes and the risk of developing atherosclerosis [64]

**Antiviral**

- In silico studies, HES has low binding energy with the main protease and spike proteins of SARS-CoV-2, showing a strong and specific bond [61]
- In vitro, HES showed a moderate efficacy (41%) against Hepatitis B virus replication [71]
- In vitro, glucosyl HES inhibited Influenza A Virus replication [70]

**Anti-aging**

- HES derivates are inhibitors of hyaluronidase
- Potential topical UV-protective agent
- Anti-photoaging factor by regulating the metalloproteinases MMP-9 via the MAPK pathway [60]

**Anti-obesity**

- Regulates lipid and glucose metabolism by mediating AMPK and PPAR signaling pathways
- HES-enriched dietary supplements can improve postprandial hyperglycemia and hyperlipidemia symptoms [72]

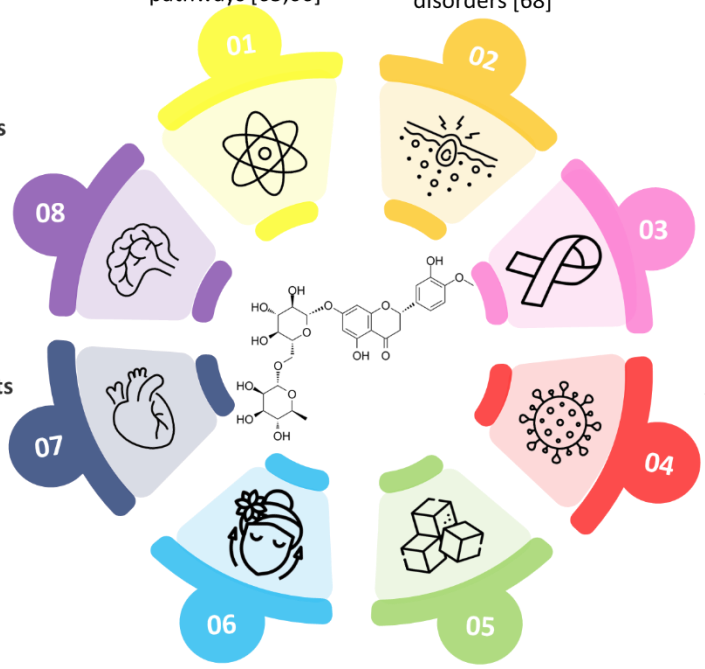


Figure 7 – HES biological and pharmacological properties.

#### 2.1.1.1. Extraction Methods

As a result of extensive research into the potential applications of HES in different industries, special attention has been given to developing and optimizing high-yielding extraction methodologies that lead to the highest purity possible. Several methods have been explored for HES extraction, most of which use citrus peel waste as HES source. These methods differ from one another in terms of the chosen citrus matrix, pre-treatment, extraction technique (conventional or non-conventional), solvent, and physical-chemical parameters applied. Several solvents have been proposed as extractants, including organic solvents, generally recognized as safe solvents (GRAS), ionic liquids, and deep eutectic solvents (DES). Time, temperature, liquid-to-solid ratio, frequency, and pressure are among the extraction parameters that are usually optimized to increase efficiency.

Conventional methods, such as maceration and Soxhlet extraction, are among the most commonly reported techniques for HES. These are often associated with organic solvents, especially methanol, and ethanol (also a GRAS). For example, Victor *et al*. [52] performed hot methanol maceration for dry and fresh orange albedos, obtaining the highest yield and purity (2.8 and 89.4%, respectively) with the raw samples. Cypriano *et al*. [78] described a low-yielding Soxhlet extraction (1.2%), performed with methanol and dry orange pulp, and high purity after recrystallization (92.6%). While effective, methanol and other organic solvents are highly toxic and harmful to the environment. Researchers have lost interest in conventional methods due to long extraction times, large volumes of solvents, high energy costs, and the necessity to perform repeated crystallizations to achieve higher purity. Green extraction techniques have gained attention from the scientific community for their eco-friendlier impact, owing to solvent volume reduction, low energy consumption, higher performance, and automatization [79]. As a result, recent studies have focused on developing HES extraction methodologies based on greener techniques and solvents.

Ultrasound-assisted extraction is one of the most frequently described methods for HES extraction because it is non-laborious and effective with short extraction times. Ma *et al*. [80] studied the effect of different solvents, frequencies, extraction times, and temperatures on HES yields from tangerine peel. The authors verified that the best conditions were methanol as the solvent, a frequency of 60 kHz, an extraction time of 60 minutes, and a temperature of 40 °C. Due to this technique's flexibility in terms of the type of solvent employed, DES were recently suggested as HES extractors and potential replacements for methanol. Xu *et al*. [81] performed an extensive comparative study of the flavonoid extraction efficiency using different types of DES and organic solvents. This study demonstrated that an 80% aqueous solution of choline chloride-levulinic acid-n-methyl urea exhibited better performance in extracting flavonoids, including HES, than methanol under the same ultrasonic conditions (25 min, 50 °C, and 1:50 g/mL of peel). Besides this technique, other methods combining different solvents have been described

for HES extraction, these being microwave-assisted extraction [82,83], supercritical fluid extraction [84], pressurized liquid extraction [85], and pulsed electric field [86].

HES alkaline extraction from citrus waste has been suggested as a greener alternative to the aforementioned methodologies. These extractions are based on the addition of an aqueous sodium hydroxide solution to the citrus peel to pH 11. Depending on the method used, this mixture can be left on the bench for 24h or heated at 40-45 °C for 1h. Then, the mixture is filtrated, and the pH of the filtrate is lowered to 4-6 by the addition of an aqueous hydrochloric acid solution. This solution can be left on the bench for several hours or heated at 40-45 °C for 12-24h, which triggers HES precipitation [87,88]. Other protocols mention the addition of aqueous calcium chloride solutions to citrus peel before increasing the pH to disrupt the interactions between HES and pectin [60]. Even though this method avoids the use of organic solvents, it requires significant amounts of alkali and acidic solutions and is time-consuming. Similar to other reported methodologies, other compounds, such as other flavonoids, are equally extracted alongside HES [87].

#### 2.1.1.2. Purity Determination

HES purity is mainly determined by chromatographic methods, especially high-performance liquid chromatography coupled with a diode array detector (HPLC-PDA). These analyses are carried out in the reverse stationary phase, such as C18 columns, but usually differ in mobile phase composition and type of elution. The most common eluent mixtures are methanol and water, with or without phosphoric acid, and methanol, water, and acetic acid [52,60,80,89]. Even though this analytical technique is considered accurate and reliable for purity analysis, one of its major drawbacks is that some methods require large amounts of organic solvents for the mobile phase.

Quantitative nuclear magnetic resonance (qNMR) has been explored as an attractive technique for purity analysis of a wide range of organic molecules, agrochemicals, and pharmaceuticals. In this quantification method, the peak area of a signal is directly proportional to the number of nuclei, which allows direct measurement of analyte purity through the integration of the analyte and internal or external standard signals [90,91].The use of an adequate internal standard (IS) facilitates the overall purity determination procedure as calibration curves are unnecessary. However, external standards and electronic reference methods have been described as well. Moreover, this technique can be applied to a wide variety of nuclei, for instance, $^{19}F$ and $^{31}P$, allowing the purity assessment of a wide range of organic molecules [91]. Nonetheless, $^{1}H$-qNMR is more commonly used given its rapid acquisition. qNMR offers significant advantages over chromatographic methods because it requires low amounts of sample and solvent, is nondestructive, operates at low temperatures, and reduces the analysis time, especially when working with $^{1}H$ nuclei [92]. To date, no qNMR method has been proposed for HES purity assessment.

## 3. Pesticide Encapsulation

Even though pesticides' noxiousness to the environment and human health is well known, their utilization is indispensable in agriculture for crop viability. Although biopesticides have emerged as potential complements for conventional pesticides, they lack efficiency and stability. In this regard, researchers have focused on developing new formulations and pesticide encapsulation has been proposed to overcome this issue. Enclosing a pesticide within a material allows for controlled release over time and protects the pesticide from premature degradation by, for example, photolysis and microbial degradation [93]. This controlled release also reduces the loss of pesticides through leaching and evaporation, and the exposure of non-target organisms to pesticides, making them safer for the environment and human health. Other advantages include targeted delivery, increased shelf life, improved stability, and increased water solubility. As a result, encapsulation allows for increased treatment efficiency while reducing the need for reapplications, pesticide concentration, and toxicity to non-target organisms [4,13].

Owing to advances in nanotechnology, the nanoencapsulation of pesticides has gained the interest of the scientific community because pesticide-loaded or -entrapped nanomaterials exhibit more beneficial physicochemical properties (crystallinity, thermal stability, stiffness, etc.) than traditional formulations. In addition, nanopesticides are easily absorbed into the lipid layers of insects, disrupting the water protection barrier and causing insect death via desiccation [93]. Although nanoparticles usually have a size range of 1-100 nm, most of the so called nanopesticides are larger than 100 nm [94–96]. Some reasons include the complexity of the pesticide (particularly for essential oils and extracts), the physicochemical properties of the encapsulating material, the method used to perform the nanoparticle preparation, and the technique used to determine the size. Nonetheless, several materials have been reported, including those based on polymers and lipids, inorganic porous materials, and clays. Nanocapsules, nanospheres, micelles, and other structures have also been explored for pesticide encapsulation [97].

### 3.1. Polysaccharides as Safe Release Systems

Polysaccharides are natural polymers consisting of repeated units of monosaccharides linked by glycosidic bonds. Depending on the type of monosaccharides present, they are categorized as homopolysaccharides or heteropolysaccharides. They can be linear, branched, or cyclic in structure, and have different charges (neutral, positive, or negative). Polysaccharides are abundant in nature, particularly in higher plants, algae, animals, and microorganisms, where they play two essential roles: energy storage and structural support [98,99]. They offer significant advantages over other materials in the controlled release of pesticides. They are widely available in nature and are biodegradable, biocompatible, non-toxic, and cheap. Their high versatility in functional groups makes them appealing as encapsulating materials because these groups can be chemically modified to improve the mechanical strength and better control of pesticide release [98]. Consequently, it is common to find studies using these natural polymers in

different encapsulating forms, either alone or in combination with other polysaccharides or materials [30,100].

Several mechanisms have been proposed for the release of pesticides from natural polymers, with the most common ones illustrated in Figure 8. Desorption occurs when pesticide molecules are adsorbed or weakly bound to the nanoparticle surface, resulting in a high initial release [101]. The diffusion mechanism relies on the mass transfer of pesticide molecules from one part of the system to another. This method depends on different diffusion rates and polymer chain relaxations [102]. Pesticide release by matrix erosion occurs when polysaccharides undergo chemical and/or mechanical reactions that lead to matrix disruption [101]. Pesticide release can be triggered by various external factors independently of the mechanism. These include variations in temperature, soil pH, light, solvents, mechanical stresses (pressure, shaking, and ultrasonic waves), and enzymes [103].

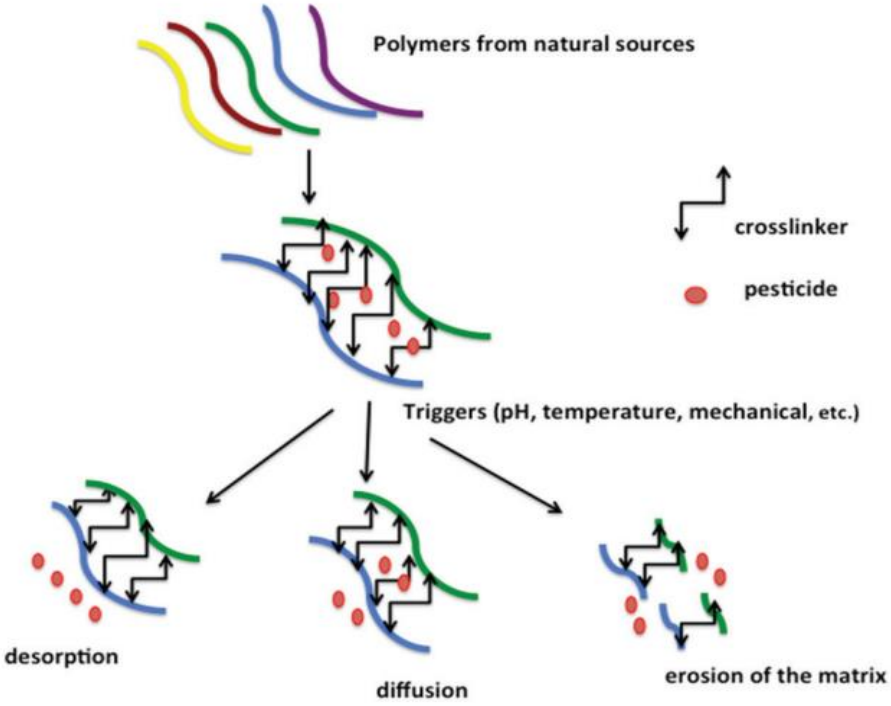


Figure 8 - Mechanisms of pesticide release from polymer matrix [102].

Several polysaccharides have been explored as encapsulating materials for controlled release of pesticides. Although alginate is the most commonly reported, chitosan and pectin have also been proposed for this purpose.

### 3.1.1. Chitosan

Chitosan (Figure 9) is a deacetylated linear copolymer of chitin, a structural polysaccharide found on crustaceans' shells, crabs' and shrimp exoskeletons, and mushrooms' cell walls [104–106]. Alkaline treatment of chitin influences the physicochemical properties of this polysaccharide, such as the deacetylation and polymerization degree, viscosity, and molecular mass. Chitosan is a positively charged polymer in acidic pH, that interacts with molecules or adheres to surfaces with opposite charges [107].

Consequently, chitosan-based drug delivery systems have been widely studied in biomedicine. It is commonly used as a biostimulant in agriculture because it induces plant defense responses and enhances plant growth and resistance to abiotic factors [104,108]. Owing to its antimicrobial activity, chitosan has been used to inhibit the growth of pathogenic fungi on, for example, postharvest fruits [109]. Another common application of chitosan is as a carrier of plant growth regulators, nutrients, and agrochemicals such as pesticides and fertilizers [106].

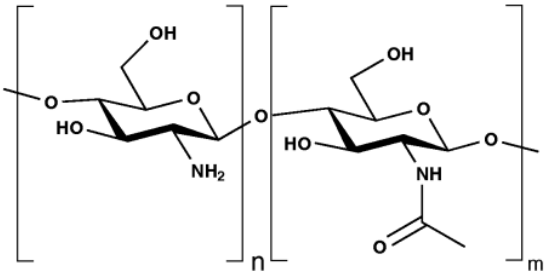


Figure 9 - Chemical structure of chitosan (adapted from [106]).

### 3.1.2. Pectin

Pectin is an anionic structural heteropolysaccharide found in plant cell walls. This linear polymer has a main chain or backbone composed of hundreds to thousands of D-galacturonic acid monomeric units (approximately 70% of the total pectin) linked by α-(1→4) glycosidic bonds [110]. As shown in Figure 10, side chains composed of different sugars can be attached to the main structure, resulting in different types of pectin. Due to its gelling properties, pectin has been used as an encapsulation material in the food and pharmaceutical industries and for the delivery of agrochemicals and plant growth factors [94]. Their degradation results in D-galacturonic acid oligosaccharides, which promote fruit ripening and growth [111]. Pectin's role in plants is extensive, with some examples of its involvement in responses against abiotic factors (like saline and osmotic stress), intercellular communication, and morphogenesis [112].

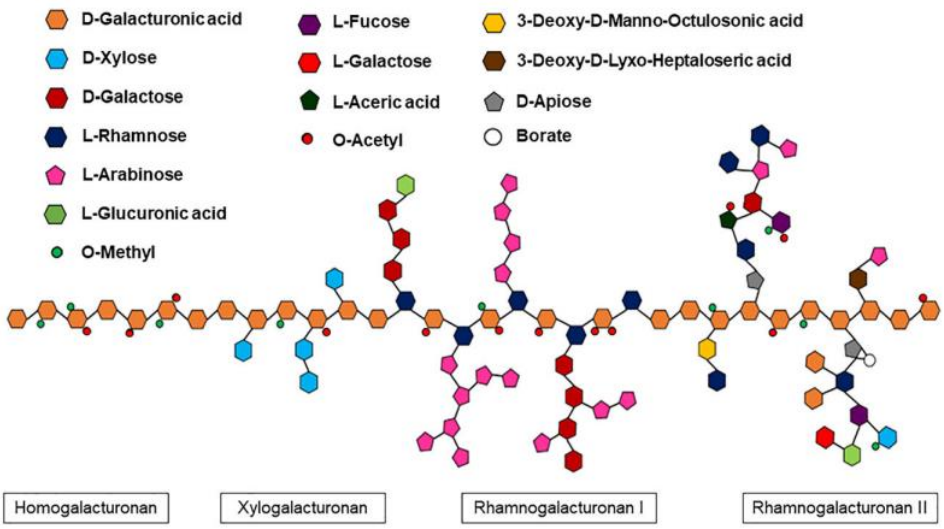


Figure 10 - Chemical structure of four pectin polysaccharides [109].

## 3.2. Encapsulation Approaches

Different encapsulation methods have been described for the preparation of nano-controlled-release systems for bioactive molecules based on polysaccharides. The chosen technique depends on several factors, such as the nature of the bioactive molecule, particle size, stability, kinetic release profile, and toxicity [113]. Some of these methods include complex coacervation, desolvation, emulsification, ionotropic gelation, nanoprecipitation, and spray-drying, which are briefly described below. Their main advantages and drawbacks are summarized in Table 2.

Complex coacervation is a physicochemical method based on the ionic interactions between two or more polysaccharide solutions with opposite charges. This interaction in aqueous medium results in two immiscible liquid phases: a polymer-rich dense phase, also known as a coacervate, and a dilute continuous phase. This method is divided into three steps: emulsion formation, coating formation, and stabilization. First, two or more polysaccharides are dissolved in an aqueous medium. A hydrophobic solution containing the core material (such as oil) is dispersed in this medium, and the resultant mixture is homogenized to produce a stable emulsion. Coacervation and phase separation are induced by changes in pH, temperature, or the addition of salt. The coating of the resulting particles, known as coacervates, is hardened, and stabilized by thermal treatment, crosslinking, or addition of desolvation agents. Their physical properties are affected by a wide variety of factors, such as the molecular weight and ratio of polysaccharides, charge density, solution pH, pressure, temperature, ionic strength, and stirring speed [114–116].

The desolvation method is frequently used to formulate polymer-based nanoparticles. In this technique, desolvating agents (including salts, alcohol, or other solvents) are added dropwise to an aqueous solution of one or more polysaccharides. This addition induces polymer precipitation to form nanoparticles owing to the dehydration of the polymer chains [113]. The selection of an appropriate

desolvation agent depends on the nature of the active compound. Finally, these particles are often stabilized by crosslinking [117].

Emulsification is a two-step procedure that is commonly used to prepare polysaccharide-based nano- and microparticles. In this method, polysaccharides are dissolved in water or other solvents and dispersed in an oil phase, forming an emulsion through stirring or sonication [113,115]. Different emulsions can be prepared, particularly direct emulsions (oil-in-water), inverse emulsions (water-in-oil), and other more complex emulsions. Several methods are known to formulate nanoparticles from emulsions, including solvent evaporation, solvent diffusion, and salting-out [117].

Ionotropic gelation is one of the most commonly described methods for the synthesis of nano- and micro-particles. This physicochemical method relies on the formation of a three-dimensional network structure via electrostatic interactions between two opposite charged compounds. In this technique, these compounds can be two polysaccharides (for example, chitosan and alginate) or one polymer and a metal ion (for example, pectin and calcium ion ($Ca^{2+}$)). The bioactive molecules are dissolved in the polymeric solution, and the counterion solution is added dropwise. Another way is to add this polymeric solution to the counterion solution [116,118]. This typically occurs in an aqueous medium at room temperature under high mechanical stirring, forming an opaque solution. Particles are collected by centrifugation. The physicochemical properties of the resultant particles are influenced by the concentration/ratio of the two ionic species, pH, and stirring speed [119].

Nanoprecipitation, also known as solvent displacement and interfacial deposition, is a one-step method for formulating nanoparticles in a colloidal suspension. Polysaccharides and bioactive compounds are dissolved in an organic solvent that is highly soluble in water and easily evaporated (for example, acetone) [117]. This solution is added dropwise into an aqueous phase containing a surfactant. Drug-loaded nanoparticle precipitation occurs through the rapid diffusion of the organic solvent into the aqueous phase. Particles are collected by solvent evaporation, dialysis, or lyophilization [115]. The organic solution injection rate, aqueous phase's stirring speed, and ratio between these phases influences the nanoparticles physicochemical properties [119].

Spray-drying is a physical method commonly used to convert liquids into powders, granules, or agglomerates. In this technique, a liquid containing the coating agent and bioactive molecules is atomized into small droplets through a nozzle. Then, the aerosol droplets are rapidly dried owing to their contact with hot gas that evaporates their solvent or moisture, which leads to particle formation [116]. The spray flow rate, atomization pressure, nozzle size, inlet air temperature, and extent of crosslinking are among the parameters that affect particle size [115,119].

Table 2 - Advantages and disadvantages of some methods for nanoencapsulation of bioactive molecules.

| Encapsulation Method | Advantages | Disadvantages | References |
|---|---|---|---|
| **Complex Coacervation** | Ideal for high-value active molecules or unstable substances | Low control over particles size and shape<br>High production cost | [115,116] |
| **Desolvation** | Simple procedure | Purification step needed<br>Possible toxicity of desolvating agents | [115] |
| **Emulsion-based** | Controlled size and drug loading | Additional steps required when compared with other methods<br>Requires large amounts of organic solvents | [115] |
| **Ionotropic Gelation** | High encapsulation efficiency<br>Cheap, easy, and fast (<10h)<br>Avoid organic solvents | Large particles (100-300 nm) with high polydispersion indices (usually >0.1 and <0.5) | [118] |
| **Nanoprecipitation** | Well-defined size and distribution<br>Easy, fast, and simple procedure | Low productivity<br>System blocking<br>Use of organic solvents | [115,119,120] |
| **Spray-drying** | Rapid drying method Low cost, flexible, high-yielding, and stable particles | Large size distribution | [116] |

## 4. Objectives of the Dissertation

The main goals of this dissertation fall within some of the main goals of the MACBIOPEST project research fellowship. In this regard, this dissertation can be divided into two major objectives:

(a) To develop a new consecutive extraction scheme for two bioactive compounds from citrus waste, pectin and HES, following the principles of green chemistry and circular economy. In more detail, to develop a sustainable extraction scheme focused on solvent recovery and maximization of the waste potential, and to create a new HES extraction and precipitation methods.

I. Compare the new method with one of the standard HES extraction methods (methanolic extraction) to evaluate efficiency, selectivity, and environmental impact by characterizing the isolated HES by diverse techniques such as Fourier transform infrared spectroscopy coupled with a universal attenuated total reflection (FTIR-UATR), nuclear magnetic resonance (NMR), HPLC-PDA, and melting point;

II. To perform preliminary studies with qNMR (using the internal standard method) to evaluate the possibility of using this technique to assess HES purity.

(b) To study and compare two different polysaccharides, chitosan and pectin, as HES controlled release systems for biopesticide application. More specifically, the encapsulation of HES in nanoparticles of these polysaccharides and characterization of their physicochemical (by dynamic light scattering (DLS)), chemical groups (using FTIR-UATR), antioxidant activities (by two different methods), in vitro release profiles, water absorption capacities, and stability studies (at two different temperatures in the dark).

# II. Methods and Experimental Procedure

# 1. Materials and Equipment

## 1.1. Orange Raw Material

Orange peel was collected from the university student bar. Fresh peels were washed with water to remove possible detritus, and flavedos and excess pulp were removed. After that, albedos were cut into coarse pieces and, if not immediately used, stored at -18 °C.

## 1.2. Reagents, Standards, and Equipment

Information regarding the reagents, standards, and solvents used is described in Supplementary Information Section Table S1, while the equipment and materials are listed in Table S2.

# 2. Hesperidin Extraction Methodology

## 2.1. Extraction Method I: Methanolic Extraction

Hot methanolic extraction was carried out according to the procedure described by Victor *et al*. [52], although with some modifications. 13.4 g of oven-dried albedos (at 40 °C until constant weight) were weighed in an Erlenmeyer and 60 mL of methanol were added. The mixture was magnetically stirred for 3 hours at 55 °C in a thermostatic water bath with submerged stirring plates. After this period, the organic solvent was filtered by gravity into a distillation flask and a new extraction took place using the same sample material and 60 mL of methanol. This second extraction step was performed at the same temperature for 30 minutes. The organic phases were combined and evaporated in the rotary evaporator, at 40 °C, to a viscous brownish extract. For HES crystallization, 20 mL of water were added to the extract, and this mixture was stirred at 65 °C for 30 minutes. Subsequently, 3 mL of dichloromethane were added to the hot mixture. During this addition, the formation of bubbles and yellowish powder were observed. The HES crystals were isolated by vacuum filtration using a sintered glass Buchner funnel, washed with cold distilled water and acetone, and transferred to a Petri dish for drying until a constant weight was achieved.

## 2.2. Extraction Method II: Consecutive Extraction

Fresh albedos were submitted to two treatments before pectin and HES extractions to remove or inactivate possible interferents, such as monosaccharides, pigments, organic molecules, and pectic enzymes [121]. These pretreatments followed a previously described method with some modifications [121]. The first one consisted of blanching albedos in water at 90 °C for 1 hour at a solid-to-liquid ratio of 1:25 (w/v) using a heating and stirring plate. The mixture was allowed to cool to room temperature, filtered through a food-grade filter, and dried in a food dehydrator at 50 °C until constant weight. Blanched albedos were mechanically ground using a domestic grinder and subjected to a second pretreatment, which consisted of removing alcohol-soluble solids (AIS). In this step, albedo powder was washed with ethanol at 70 °C for 20 minutes at a solid-to-liquid ratio of 1:10 (w/v). The mixture was filtered using a Buchner

funnel and the resulting solid was dried under the same conditions as previously mentioned. The ethanol was reused for further pretreatment and extraction after distillation.

Pectin was extracted according to the method described by Labrada *et al*. [122]. Pretreated albedos were placed in acidic conditions (pH around 1.5) with a 0.1 M aqueous hydrochloric acid (HCl) solution at a solid-to-liquid ratio of 1:15 (w/v). The mixture was mechanically stirred for 1 hour at 75 °C on a heating plate. After cooling and filtration, pectin precipitation was achieved by adding 3 volumes of ethanol. The suspension was incubated overnight at 8 °C, followed by centrifugation at 4000 rotations per minute (rpm) for 15 minutes. The supernatant was discarded, and the pectin pellet was washed with distilled water and twice with two volumes of acetone to remove the remaining pigments and other impurities. Similar to ethanol, acetone was reincorporated in the washing steps after distillation. Pectin and the solid residue obtained from the extraction were dried and ground in the same conditions as stated before. Solid pectin was dissolved in water and re-precipitated with ethanol for further purification.

HES extraction was conducted under alkaline conditions (pH around 9 and 12) using the solid fraction obtained from the pectin extraction. First, the solid was suspended in ethanol, and this mixture was basified by adding a 0.1 N aqueous solution of sodium hydroxide (NaOH) and the pH was controlled using a pH meter. The extraction parameters of temperature (50–80 °C), extraction time (30–180 minutes), and solid-to-liquid ratio (1:10-1:20 (w/v)) were assessed for this extraction, and therefore, each condition was performed in triplicate. After extraction, the mixture was vacuum-filtered using a Büchner funnel, and the filtrate was neutralized with a 0.25 N aqueous HCl solution to pH 6-7. HES precipitation occurred during ethanol recovery in the rotatory evaporator, at 50 °C, and the suspension was kept overnight at 8 °C. HES was recovered by vacuum filtration with a sintered funnel and washed with cold distilled water and acetone. The obtained HES powder was dried under the same conditions as described previously.

## 2.3. Characterization

### 2.3.1. Extraction Yields

Pectin and HES yields were determined gravimetrically. Pectin yields were estimated by comparing the mass of extracted pectin to the mass of dry albedo obtained before the pretreatment. HES yields were calculated by comparing the mass of the isolated HES with the initial mass of the dry albedo. For Extraction Method II, this mass corresponded to the value obtained before the pretreatments, which was estimated using the fresh albedo weight and the corresponding moisture percentage (of 82.43 ± 0.46 %, determined with a moisture analyzer).

### 2.3.2. FTIR-UATR

Samples of extracted and standard pectin and HES were characterized by FTIR-UATR. All spectra were acquired by scanning in the range of 4000-400 $cm^{-1}$ and performing 32 scans, with a resolution of 4 $cm^{-1}$.

### 2.3.3. Equivalent Weight, Methoxyl Content, Anhydrouronic Acid Content, and Degree of Esterification

The pectin equivalent weight (EW) was determined (Equation 1) by adapting to previous works [123]. Pectin (0.5 g) was moistened in 5 mL of ethanol, followed by the addition of 1 g of NaCl and 100 mL of distilled water. This mixture was mechanically stirred until it reached homogeneity, and six drops of phenol red solution were added. The solution was titrated against a 0.1 NaOH aqueous solution until it reached the endpoint (transparent solution turned bright pink). For more rigorous measurement, this step was controlled using a pH meter.

$$\text{EW (g/mol)} = \frac{\text{Pectin mass (g)}}{V_1\ \text{(Volume of NaOH)} \times 0.1\ \text{(Normality of NaOH)}} \quad (1)$$

To determine the pectin methoxyl group content (MeO), 25 mL of 0.25 N NaOH aqueous solution was added to the previous solution. After stirring for 30 minutes at room temperature, 25 mL of 0.25 N HCl aqueous solution was added, and this solution was titrated against 0.1 N NaOH [123]. The titration was controlled using a pH meter. MeO was calculated using Equation 2, where 31 is the molecular weight of MeO (g/mol).

$$\text{MeO (\%)} = \frac{V_2\ \text{(Volume of NaOH)} \times 0.1 \times 31}{\text{Pectin mass (g)} \times 0.1} \quad (2)$$

The anhydrouronic acid content (AUA) and degree of esterification (DE) were calculated using Equations 3 and 4, respectively [124]. 176 represents the molecular weight of anhydrouronic acid (g/mol).

$$\text{AUA (\%)} = \frac{176 \times 0.1V_1}{\text{Pectin mass (g)} \times 0.1} + \frac{176 \times 0.1V_2}{\text{Pectin mass (g)} \times 0.1} \quad (3)$$

$$\text{DE (\%)} = \frac{176 \times \text{MeO (\%)}}{31 \times \text{AUA (\%)}} \times 100 \quad (4)$$

### 2.3.4. Melting Point

The melting point of standard and extracted HES was determined by establishing a temperature range of 210 to 260 °C, with a temperature gradient of 3 °C per minute.

### 2.3.5. HPLC-PDA analysis

The purity of standard and extracted HES was determined by HPLC-PDA analysis[1]. HES solutions at 0.1 mg/mL concentration were prepared in triplicate in a solvent mixture of dimethyl sulfoxide (DMSO) and ultrapure water (UPW; 1:9, respectively), followed by filtration with 0.45 µm cellulose acetate filters. The chromatographic conditions were as follows: Kinetex 5u $C_{18}$ LC column (150 x 4.60 mm); mobile phase A: acetonitrile, B: distilled water with 0.1% acetic acid; flow rate of 600 µL/minute; injection volume of 20 µL; UV wavelength: 290 nm. Table 3 resumes the elution gradient applied, the return and equilibrium periods.

Table 3 – Chromatographic conditions for HPLC-PDA analysis used for HES purity determination.

| Parameter | Time (minutes) | Phase B concentration (%, v/v) |
|---|---|---|
| **Elution Gradient** | 0 | 80 |
| | 3 | 70 |
| | 5 | 40 |
| | 7 | 40 |
| | 10 | 10 |
| | 12 | 10 |
| **Return** | 14 | 80 |
| **Equilibrium** | 22 | 80 |

### 2.3.6. NMR

#### 2.3.6.1. Qualitative NMR

Standard and extracted HES were analyzed by $^{1}$H-NMR for structure elucidation. A known amount of each sample, in triplicate, was weighted in an analytical balance. Samples were dissolved in 540 µL of deuterated dimethyl sulfoxide (DMSO-d6) and transferred to the NMR capillary tube for further analysis.

NMR spectra were obtained on an NMR spectrometer, running at a frequency of 400 MHz for $^{1}$H. The time of acquisition was 50 seconds and all spectra were acquired with eight scans.

#### 2.3.6.2. Development of a qNMR method for purity determination

A qNMR method to assess the purity of HES was developed. The criteria for the selection of the ideal internal standard (IS) were to have only a few signals to minimize overlapping, to be soluble in DMSO-d6 (since HES is highly soluble in this solvent), to be free of residual water, non-hygroscopic, non-volatile, and highly pure [90]. Bearing this in mind, benzoic acid was chosen as the most suitable IS. Known amounts of HES and benzoic acid (Supplementary Information, Table S3) were accurately weighted in an analytical microbalance. Samples were dissolved just as described in 2.3.6.1. Each analysis was performed in sextuplicate and prepared immediately before analysis.

[1] This analysis was performed by the company MadeBiotech with an already established method for polyphenols. The method validation by determining the limit of detection and quantification was not assessed.

$^{1}$H-NMR spectra were obtained at the same frequency as mentioned previously. qNMR data were acquired with the following acquisition parameters: 30 °C pulse width, an acquisition time of 5.453 seconds, relaxation delay of 1 second for 64K data table with a spectral width of 6009 Hz (15 ppm), and temperature of 300K. Eight scans with a pre-scan delay of 6.5 seconds were performed for each sample.

After the acquisition, spectra were processed in TopSpin NMR Software (version 4.1.4). Manual phase and baseline correction were performed, followed by manual signal integration of the proton signals of interest. Sample purity was calculated for each individual analysis, using Equation 5.

$$P_{HES} = \frac{I_{HES}}{I_{IS}} \times \frac{N_{IS}}{N_{HES}} \times \frac{M_{HES}}{M_{IS}} \times \frac{m_{IS}}{m_{Sample}} \times P_{IS} \quad (5)$$

Where,

$P_{HES}$ = Purity of HES

$P_{IS}$ = Purity of IS

$I_{HES}$ = Integral value of the HES signal (δ 6.14 ppm)

$I_{IS}$ = Integral value of the IS signal (δ 7.95 ppm)

$N_{HES}$ = Number of HES protons

$N_{IS}$ = Number of IS protons

$M_{HES}$ = Molecular mass of HES

$M_{IS}$ = Molecular mass of IS

$m_{IS}$ = Mass of IS

$m_{sample}$ = Mass of sample

## 3. Synthesis of Hesperidin Nanoparticles

### 3.1. Preparation of Chitosan-Hesperidin Nanoparticles

Chitosan nanoparticles (ChNPs) were synthesized similarly to those described by Gomathi *et al*. [125]. A chitosan solution (5 mL, 10 mg/mL) was left overnight to dissolve in 2% (v/v) aqueous acetic acid. Subsequently, an aqueous solution of sodium tripolyphosphate (TPP; 5 mL, 0.8% (w/v)) was slowly added dropwise, and the mixture was constantly stirred at 550 rpm for 1 hour. The resulting nanoparticle suspension was centrifuged at 12000 rpm for 45 minutes, resuspended and washed with UPW, and re-centrifuged at the same rpm for 10 minutes. The supernatant was decanted and preserved to determine the HES encapsulation efficiency, while the pellet was lyophilized. For the HES loading experiments, 5 mL of HES solutions at different concentrations (0.02, 0.06, and 0.1 mg/mL) were added to the chitosan solution and mixed for 30 minutes before TPP addition. The addition steps were performed using a Pasteur glass pipette 1 cm above the mixture.

### 3.2. Preparation of Pectin-Hesperidin Nanoparticles

Pectin nanoparticles (PecNPs) were formulated as proposed by Chittasupho *et al*. [126]. PecNPs and HES-PecNPs preparation was made using the final products obtained from the consecutive extraction scheme, reported in section 2.2. The solutions were prepared in UPW immediately before nanoparticle synthesis and filtered with 0.45 µm cellulose acetate filters, with the exception of pectin solution. Briefly,

magnesium chloride heptahydrate (4 mL, 10 mg/mL) was slowly dropped into the citric pectin solution (4 mL, 20 mg/mL). Subsequently, 2 mL of sodium bicarbonate solution (10 mg/mL) were slowly added to the mixture. These additions were performed using a Pasteur glass pipette at the 1 cm top of the mixture. The mixture was agitated for 30 minutes at room temperature and centrifuged at 13000 rpm for 10 minutes. The resulting nanoparticles were lyophilized. For HES encapsulation, 2 mL of HES solution at different concentrations (0.1, 0.3, and 0.5 mg/mL) were slowly added to the citric pectin solution and agitated for 30 minutes. HES solution was dissolved in the same solvent proportion as mentioned in 2.3.5. After this step, the procedure was performed the same way as previously described for pectin nanoparticles.

## 3.3. Characterization

PecNPs and HES-PecNPs at different HES concentrations were characterized in terms of their average size and zeta potential (3.3.1), encapsulation efficiency, loading content, and yield of production (3.3.2), FTIR-UATR (2.3.2), and antioxidant activity (3.3.3). The most suitable HES-PecNPs were further characterized by determining their release profile (3.3.4), stability (3.3.5), and water absorption capacity (3.3.6). Throughout the following text, HES-PecNPs formulated with a HES concentration of 0.1, 0.3, and 0.5 mg/mL will be further identified by the abbreviations HES-PecNPs 0.1, HES-PecNPs 0.3, and HES-PecNPs 0.5, respectively.

### 3.3.1. Hydrodynamic Size and Zeta Potential

The resultant particles were suspended in UPW at 0.1 mg/mL and sonicated for 3 minutes at 40 KHz with a sonication probe. The hydrodynamic diameter and polydispersity index (PDI) of the prepared suspensions were determined in a disposable cuvette using a zeta sizer equipment. The suspensions were transferred to a DTS1060 folded capillary cell to determine the zeta potential. All measurements were performed in triplicate with a minimum of 12 scans, at 25 °C, and a 173-degree scattering angle.

### 3.3.2. Encapsulation Efficiency, Drug-Loading Content, and Yield of Production

The encapsulation efficiency (EE) was determined by measuring the concentration of free HES in the supernatant. A linear standard curve for HES was designed by measuring the absorbance of a range of different concentrations (Supplementary Information, Figure S1). These measurements were performed in triplicate using UV/Vis spectrophotometry at a fixed wavelength of 284 nm. The encapsulation efficiency was calculated using Equation 6.

$$\mathrm{EE}(\%) = \frac{\text{Weight of added HES} - \text{Weight of non} - \text{entrapped HES}}{\text{Weight of added HES}} \times 100 \quad (6)$$

The drug-loading content (DLC) and yield of production (YP) were calculated using Equation 7 and Equation 8, respectively.

$$\text{DLC}\ (\%) = \frac{\text{Weight of HES in nanoparticles}}{\text{Weight of nanoparticles}} \times 100 \quad (7)$$

$$\text{YP}\ (\%) = \frac{\text{Weight of HES} - \text{PecNPs}}{\text{Weight of pectin} + \text{Weight of HES}} \quad (8)$$

### 3.3.3. Antioxidant Activity in vitro

The antioxidant activity of obtained HES-PecNPs, HES and pectin were determined by the two following assays, the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{+\bullet}$). HES-PecNPs dispersions were prepared in distilled water while HES solutions prepared in DMSO. Appropriate negative and positive controls were used. For each sample, these assays were carried out in triplicate and the scavenging activity (%) was calculated using Equation 9.

$$\text{Scavenging activity}\ (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \quad (9)$$

#### 3.3.3.1. $DPPH^{\bullet}$ scavenging activity assay

The $DDPH^{\bullet}$ scavenging activity assay followed previously reported methods [127]. Briefly, a 0.250 mM $DPPH^{\bullet}$ solution was prepared in absolute ethanol. 500 µL of this solution were added to the same volume of sample in an eppendorf, vortexed and incubated in the dark at room temperature for 1 hour. After this period, sample absorbance was measured at 517 nm in spectrometer equipment. Absolute ethanol was used for the blank measurements.

#### 3.3.3.2. $ABTS^{+\bullet}$ scavenging activity assay

The antioxidant activity of each sample against $ABTS^{+\bullet}$ was measured according to the described by Spínola [127]. The radical solution was obtained by reacting 7 mM aqueous ABTS solution with 2.45 mM aqueous potassium persulfate solution, followed by an incubation of 12-16h in the dark at room temperature. Then, the $ABTS^{+\bullet}$ solution was diluted with 0.1 M phosphate buffer saline (PBS) at pH 7.4 to an absorbance of 0.700 at 734 nm. For the assay, 40 µL of each sample were added to 1.96 mL of $ABTS^{+\bullet}$ solution and the sample absorbance was measured in a spectrometer equipment after 6 minutes at 734 nm for the Trolox and HES standard solutions. For the nanoparticles samples, the absorbance was measured after 1 hour. The blank measurements were performed with PBS.

The antioxidant activity of each sample subjected to a previous oxidative stress was measured as well. For this purpose, an oxidant was prepared in accordance with the reported by Roy *et al*. [128], consisting of a solution of 2 mM potassium persulfate in presence of 200 µM cobalt acetate in 10 mM PBS

(pH 7.4). The assay consisted of adding 40 µL of oxidant to 40 µL of each sample in an eppendorf, followed by incubation for 24 hours at 37 ºC. The antioxidant activity of the incubated samples was determined as mentioned above.

### 3.3.4. In vitro Release Studies

HES release from the nanoparticles was studied in vitro using the dialysis method. Briefly, dialysis membranes containing 2 mL of HES-PecNPs dispersion, prepared in PBS (pH 6.6), were immersed in the same buffer at room temperature with constant agitation. At indicated times, 2 mL of the external medium was removed and replaced with 2 mL of fresh buffer. Each experiment was performed in triplicate. A control was performed using a solution of HES prepared in DMSO at the same concentration of the encapsulated in the particles. HES concentration on each sample was determined by UV-Vis spectrophotometry at 284 nm using the respective standard curve (Supplementary Information, Figure S2). The cumulative percentage amount of drug release was calculated and plotted against time.

### 3.3.5. Stability studies

The stability of the lyophilized HES-PecNPs was studied at two different temperatures. The nanoparticles were maintained in the dark for 30 days, and their physicochemical properties were determined at certain days (days 0, 7, 14, 21, and 30). These measurements were performed using the same conditions as mentioned in section 3.3.1 and each analysis was performed in triplicate. The temperatures were monitored with a mercury thermometer.

### 3.3.6. Water Absorption Capacity

The Water Absorption Capacity (WAC) of pectin and respective nanoparticles was determined as reported by Williams *et al*. [129] with some modifications. 10 mg of each sample were added to a pre-weighted 2 mL centrifuge tube, followed by adding and weighing 2 mL of UPW. The suspension was vortexed for 5 minutes, followed by centrifugation at 3500 rpm for 30 minutes. The supernatant was decanted and weighed. This experiment was conducted in triplicate, and the WAC was calculated using Equation 10.

$$\mathrm{WAC\ (g/g)} = \frac{\text{Weight of absorbed water}}{\text{Weight of sample}} \quad (10)$$

## 4. Statistical Analysis

The results of samples analyzed in several replicates are expressed as the mean ± standard deviations. The data obtained were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test using SPSS for Windows, IBM SPSS Statistic 28 (SPSS, Inc., USA). A value of $p < 0.05$ was considered statistically significant.

# III. Results and Discussion

## 1. Hesperidin Extraction from Citrus Waste

HES extraction from citrus waste has traditionally been performed using organic or alkaline solvents via conventional methods. These methodologies are non-selective for HES, low efficient, time-consuming, and require large amounts of solvents, which may be detrimental to the environment. Moreover, the efficiency and quality of obtained HES could be improved if certain compounds were previously extracted, such as essential oils and sugars. There is a lack of knowledge on how these methodologies can be introduced into extraction schemes of bioactive compounds from citrus waste, maximizing the residue potential itself, and whether they are economically and energetically viable for the industry. Zhou *et al*. [88] reported a consecutive extraction of pectin and HES from *Citrus aurantium* L., where pectin and HES were extracted with citric acid and sodium hydroxide, respectively. Despite the excellent properties of pectin and the high purity of HES, this work has significant drawbacks because it uses the whole fruit instead of the considered waste and employs citric acid, which can be obtained from the same matrix and is a greener solvent than HCl, but its extraction and purification are highly dispendious. Bearing this in mind, a consecutive extraction of these two compounds from orange waste, taking efficiency, environmental impact, and economic viability into consideration, was developed. The other method applied was a hot methanolic extraction (Extraction Method II), which is one of the most reported methods in the literature, to compare the efficiency and selectivity of both methodologies.

### 1.1. Development of Extraction Method II

The development of the proposed extraction scheme took into consideration the differences in the solubility and degradation of pectin and HES in water. Pectin extraction is favored under acidic conditions in which HES is insoluble. HES extraction is promoted in high alkali conditions because its phenol groups deprotonate, increasing its solubility in water [60]. By decreasing the pH with HCl, these groups are protonated, decreasing HES solubility, and causing precipitation. Given their different solubilities, they can be easily isolated from the same matrix. However, pectin should be extracted first because a high pH promotes saponification and β-elimination, and HES does not deteriorate in acidic solutions [89,130]. The methodology also allowed for the development of a new precipitation method for HES, which is less time-consuming than most reported alkali methods because the extraction is performed with a hydroalcoholic mixture. Ethanol may not only act as an extracting solvent but also as a HES solubilizer because, when ethanol was recovered in the rotatory evaporator, HES immediately precipitated in the neutralized liquid owing to supersaturation.

#### 1.1.1. Pectin Extraction and Characterization

Pectin extracted under acidic conditions with HCl showed satisfactory yields of 11.89 ± 0.36 %. These yields are similar to those reported by Kute *et al*. [131] (8.78-15.79 %) and Kamal *et al*. [132] (12.50-22.45

%). The differences may be related to the degree of maturation, growing conditions, pretreatments, extraction methods applied, and yield formula calculation [133].

#### 1.1.1.1. Physicochemical Properties

Extracted pectin showed satisfactory physicochemical properties (Table 4). EW represents the content of non-esterified galacturonic acid present in pectin and indicates its capacity to form a gel, where high values correspond to a better gel-forming ability [134,135]. In this study, the EW of obtained pectin from orange albedo was 655.00 ± 21.41 g/mol (Table 4), which is slightly higher than the values reported by Kute *et al*. [131] (381-485 g/mol) but lower than the citric standard and the ones stated by Kamal *et al*. [132] (1744-1899 g/mol).

MeO characterizes pectin distribution ability in water and its capacity to form firm gels [134,135]. By this parameter, isolated pectin was classified as low-methoxyl and presented a higher MeO than the citric standard (Table 4). These values are in line with the ones mentioned by Kute *et al*. [131] (5.75-6.99%) but higher than Kamal *et al*. [132] (5.19-5.48%) for citric pectin.

AUA reflects the purity and quality of pectin, which should not be less than 65% for industrial purposes as recommended by the Food Chemical Codex [132]. Pectins with an AUA < 65% may indicate high amounts of protein. The data revealed that extracted pectin was of high purity and quality, since the AUA was within the recommended value, with 66.20 ± 1.25%. This value was higher than the ones stated by Kamal *et al*. [132] (38.47-41.30%) and the standard did not meet the criteria to be applied for industrial purposes (Table 4) [132].

DE describes the extent to which the galacturonic acid carboxyl groups in pectin molecules are methyl esterified [135]. This polysaccharide is classified into high-methoxyl if a DE > 50% and low-methoxyl if a DE < 50%. Bearing this in mind, extracted pectin was categorized as high-methoxyl (59.37 ± 0.75 %) but presented a lower DE than the standard. This value is also lower than the ones reported by Kamal *et al*. [132] (73.26-75.38%) but higher than the ones stated by Kute *et al*. [131] (35.48-42.85%) for citric pectin.

Table 4 - Physicochemical properties of standard and extracted pectin.

| Pectin Sample | EW (g/mol) | MeO (%) | AUA (%) | DE (%) |
|---|---|---|---|---|
| **Citric Standard** | 5060.00 ± 11.78 | 1.63 ± 0.42 | 12.75 ± 2.40 | 71.68 ± 5.58 |
| **Extracted** | 655.00 ± 21.41 | 6.92 ± 0.11 | 66.20 ± 1.25 | 59.37 ± 0.75 |

#### 1.1.1.2. FTIR-UATR

The extracted pectin exhibited polysaccharide characteristic bands, although with different intensities when compared with the standard, as shown in Figure 11. The single bond stretch region of the spectra showed the presence of a broad band between 3500 and 3200 $cm^{-1}$, indicating the presence of

hydroxyl groups, and all the stretching vibration bands of C-H bonds between 3000 and 2800 $cm^{-1}$. The double bond region of the spectra revealed two bands in 1730 and 1640 $cm^{-1}$, corresponding to the stretching vibrations of the C=O bonds in esterified and non-esterified carboxyl groups, respectively. Infrared (IR) spectroscopy methods have been developed to determine the DE of pectin based on these two bands, by calculating the ratio of the areas of the 1730 $cm^{-1}$ band and the sum of the areas of the two bands 1730 and 1640 $cm^{-1}$ [136]. For this purpose, calibration curves are necessary and, given the scope and goals of this dissertation, the determination of the DE by this technique was not attempted. The fingerprint region of the spectra indicated the bending vibrations of the pyranose ring and stretching vibrations of $COO^-$ of the ester groups at approximately 1200-1350 $cm^{-1}$. The bands around 1200-1000 $cm^{-1}$ were attributed to the vibrations of the C-O and C-C bonds owing to the overlapping peaks of the glycoside bonds and pyranose cycles. In this region, it was also possible to verify the α- and β-configurations of pectin using the bands at 830 and 500 $cm^{-1}$.

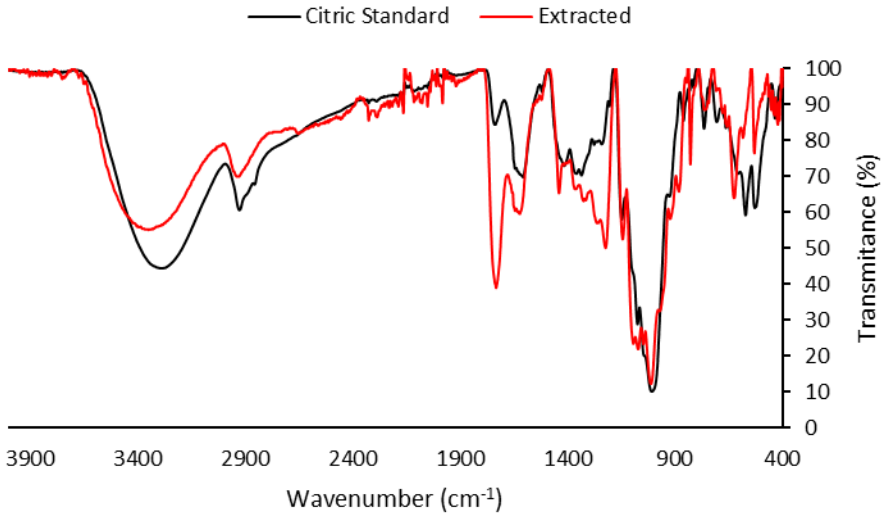


Figure 11 - FTIR-UATR analysis of pectin standard and extracted with Extraction Method II.

### 1.1.2. Hesperidin Extraction

HES hydroethanolic extraction was subjected to improvement by studying the effect of different extraction times, temperatures, and ratios on HES yields (Figure 12). Three extraction times (30, 60, and 180 minutes) were studied to evaluate whether the yield increased with increasing contact time between the matrix and solvent. To evaluate the effect of time, the temperature and solid-to-liquid ratio were fixed at 70 ºC and 1:10 (w/v), respectively. The present work showed that no statistically significant differences ($p > 0.05$) were detected for extraction times greater than 30 minutes. This observation demonstrates that 30 minutes of extraction is sufficient to reach an equilibrium of solute concentration between the solid and liquid phases, which follows Flick's second law. These findings are also supported by Kim and Lim [137], who studied through response surface optimization the influence of some extraction parameters,

including time, on HES yields from immature *Citrus unshiu* pomace under fixed conditions (60% ethanol concentration, 60 ºC, and 30 mL/g dry sample). They verified that after half an hour of extraction, there were no significant differences between yields.

The effect of four temperatures (50, 60, 70, and 80 ºC, Figure 12) on HES yields was also analyzed. For that purpose, time and solid-to-liquid ratio were fixed at 30 minutes and 1:10 (w/v), respectively. The statistical tests applied only verified significant differences ($p < 0.05$) between 50, 60, and 70 ºC. At 80 ºC, was observed a decrease in HES yields, which was not statistically different from the yield obtained at 60 ºC. This result was expected since ethanol has a low boiling point (around 78°C) and therefore, at this temperature, higher quantities of solvent would evaporate, resulting in less HES extracted or even loss by precipitation due to solvent supersaturation. These findings are once again supported by Kim and Lim [137], which constated that 75 ºC was the ideal extraction temperature and verified a decrease in yield at 90 ºC.

The effect of solid-to-liquid ratio (1:10, 1:15, and 1:20 (w/v), Figure 12) on HES yields was the last parameter evaluated. HES yields decreased with increasing amounts of solvent, which was not expected. The statistical post-hoc tests applied verified significant differences ($p < 0.05$) between 1:10 and 1:20 (w/v), but no significant differences ($p > 0.05$) between 1:10 and 1:15 (w/v) and 1:15 and 1:20 (w/v). This phenomenon could be explained by a higher dissolution and extraction of impurities, such as other alcohol-soluble and lipophilic compounds, which could have reduced HES solubility in the solvent. Moreover, higher amounts of NaOH were needed to maintain an alkaline pH during extraction, which could have as well resulted in the deprotonation of impurities and even their consequent aggregation with deprotonated HES.

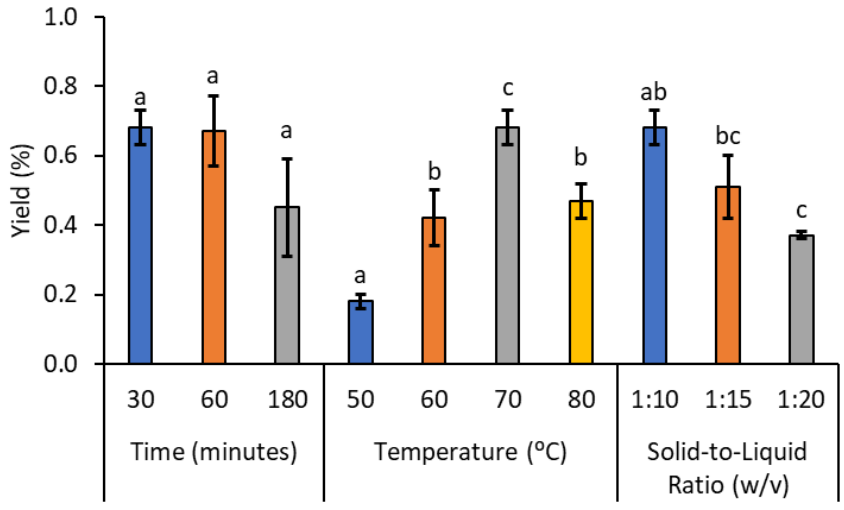


Figure 12 - Effects of extraction parameters (time, temperature, and ratio) on HES extraction yields. The statistical differences were analyzed using one-way ANOVA followed by Tukey's multiple comparisons tests. For each condition, each bar followed by the letters (a-c) is significantly different (n=3; $p < 0.05$).

## 1.2. Extraction Method I VS Extraction Method II

Both HES extraction methodologies were compared in terms of their efficiency. HES extracted under a hot methanolic extraction (Extraction Method I) showed yields of 1.73 ± 0.12%, higher than those obtained with Extraction Method II under the best conditions tested (0.68 ± 0.05%, Figure 12). One plausible explanation for the lower yields in Extraction Method II may be the loss of HES in the second pretreatment applied to remove ethanol-soluble solids to increase pectin quality. The final products from both methodologies were characterized to study the method's selectivity for HES.

### 1.2.1. FTIR-UATR

In the analysis of the FTIR-UATR spectrum of extracted HES by both methodologies (Figure 13), the hydroxyl stretching region shows a broad band at 3404 $cm^{-1}$, associated with intermolecular hydrogen bonds owing to the multiple hydroxyl groups present in the sugar moiety. However, it was also possible to identify sharper bands at 3543 and 3473 $cm^{-1}$ that represent the intramolecular hydrogen bonds between the hydroxyl and the ortho methoxy groups in ring B, and the hydroxyl group of ring A and the carbonylic oxygen of ring C. Furthermore, it was observed all stretch bands referring to the CH bonds at 3083, 2983, 2938, and 2919 $cm^{-1}$, and the HES characteristic carbonylic bond located at 1645 $cm^{-1}$. The bands at 1605, 1519, 1468, and 1443 $cm^{-1}$ were attributed to the stretching of the C=C bonds from the phenolic rings, while the ones referring to the stretching of the C-O bonds were identified at 1275 and 1205 $cm^{-1}$. The comparison of the FTIR-UATR spectra of the extracted samples with the standard spectrum corroborated that the isolated products from both extraction methodologies had the same characteristic bands as the HES standard. The FTIR-UATR spectra of extracted samples did not show the clear presence of impurities.

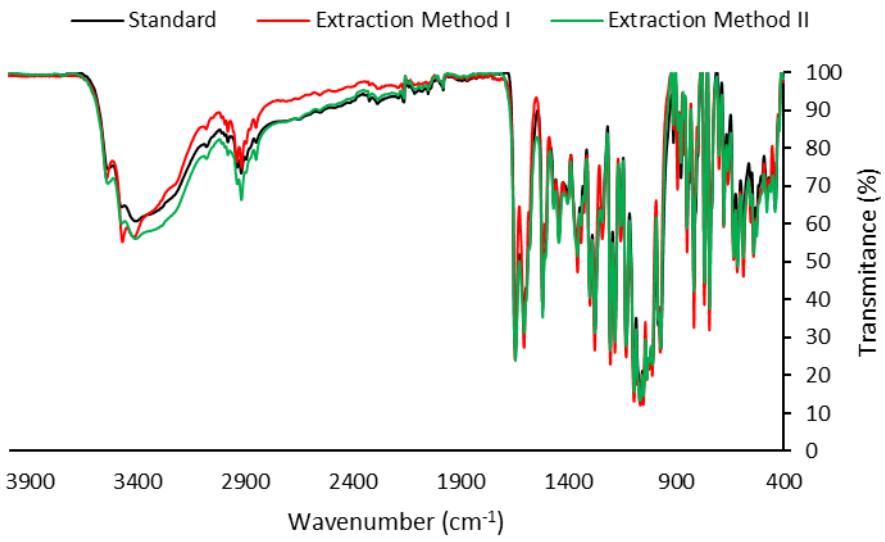


Figure 13 - FTIR-UATR analysis of HES standard and isolated from two extraction methodologies.

### 1.2.2. Melting Point

HES obtained by Extraction Method I and Extraction Method II presented an identical melting point range of [238.1- 243.1] and [238.2- 241.8] ºC, respectively. Nonetheless, both samples indicate the presence of impurities, probably other compounds or residual amounts of solvent, owing to a lower fusion point than the standard (249.8 ºC).

### 1.2.3. Qualitative NMR

#### 1.2.3.1. Hesperidin

Structural analysis of standard HES was performed using $^{1}$H-NMR. Given the poor solubility of hesperidin in most solvents, this analysis was conducted in DMSO-d6, which difficulted the identification of the sugar moiety protons due to overlap with the residual and moisture proton signals of the solvent (Figure 14). For a better understanding and identification of the protons, the theoretical chemical shifts of the aglycone protons were calculated and the linkage between rutinose and hesperidin was corroborated by the identification of the only methyl group of rutinose.

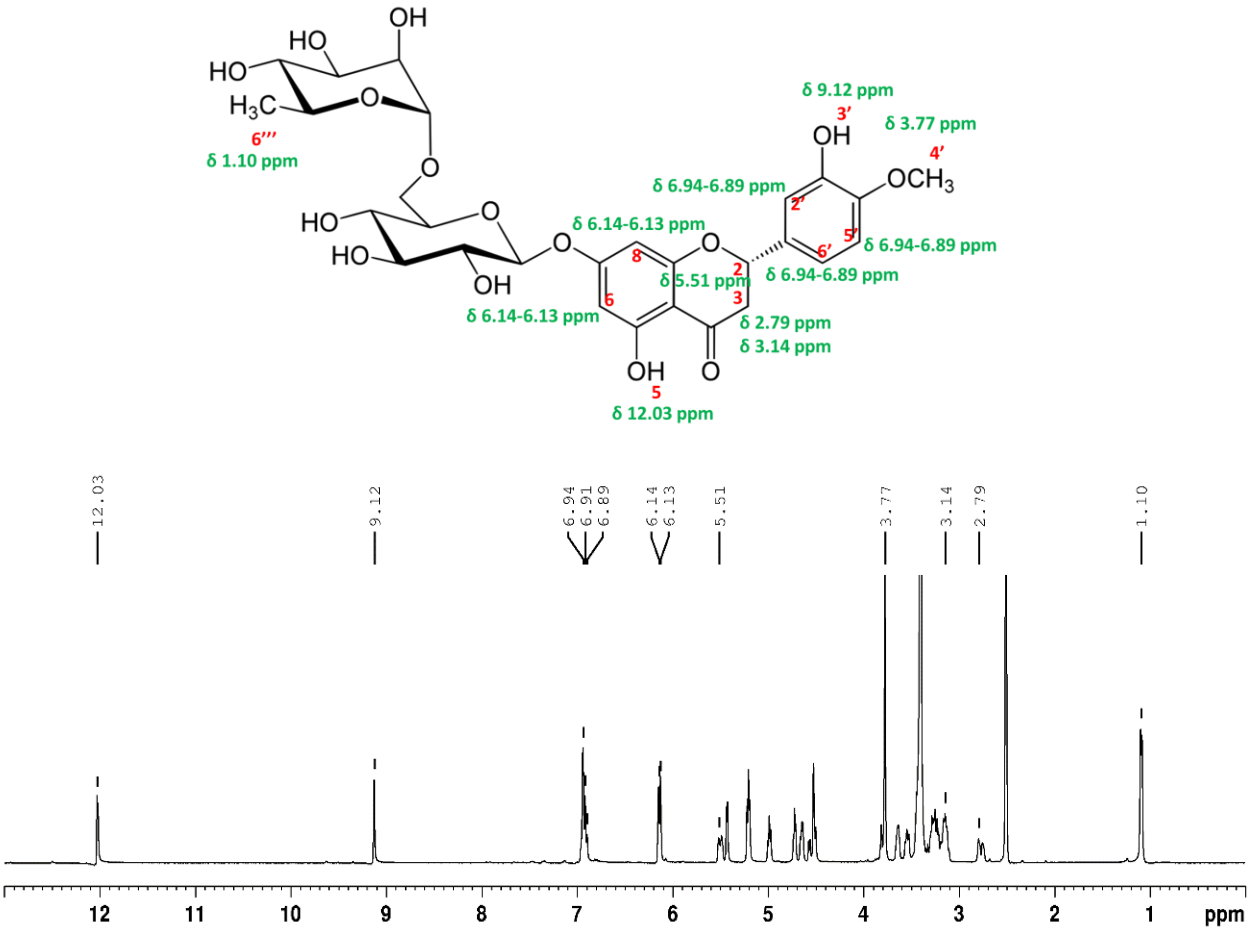


Figure 14 - $^{1}$H-NMR spectra of standard HES.

The $^{1}$H-NMR spectra of extracted samples (Figure 15) confirmed that the final products were HES due to the presence of its characteristic proton signals, supporting as well the previously analysed by FTIR-

UATR. The $^{1}$H-NMR spectra of HES from Extraction Method I (Figure 15A) and Extraction Method II (Figure 15B) showed the clear presence of impurities owing to the presence of an extra proton signal at 1.3 ppm. However, it was verified a loss of signal definition, specially between 4.5 and 7.0 ppm, for HES from Extraction Method II. These results may suggest that these impurities may be other flavonoids since extraction with NaOH followed by neutralization can allow the formation of flavonoid complexes [87]. Nonetheless, this might as well indicate the presence of residual amounts of solvent in the sample, supporting the results obtained in section 1.2.2.

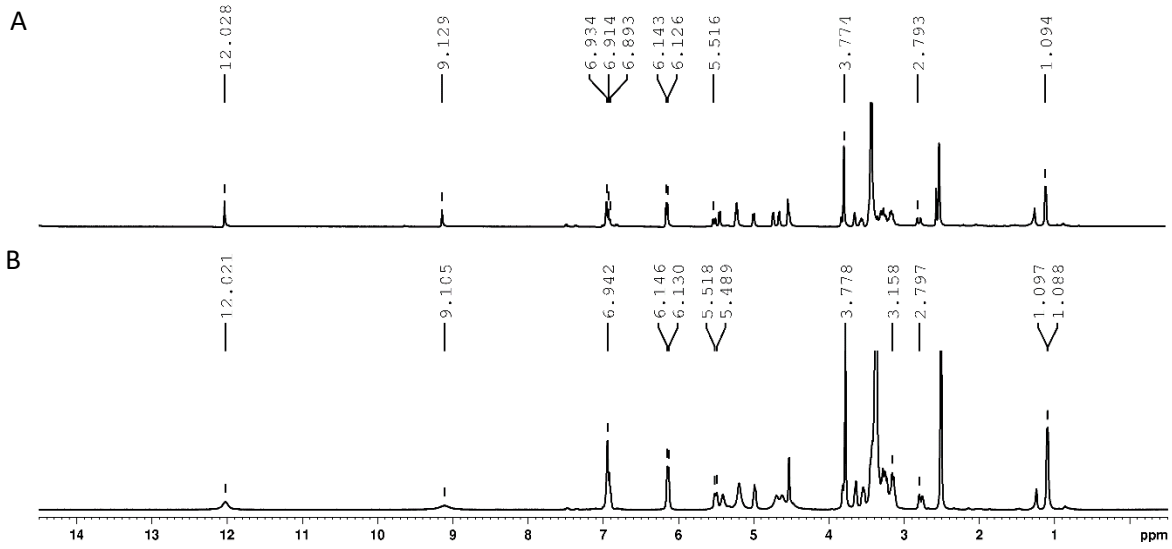


Figure 15 - $^{1}$H-NMR spectra of extracted HES with Extraction Method I (A) and II (B).

### 1.2.3.2. Benzoic Acid

Qualitative analysis of the available benzoic acid was performed in DMSO-d6 to study if it would be a suitable IS to determine the purity of HES by $^{1}$H-qNMR (Figure 16).

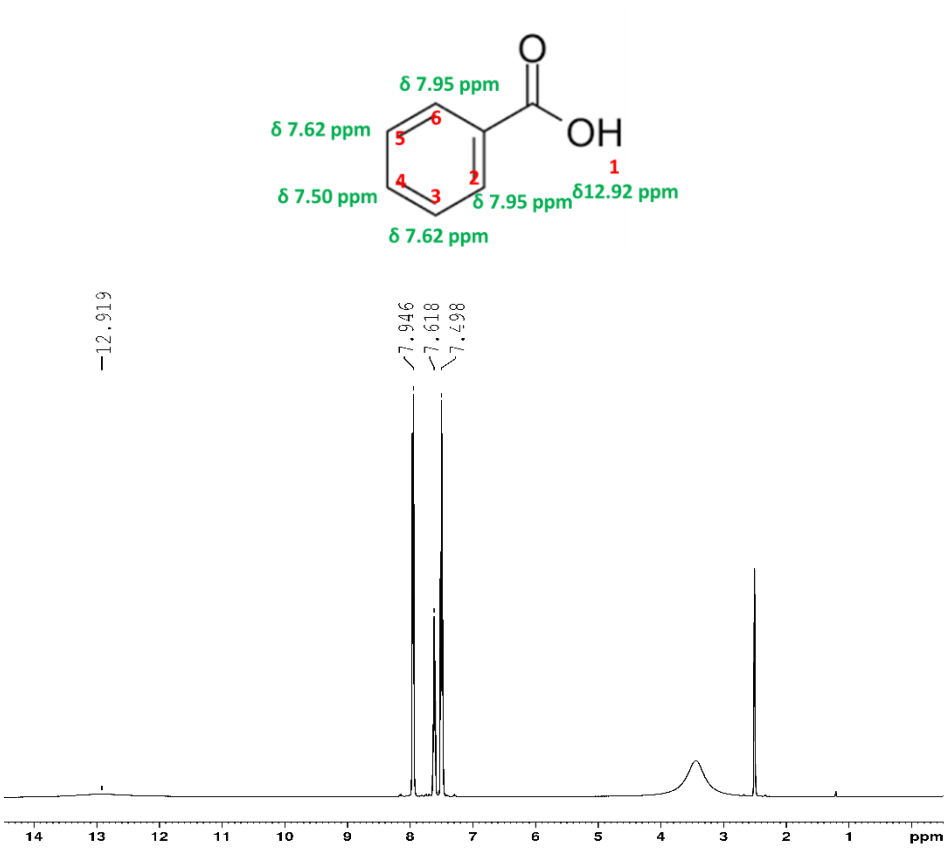


Figure 16 - $^{1}$H-NMR spectra of benzoic acid.

### 1.2.4. HPLC-PDA vs qNMR

In the present study, HES purity was estimated by the standard technique HPLC-PDA and qNMR. Table 5 summarizes the results obtained for the standard and extracted samples for each technique. Considering the purity determined by the chromatographic method, HES isolated from both methodologies showed similar purities, proving that Extraction Method II was effective in obtaining HES with equivalent quality to Extraction Method I using greener and safer solvents and less time-consuming extraction processes. The HPLC-PDA analysis of each sample showed the same major peak (Figure 17), at 8.7 minutes retention time and, interestingly, it was also possible to verify impurities that overlapped for all samples, including the standard.

Table 5 - HES purity assessed by HPLC-PDA and qNMR. The statistical differences were analyzed using one-way ANOVA followed by Tukey's multiple comparisons tests. Letters (a, b) in each bar represents the statistically significant differences detected ($p < 0.05$) between purity of samples determined by the same method. * Represents statistically significant differences ($p < 0.05$) between the purity calculated by both methods for the same sample.

| HES Sample | Purity (%) | |
|---|---|---|
| | HPLC-PDA (n=3) | qNMR (n=6) |
| **Standard** | 88.36 ± 0.03[a] | 89.65 ± 0.97[a] |
| **Extraction Method I** | 85.43 ± 0.02[b] | 86.30 ± 1.08[b] |
| **Extraction Method II** | 84.01 ± 0.00[b,*] | 67.75 ± 1.64[c,*] |

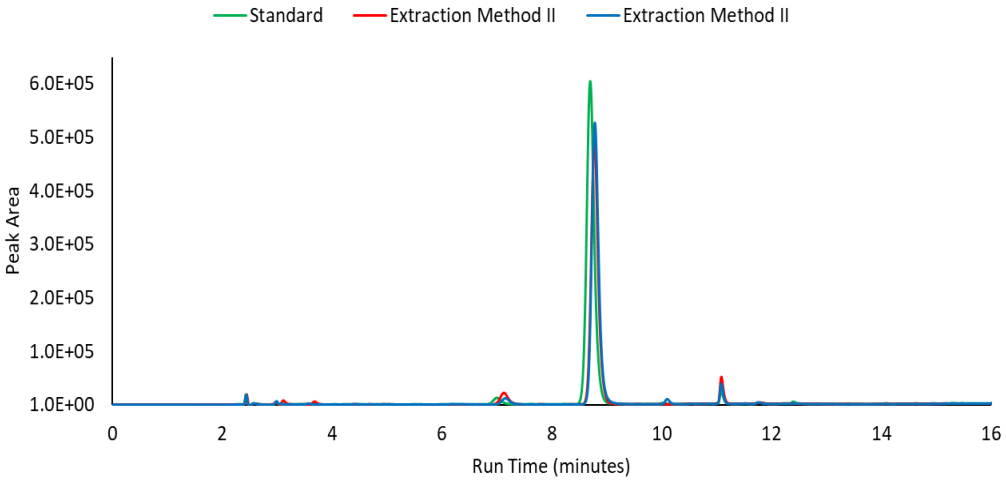


Figure 17 - HPLC-PDA chromatogram analysis of standard and extracted HES samples.

In the present study, preliminary studies were conducted to evaluate the possibility of using qNMR as a technique for purity determination of HES. For this purpose, the IS method was chosen given its reported higher accuracy over the external and electronic reference ones. Benzoic acid was selected as the most appropriate IS for having very few proton signals, minimizing the possibility of overlap with the ones of HES; high solubility in DMSO-d6, and its inert property towards HES [90]. Figure 18 shows the resultant $^{1}$H-NMR spectrum of combined IS and standard HES. No significant chemical shifts were detected and therefore, benzoic acid was selected as IS. Quantification was performed considering the signal at δ 6.14 ppm, correspondent to HES (identified in section 1.2.3.1), and 7.95 ppm, referring to the IS (identified in section 1.2.3.2). These signals were selected due to their easier integration, isolation from other HES proton signals, and lower probability of overlapping with other proton signals attributed to impurities.

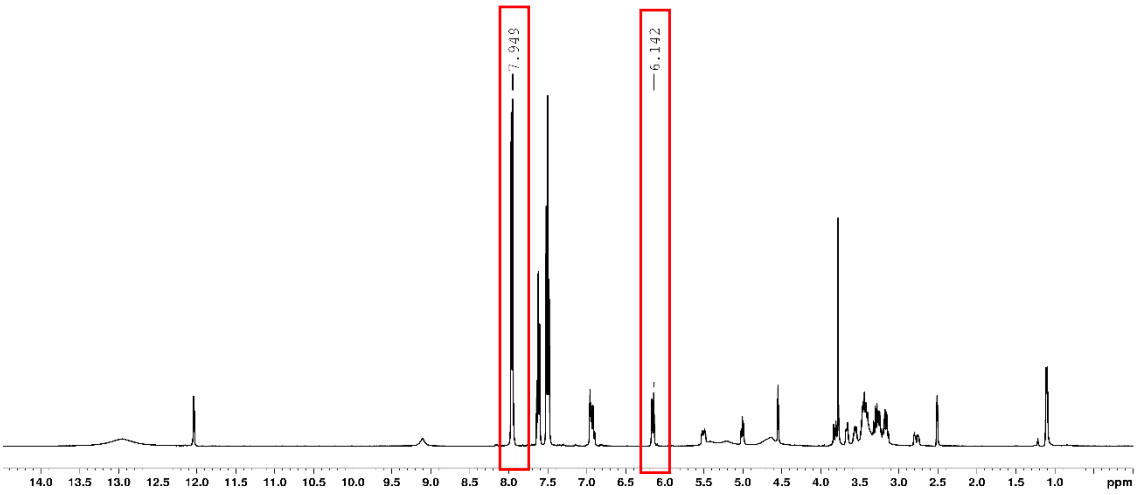


Figure 18 - $^{1}$H-NMR spectra of IS with standard HES.

The preliminary results were promising since the values of the standard and extracted HES with Extraction Method I were not statistically different from those obtained with HPLC-PDA (Table 5). However, the value obtained for HES using Extraction Method II was very different from these two samples. Figure 19 shows the $^{1}$H-NMR spectra of IS with extracted HES from both methodologies. Considering that the NMR equipment parameters were maintained equal for each analysis, this result may be justified by the interference of impurities (previously discussed in 1.2.2 and 1.2.3.1) in the same signals used to perform the quantification and/or by the presence of paramagnetic materials in the final product, which may have caused the broadness of the signals (Figure 19B). Unfortunately, the combined uncertainty of the purity calculated was not possible to determine given insufficient information about the uncertainty of the purity reported from the benzoic acid manufacturer. Although these preliminary studies were promising in showing this technique potential for HES purity determination, qNMR method optimization and validation is needed. The determination of the longitudinal relaxation time ($T_1$) and, consequently, the relaxation delay ($d_1$) is crucial when working with qNMR, along with accurate sample preparation and spectra processing [90]. $d_1$ corresponds to the time (in seconds) for the equilibrium magnetization to be achieved between pulses when accumulating co-added FIDs. This value should be 5 to 7 times higher than $T_1$, which corresponds to the value of the proton with the longest relaxation time in the mixture [92]. In the present study, their determination was not attempted.

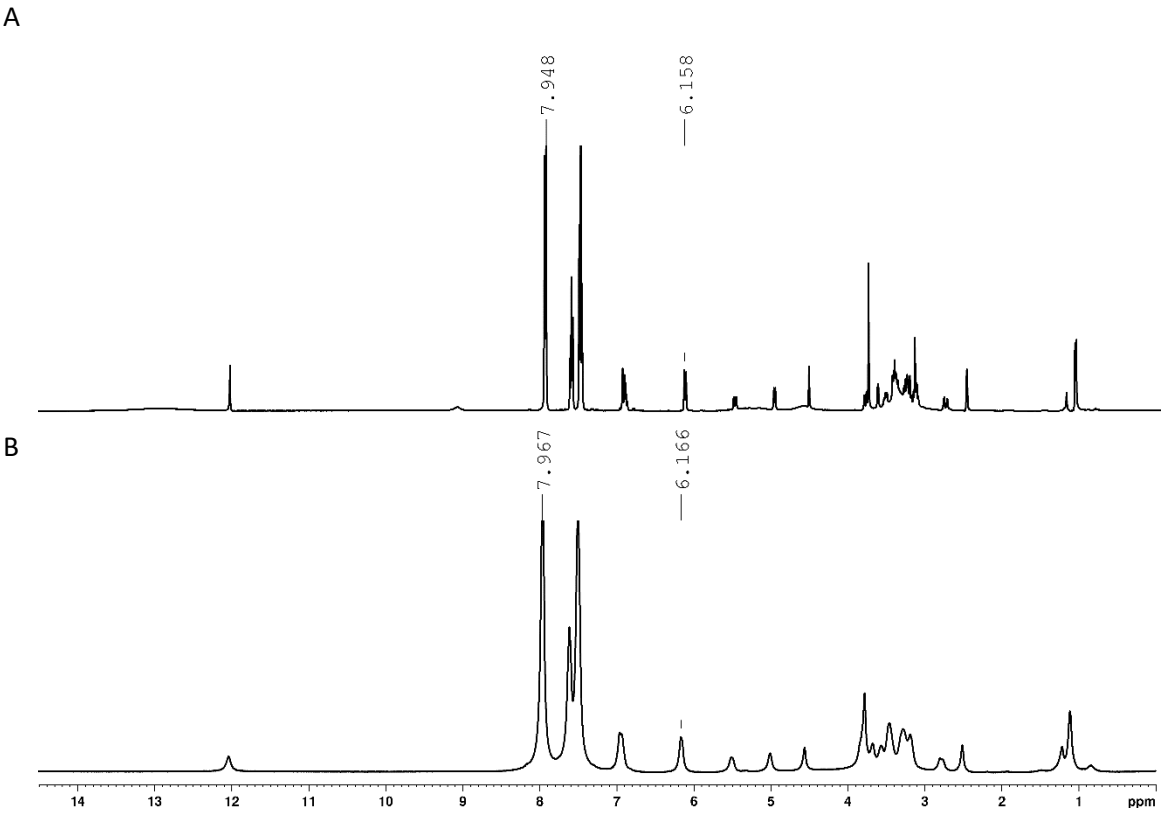


Figure 19 - $^{1}$H-NMR spectra of IS and HES from Extraction Method I (A) and II (B).

## 2. HES-ChNPs Preparation and Characterization

Nanoparticle preparation was accomplished by ionotropic gelation, which is a simple, straightforward, cost-effective, and environmentally friendly method. Several methods have been proposed for ChNPs preparation using this approach and TPP as a crosslinker. Interactions of the amino groups in chitosan with the polyanions through intermolecular and intramolecular cross-linkages are responsible for nanoparticle formation [125].

Initial ChNPs presented a hydrodynamic size, PDI, and zeta potential of 464 ± 95 nm, 0.264 ± 0.061, and 5.88 ± 0.4 mV, respectively. However, the DLS characterization results of HES-PecNPs revealed very low reproducibility owing to the constant Z-average and distribution size variation between experiments, with particles ranging in Z-average from 1 µm to 2 µm (data not shown). These results suggest that the particle increased variably in size with HES encapsulation or reflected the aggregation of the nanoparticles owing to a decrease in their zeta potential. At the time these experiments were conducted, there was no imaging equipment available to determine which hypothesis was correct. Bearing this in mind, HES-ChNPs were not considered for the following work.

## 3. HES-PecNPs Preparation and Characterization

PecNPs preparation was performed using the ionotropic gelation method. In this case, nanoparticle formation occurs because of the electrostatic interaction between the negatively charged groups of pectin

and the positively charged crosslinker. Initial studies to select the most appropriate crosslinker were performed to evaluate the effect of divalent calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) cations on nanoparticle size and PDI. Smaller particles can easily penetrate the lipid layers of insects and permeate the protein and ion channels of microbial cell walls [30,93]. These crosslinkers were chosen because of their crucial roles in plants. For example, $Mg^{2+}$ is involved in chlorophyll synthesis, whereas $Ca^{2+}$ acts as an intracellular messenger in the cytosol. The studies performed with apple pectin revealed that the nanoparticles formed via electrostatic interactions with the $Mg^{2+}$ cation had a slightly lower hydrodynamic size (363.6 ± 57.56 nm) than those produced with $Ca^{2+}$ (425.6 ± 2.14 nm). These findings were supported by Opanasopit *et al*. [138], who showed that the crosslinking species highly influenced the particle size and that $Mg^{2+}$ and manganese yielded smaller pectin particles than $Ca^{2+}$. In this study, the effect of crosslinkers on the PDI of a colloidal solution of nanoparticles was studied. This parameter is an indicator of the width of the particle size distribution, where values < 0.2 show a very well monodispersed colloidal and values > 0.7 indicate heterogeneity in size, with possible nanoparticle aggregation. Interestingly, PDI did not change significantly (0.478 ± 0.039 and 0.429 ± 0.082 with $Ca^{2+}$ and $Mg^{2+}$ as crosslinkers, respectively). Due to these reasons, $Mg^{2+}$ was chosen as the ideal pectin crosslinker. In this study, sodium bicarbonate was added after polyelectrolyte complex formation, given that some reports mention that the interaction between carbonic anions and $Mg^{2+}$ results in a higher stabilization of the spherical shape of the nanoparticles [126].

Pectin and HES interaction in orange juice clouds is well-documented. Ben-Shalom and Pinto [139] studied the interaction between these two compounds in a model juice cloud system. The authors verified that hesperetin was unable to interact with pectin, demonstrating that pectin and HES specific interactions rely on hydrogen bonds established by the neutral sugars of both compounds. It was also demonstrated that pectins with a higher neutral sugar content interacted more strongly with HES. In another study published by these authors, they evaluated the effect of a range of pectins with different DE (10-73%) on this interaction and concluded that this parameter was irrelevant, as it did not affect the interaction and the formation of colloidal particles [140]. However, molecular weight influenced the stability of the final particles.

## 3.1. Physicochemical Properties and Encapsulation Parameters

Three concentrations of HES were tested to evaluate their effect on the particle's physicochemical properties and encapsulation parameters (EE, DLC, and YP) and to select the most appropriate formulation for environmental applications. Table 6 summarizes the main results obtained from nanoparticle characterization. PecNPs presented a hydrodynamic size of around 385.7 nm with a very low PDI and narrow size distribution (Supplementary Information, Figure S4), which is in accordance with the results reported by Chittasupho *et al*. [126] (391 ± 47 nm, 0.27 ± 0.11). These nanoparticles were negatively charged (Table 6; Supplementary Information, Figure S4), suggesting that the carboxylic groups are at the

nanoparticle surface and prevent aggregation. The addition of increasing concentrations of HES demonstrated a tendency to a higher hydrodynamic size, although not statistically different (Table 6; Supplementary Information, Figure S5). For all the formulations, the PDI did not significantly change, demonstrating a great homogeneity in nanoparticle size. The zeta potential remained constant through the HES-PecNPs formulations but was statistically significantly different for the PecNPs and HES-PecNPs formulated at the highest HES concentration (Table 6; Supplementary Information, Figure S6). Moreover, HES-PecNPs 0.5 presented the highest values of EE and DLC. YP remained constant for the HES-PecNPs formulations. Bearing this in mind, HES-PecNPs 0.5 were chosen as the most advantageous for biopesticide applications.

Table 6 – Physicochemical properties and EE, DLC, and YP of PecNPs and HES-PecNPs formulated at HES different concentrations. The statistical differences were analyzed using one-way ANOVA followed by Tukey's multiple comparisons tests. For each condition, each bar followed by the letters (a-c) is significantly different (n=3; $p < 0.05$).

| Sample | Hydrodynamic size (nm) | PDI | Zeta Potential (mV) | EE (%) | DLC (%) | YP (%) |
|---|---|---|---|---|---|---|
| **PecNPs** | $385.7 \pm 10.3^{a}$ | $0.2 \pm 0.0^{a}$ | $-19.7 \pm 0.2^{a}$ | - | - | $16.3 \pm 0.5^{a}$ |
| **HES-PecNPs 0.1** | $407.7 \pm 14.9^{ab}$ | $0.3 \pm 0.1^{a}$ | $-19.3 \pm 1.2^{ab}$ | $66.9 \pm 2.7^{a}$ | $1.5 \pm 0.4^{a}$ | $17.7 \pm 0.0^{b}$ |
| **HES-PecNPs 0.3** | $421.6 \pm 25.8^{ab}$ | $0.3 \pm 0.1^{a}$ | $-17.3 \pm 0.8^{ab}$ | $76.8 \pm 2.0^{b}$ | $4.4 \pm 0.3^{b}$ | $17.4 \pm 0.4^{b}$ |
| **HES-PecNPs 0.5** | $452.8 \pm 22.1^{b}$ | $0.2 \pm 0.0^{a}$ | $-16.9 \pm 0.7^{b}$ | $85.0 \pm 1.5^{c}$ | $6.9 \pm 0.4^{c}$ | $19.0 \pm 1.0^{b}$ |

## 3.2. FTIR-UATR

The formation of PecNPs was confirmed using FTIR-UATR. The spectrum of PecNPs showed the characteristic bands of pectin (Figure 20), although with clear intensity changes and wavenumber shifts. In the long-wavelength region of the IR spectrum of PecNPs, the absorption band representing the stretching vibrations of the free hydroxyl groups was significantly more intense than in the IR spectrum of pectin. Sharper bands were also observed at 3544 and 3466 $cm^{-1}$. These observations indicate the formation of more and stronger intra- and intermolecular hydrogen bonds when compared to pectin. The asymmetric stretching vibration band of the carboxyl groups increased in intensity, as did the wavelength shift from 1645 to 1606 $cm^{-1}$. Moreover, the absorption band at 1520 $cm^{-1}$ in the IR spectrum of PecNPs was almost absent in the IR spectrum of pectin. These observations can be justified by the presence and interaction of carbonate ions with the crosslinker.

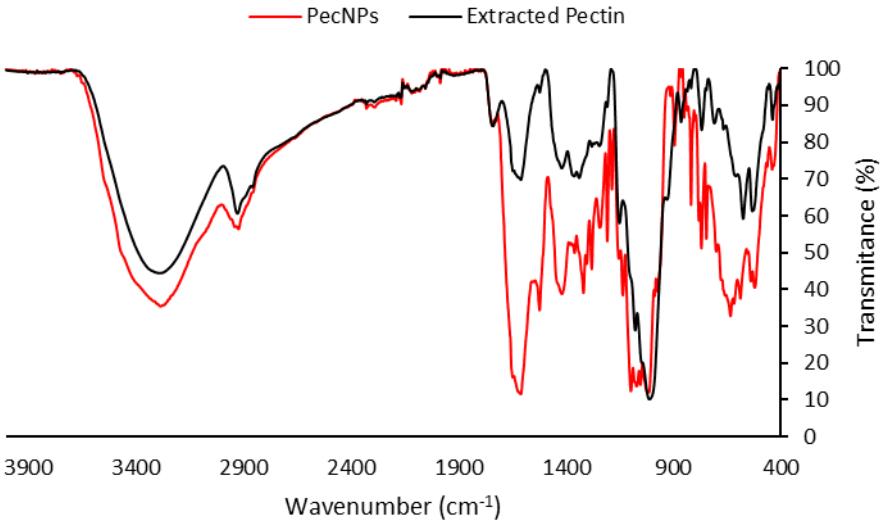


Figure 20 - FTIR-UATR analysis of pectin and PecNPs.

The encapsulation of HES at different concentrations in PecNPs caused only a slight decrease in the intensity of the hydroxyl correspondent bands (Figure 21), which may indicate the presence of weaker intra- and intermolecular hydrogen bonds than those present in PecNPs. Nonetheless, no significant changes were noted in the spectra of PecNPs and HES-PecNPs at the different concentrations. Although this technique only allows the analysis of the surface of a material, and the infrared bands of pectin and HES overlap at the same wavenumbers, the absence of band shifts may indicate that the nanoparticle surface remains intact after HES addition, suggesting that HES does not adhere to the particle surface and is encapsulated.

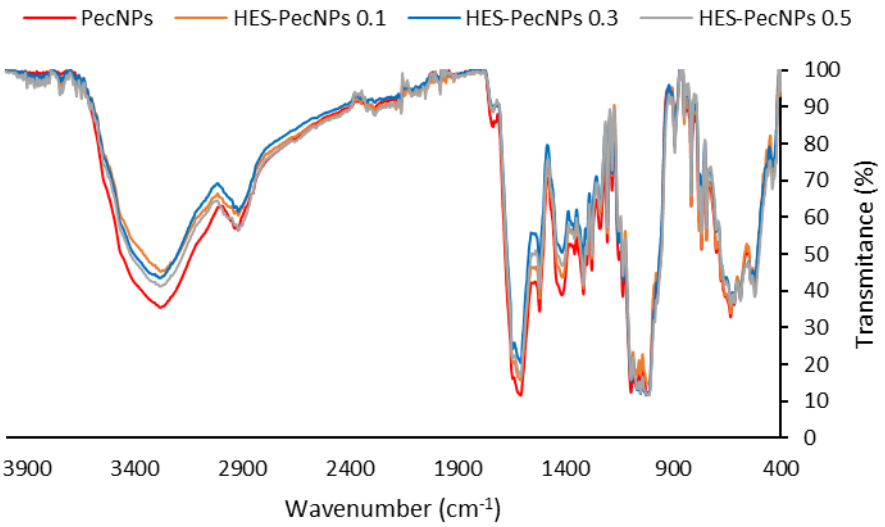


Figure 21 - FTIR-UATR analysis of PecNPs and HES-PecNPs.

## 3.3. Antioxidant Activity in vitro

The antioxidant activity of HES-PecNPs formulated at different HES concentrations was determined by $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ assays.

### 3.3.1. $DPPH^{\bullet}$ radical scavenging activity assay

The $DPPH^{\bullet}$ scavenging assay is one of the most common methods used to characterize the antioxidant activity of samples owing to its simplicity and excellent radical stability. This radical solution exhibits a dark purple color and a maximum absorbance at approximately 517 nm. In the presence of an antioxidant, the radical is reduced through electron donation, resulting in radical neutralization and solution discoloration that is accompanied spectrophotometrically at the 517 nm [127].

This assay showed to be an inadequate and unreliable method for measuring the antioxidant activity of nanoparticles as it gave unstable results (data not shown). The DPPH reagent needs to be dissolved in ethanol or methanol, which does not reflect the real behavior of antioxidants in biological systems and limits the determination of the antioxidant activity of hydrophilic compounds, such as pectin [141]. Moreover, pectin precipitation and consequent jellification are induced by these solvents. Therefore, these results were not considered.

### 3.3.2. $ABTS^{+\bullet}$ radical scavenging activity assay

The $ABTS^{+\bullet}$ method is widely used to evaluate the antioxidant activity of samples for the same reasons mentioned above for the $DPPH^{\bullet}$ assay. The radical is prepared by promoting ABTS salt oxidation with strong oxidizing agents, such as potassium persulfate. The antioxidant will promote the radical neutralization, resulting in the loss of color intensity and absorbance of the chromophore at 734 nm. However, it is highly criticized for using synthetic radicals and is, therefore, not relevant at the biological level [127].

The antioxidant activity of each sample in the absence or presence of an oxidant is shown in Figure 22. Independent of the absence or presence of an oxidant, Trolox and HES showed increasing $ABTS^{\bullet+}$ scavenging activities with increasing concentrations. In the presence of an oxidant, the scavenging activity of HES only decreased significantly ($p < 0.05$) for the concentration 0.1 mg/mL and remained statistically equal ($p > 0.05$) for the other tested concentrations. These results suggest that HES, when subjected to prior oxidative stress, can maintain its antioxidant activity in a concentration-dependent manner. These findings are supported by Roy *et al*. [128], who reported that free quercetin and myricetin presented a lower $DPPH^{\bullet}$ scavenging activity in the presence of the same oxidant. Interestingly, this was not observed for Trolox, a standard commonly used in these assays. When subjected to prior oxidative stress, Trolox exhibited significantly lower $ABTS^{\bullet+}$ scavenging activity at all tested concentrations ($p < 0.05$). This difference between these two molecules can be attributed to their different chemical structures, more specifically, the number and position of their hydroxyl groups. Regarding the nanoparticles, HES-PecNPs

0.5 presented a significantly higher antioxidant activity ($p < 0.05$) than the other formulations without and with prior oxidative stress and, consequently, was chosen for further characterization. PecNPs and HES-PecNPs 0.1 presented significantly higher scavenging activity ($p < 0.05$) against $ABTS^{\bullet+}$ in the presence of an oxidant, while the remaining particles maintained it. These results are supported by Roy *et al*. [128], who verified that quercetin and myricetin encapsulated in ChNPs presented a higher antioxidant activity in the presence of oxidative stress. Nonetheless, the present study did not allow to compare the antioxidant activity of free polyphenols and nanoparticles, given that the concentration of HES present in the weighed nanoparticles was not equivalent to the concentration of the free flavonoid.

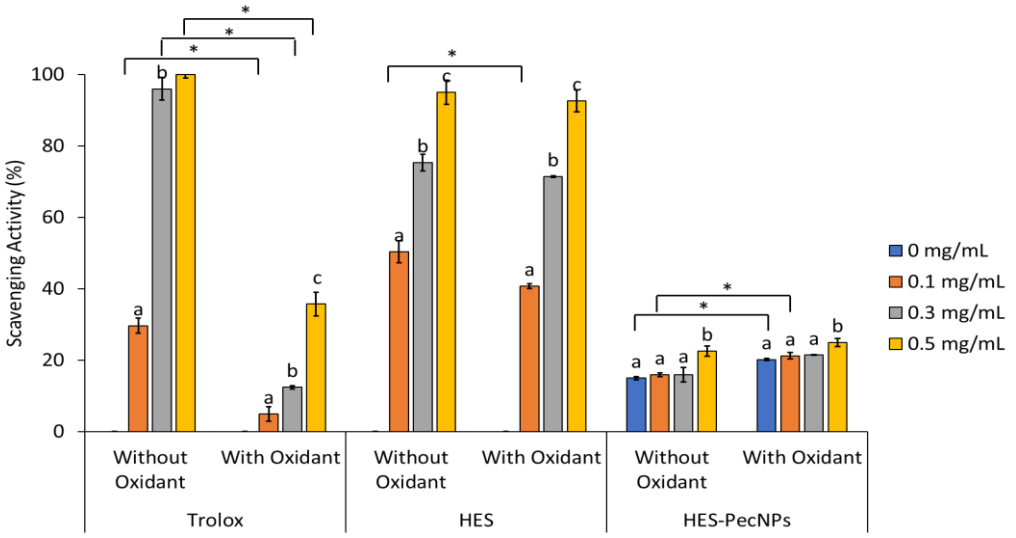


Figure 22 - Scavenging activity of Trolox, HES and HES-PecNPs in the absence and presence of an oxidant. The statistical differences were analyzed using one-way ANOVA followed by Tukey's multiple comparisons tests. Letters (a, b) in each bar represents the statistically significant differences detected ($p < 0.05$) between the concentrations of a sample. * Represents statistically significant differences ($p < 0.05$) between the same sample in the absence and presence of an oxidant.

## 3.4. In vitro Release Studies

The cumulative release profile of HES from the nanoparticles along time in PBS, at pH 6.6, is shown in Figure 23. The release profile did not reveal an initial burst release, which may indicate that HES is not adhered to the nanoparticle surface and consequently, its release mechanism may not happen through desorption. Instead, a slow and controlled release is visualized along time, with a maximum HES release around 45% within 26h. This behavior suggests that HES is encapsulated and that diffusion is the mechanism of release.

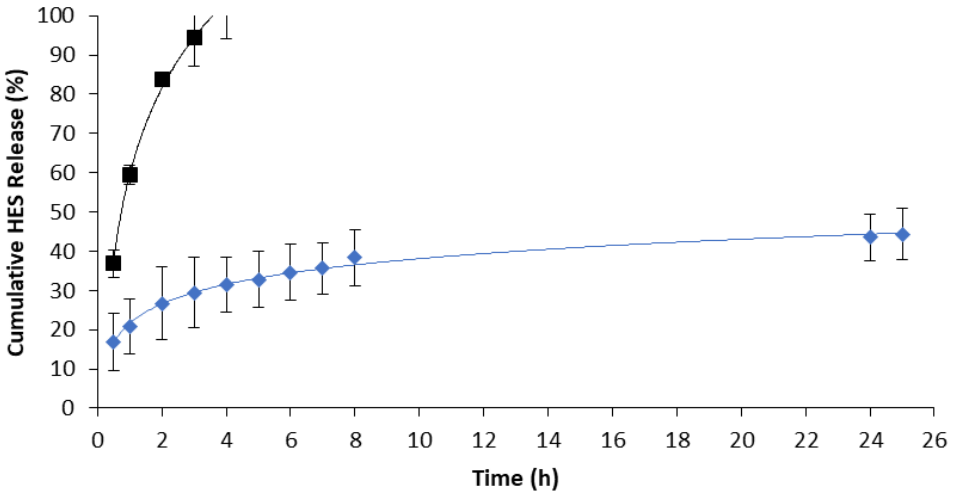


Figure 23 - In vitro release profile of free HES (in black) and HES-PecNPs 0.5 (in blue).

## 3.5. Stability studies

The stability of HES-PecNPs 0.5 at two storage temperatures was assessed for 30 days by determining their physicochemical properties (Figure 24). The hydrodynamic size of HES-PecNPs 0.5 stored at room temperature (27 ºC) showed a tendency to increase over time but was only statistically significant ($p < 0.05$) between 0 and 7 days of storage. However, at the same temperature, PDI significantly increased ($p < 0.05$) after 14 days. Changes in this physicochemical property could indicate higher heterogeneity in size, which could have been caused by nanoparticle aggregation or even degradation. Nonetheless, the zeta potential showed a tendency to decrease over time but was only statistically significant ($p < 0.05$) after 30 days of analysis. At a storage temperature of 7 ºC, the hydrodynamic size and PDI remained statistically equal ($p > 0.05$), whereas the zeta potential was significantly lower ($p < 0.05$) after 7 days but remained constant for the rest of the study ($p > 0.05$). Therefore, the most suitable storage temperature for HES-PecNPs 0.5 was determined to be 7 ºC given that no major changes were detected in their physicochemical properties.

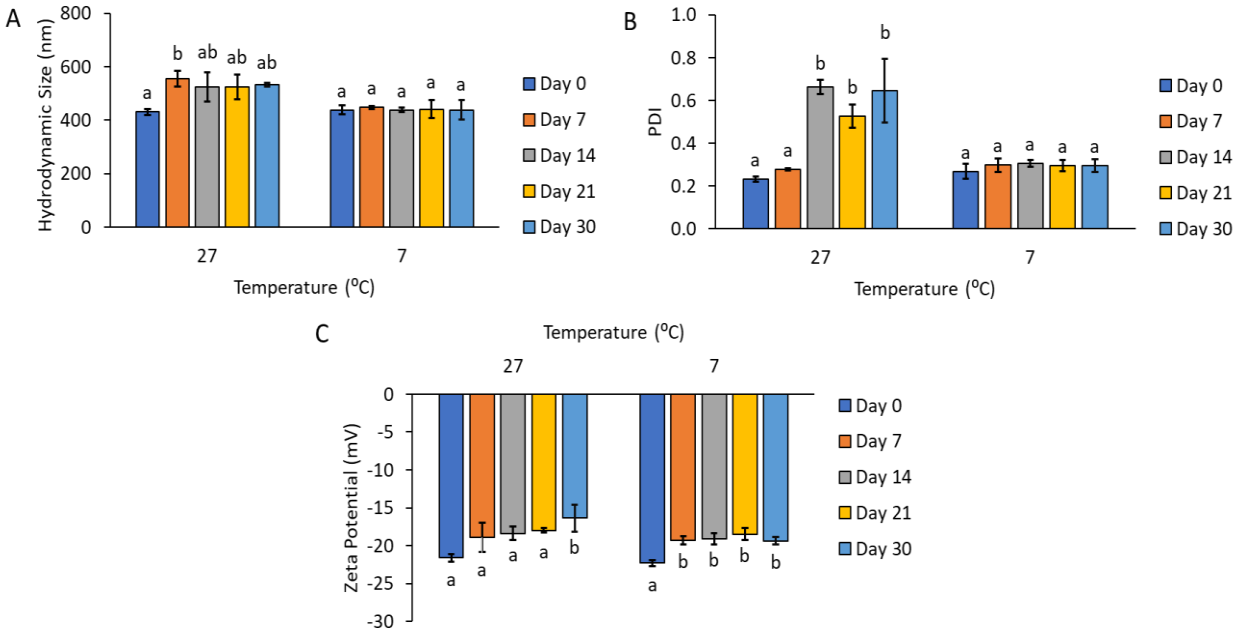


Figure 24 - Effect of two storage temperatures on HES-PecNPs 0.5 hydrodynamic size (A), PDI (B), and zeta potential (C) for 30 days. The statistical differences were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons tests. Letters (a, b) in each bar represents the statistically significant differences detected ($p < 0.05$) between the days of analysis at a given temperature.

## 3.6. WAC

Along with pest infection, drought affects crop quality and productivity by, for example, decreasing the photosynthetic and morphological rates of plants. Given that soils suffer more frequently from water scarcity due to climate change, hydrogels have been widely used as soil conditioners because of their ability to absorb and retain water [142]. These water-swelling polymers, also called superabsorbent polymers, can absorb large amounts of water without dissolving and desorbing water when exposed to mechanical stresses. In particular, they have been combined with pesticides to have a controllable and efficient release system of both pesticides and water [143].

In the present work, the WAC of pectin and nanoparticles was evaluated to determine whether conformation changed pectin absorption capacity, and if HES, a poorly soluble molecule in water, would affect the WAC of PecNPs. This method allows to measure the polymer's capacity to retain bound, hydrodynamic, and physically trapped water when subjected to a centrifugal or mechanical force [134,135]. The WAC of isolated pectin was 6.21 ± 2.21 g of water/g of sample, which is slightly lower than the reported values for pectin extracted from citrus (8-10 g/g) [144], mango peel (9.60 g/g) [135], and mixed peels of banana-papaya (8.23 ± 2.84 g/g) [134]. These dissimilarities rely on differences in the pectin chemical composition present on the fruit matrices. As expected, PecNPs presented a higher and statistically different WAC (14.42 ± 2.29 g/g) than native pectin because nanoparticles present a higher surface area compared to bulk materials, which allows a higher interaction with water. Encapsulation of HES in PecNPs (HES-PecNPs 0.5) did not significantly change this property (15.70 ± 2.29 g/g).

# IV. Conclusions and Future Work

Conventional pesticide overuse is characterized by pest resistance, soil microbiota impoverishment, loss of water quality, food contamination, and carcinogenic and teratogenic effects on humans. For these reasons, biopesticides have been advocated to complement pesticides for their rapid biodegradability and low toxicity. More recently, food waste was proposed as a potential source of botanical pesticides owing to its high abundance and easy obtention, avoiding the traditional research of these compounds in seasonal or endemic plants where, at most times, are present in low amounts. Biopesticide research using food waste focuses majorly on essential oil potential, lacking in the research of other compounds such as flavonoids for this application. When studied, flavonoids are usually extracted with resource to organic solvents or employ expensive solvents and techniques. Moreover, these methods focus on single compound extraction, lacking in maximizing the residue potential. Bearing this in mind, a major part of this dissertation focused on isolating the promising biopesticide HES from orange waste through the development of an efficient, eco-friendly, and economically viable methodology.

The developed methodology (Extraction Method II) allowed the design of a new HES extraction and precipitation method using an alkaline hydroethanolic mixture, proving to be less time-consuming than Extraction Method I. Pectin prior extraction and solvent recovery and re-incorporation in the extraction scheme did not interfere with HES extraction and quality. Isolated pectin met the criteria to be used for food industry purposes owing to a very high AUA (66.20%) and DE (59.37%). The best tested conditions for HES extraction were 30 minutes, 70 °C, and 1:10 (w/v), where the extraction yields and purity were 0.68 ± 0.05% and 84.01% (determined by HPLC-PDA), respectively. HES further characterization by NMR, FTIR-UATR, and melting point revealed the presence of some minor impurities. Based on these findings, Extraction Method II follows the principles of green chemistry and circular economy and offers valuable advantages over Extraction Method I for its low environmental impact. Moreover, it proved to be a strong starting point for citrus wastes to be converted into value-added products through efficient, economic, and eco-friendly batch methodologies.

qNMR demonstrated to be a suitable technique for HES purity determination when using benzoic acid as an internal standard. Although further method optimization and validation is necessary, this technique shows immense potential to be employed to assess the purity of flavonoids in general.

Biopesticides use in fields are associated with several limitations, including rapid biodegradability and several reapplications to achieve the desired effect. In that sense, encapsulation of this molecules in biodegradable matrices, such as polysaccharides, has been a target of interest. Bearing this in mind, a second part of this dissertation focused on attempting HES encapsulation in two polysaccharides, pectin and chitosan, whose degradation in the respective oligosaccharides is beneficial to plants. Chitosan revealed to not be a good matrice for HES nanoencapsulation, probably due to its physicochemical properties or prior degradation. HES-PecNPs 0.5 were selected as the most advantageous for biopesticide

applications from the prepared formulations, given their physicochemical properties (452.8 ± 22.1 nm and -16.9 ± 0.7 mV), high encapsulation efficiency (85.0%), and resistance to oxidative stress. The release studies in vitro demonstrated a slow and controlled release of HES over time and suggested that the mechanism of release might be by diffusion. The best storage temperature was determined to be 7 $^{o}$C for 30 days and HES encapsulation in PecNPs did not interfere with the polymer capacity to absorb water.

For future work and perspectives, it would be interesting:

- From a waste valorization perspective view, to study the possibility of extracting more bioactive compounds from citrus waste without compromising the developed consecutive extraction. This could be achieved by incorporating the extraction of essential oils, rich in D-limonene, by hydrodistillation before pectin and HES extractions.
- To perform more advanced studies about qNMR for HES purity determination by, for instance, using certified benzoic acid for qNMR (not available at the time these studies were conducted), and optimizing and validating the method by determining the $T_1$ and $d_1$;
- To determine the molecular weight of extracted pectin (by, for example, viscometry) since this physicochemical property was reported to influence nanoparticle stability in orange juice clouds by previously published articles. Moreover, to calculate its neutral sugar content given previous articles reporting the role of this property on the strength of interaction of this polysaccharide with HES.
- To perform HES-PecNPs surface and size analysis by scanning or transmission electron microscopy;
- To evaluate the effect of chitosan with different molecular weights (low- and high-molecular-weight) and origins (animal and fungi) on HES-ChNPs physicochemical properties;
- To study the inhibitory activity of free and nanoencapsulated HES in insect neuronal (such as acetylcholinesterase) and digestive enzymes (such as lipase, α-glycosidase, β-glycosidase, and α-amylase), given that the literature review on flavonoids as biopesticides revealed their tendency to impact the development and food uptake of insects in the larval state.

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# VI. Supplementary Information

Table S1 – Reagents and Standards used in the experimental procedures.

| Compounds | Manufacturer (Country) | Purity |
|---|---|---|
| **ABTS** | Fluka (Switzerland) | ≥ 99.0% |
| **Acetone** | Fisher Scientific (Finland) | Analytical reagent grade ≥99.8% |
| **Apple Pectin** | Acros Organics (Belgium) | Unknown |
| **Benzoic acid** | João Manuel Gomes Santos (Portugal) | 99.9% |
| **Chitosan, low molecular weight** | Sigma Aldrich | Unknown |
| **Citric Pectin** | Thermo Scientific (Switzerland) | Unknown |
| **Deuterated dimethyl sulfoxide** | Thermo Scientific (Switzerland) | |
| **Dichloromethane** | Sigma Aldrich (United Kingdom) | Analytical reagent grade ≥99.0% |
| **Dimethyl sulfoxide** | Fisher Scientific (United Kingdom) | Analytical reagent grade ≥99.9% |
| **Dipotassium hydrogen phosphate** | Chem-Lab (Belgium) | ≥ 99.0% |
| **DPPH** | Fluka (Switzerland) | ≥ 85.0% |
| **Ethanol** | Aga (Portugal) | 96% |
| | CQM (Madeira) | |
| **Hesperidin** | Sigma Aldrich (United Kingdom) | 92.1% |
| **Hydrochloric acid** | HoneyWell (Germany) | ACS reagent |
| **Magnesium chloride hexahydrate** | Panreac (Germany) | ACS reagent 99.0-102% |
| **Methanol** | Fisher Scientific (United Kingdom) | Analytical reagent grade 99.9% |
| **NEDA** | Sigma-Aldrich (United States) | ≥ 99.0% |
| **Phenol red** | Unknown | Unknown |
| **Phosphoric acid** | BDH (England) | 85.0% |
| **Potassium dihydrogen phosphate** | Merck (Germany) | ≥ 98.5% |
| **Potassium persulfate** | Sigma-Aldrich (Japan) | ≥ 99.0% |
| **Sodium Bicarbonate** | Fluka (Germany) | ≥99.0% |
| **Sodium chloride** | Sigma Aldrich (Denmark) | ACS reagent ≥99.8% |
| **Sodium hydroxide** | Panreac (Germany) | Analytical reagent grade 99.0% |
| **Sodium nitroprusside** | Merck (Germany) | ≥ 99.0% |
| **Sulfanilamide** | Sigma-Aldrich (United States) | ≥ 99.0% |
| **Sodium Tripolyphosphate** | ThermoFisher Alfa Aesar (Germany) | Unknown |

Table S2 – Equipment and materials used throughout the work.

| Equipment/Materials | Model, *Brand* |
|---|---|
| **0.45 µm cellulose Acetate sterile syringe filters** | *Frilabo* |
| **Analytical balance** | M124AI, *BEL Engineering* |
| **Analytical microbalance** | AT20, *Mettler Toledo* |
| **Centrifuge** | Rotofix 32 A, *Hettich Zentrifugen* |
| | 3-30ks, *Sigma* |
| **Dialysis bag, Regenerated Cellulose, 6-8 kDa MWCO** | *Spectrum™ Labs Spectra/Por™* |
| **Domestic grinder** | PC-KSW 1021 N, *PROFICOOK* |
| **Food dehydrator** | 112380, *Princess* |
| **Freeze drier** | Alpha 1-2 LD Plus, *Martin Christ* |
| **Heating and sitting plate** | Isotemp, *Fisher Scientific* |
| | MR Hei-Standard, *Heidolph* |
| **Infrared spectrometer** | Spectrum Two, *Perkin Elmer* |
| **LC column (Kinetex 5u C18, 150 x 4.60 mm)** | *Phenomenex* |
| **Melting point apparatus** | M-560, *Buchi* |
| **Microplate reader** | Victor$^3$ 1420 multilabel plate counter, *Perkin-Elmer* |
| **Moisture Analyser** | DBS 60-3, *Kern* |
| **NMR spectrometer** | Ultrashield 400 Plus, *Bruker* |
| **pH meter** | 744 pH Meter, *Metrohm* |
| **Rotary evaporator** | R-144 with Waterbath B-480, *Buchi* |
| **Sonication Probe** | Vibra Cell 72434, *Bioblock Scientific* |
| **Spectrophotometer** | Lambda 2, *Perkin Elmer* |
| **Submersible Stirring plates** | Unknown |
| **Thermostatic water bath** | GD100-S18, *Grant* |
| **Zetasizer** | Nano ZS, *Malvern* |

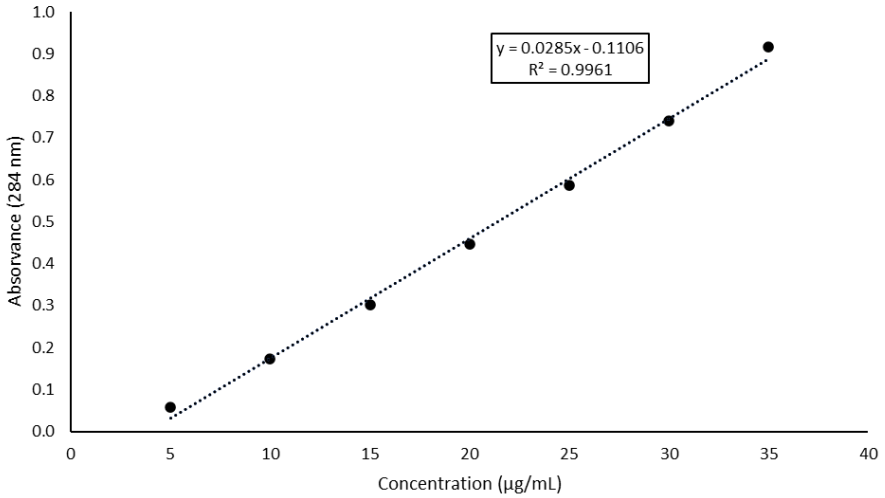


Figure S1 – Calibration curve used for encapsulation efficiency determination, obtained with different hesperidin concentrations ranging from 5 to 35 ug/mL prepared in 1 mL of DMSO: 9 mL of UPW.

Table S3 – Values of the mass of sample, mass of IS, and integration of the proton signal of HES. The value of integration of IS was maintained equal to 1 for all samples.

| HES Sample | $m_{Sample}$ (g) | $m_{IS}$ (g) | $I_{HES}$ |
|---|---|---|---|
| **Standard** | 0.0178 | 0.016824 | 0.1886 |
| | 0.021152 | 0.018284 | 0.2064 |
| | 0.01876 | 0.018252 | 0.1884 |
| | 0.019092 | 0.01784 | 0.1911 |
| | 0.01959 | 0.01914 | 0.1817 |
| | 0.0169 | 0.017412 | 0.1751 |
| **Extraction Method I** | 0.02587 | 0.02615 | 0.1669 |
| | 0.02159 | 0.02314 | 0.1627 |
| | 0.02324 | 0.02376 | 0.1713 |
| | 0.02375 | 0.02589 | 0.1571 |
| | 0.0253 | 0.0269 | 0.1635 |
| | 0.02216 | 0.02401 | 0.1597 |
| **Extraction Method II** | 0.02373 | 0.03052 | 0.1051 |
| | 0.02258 | 0.02531 | 0.1176 |
| | 0.02288 | 0.02332 | 0.1309 |
| | 0.02334 | 0.02806 | 0.1134 |
| | 0.0205 | 0.02538 | 0.1115 |
| | 0.02158 | 0.0232 | 0.1291 |

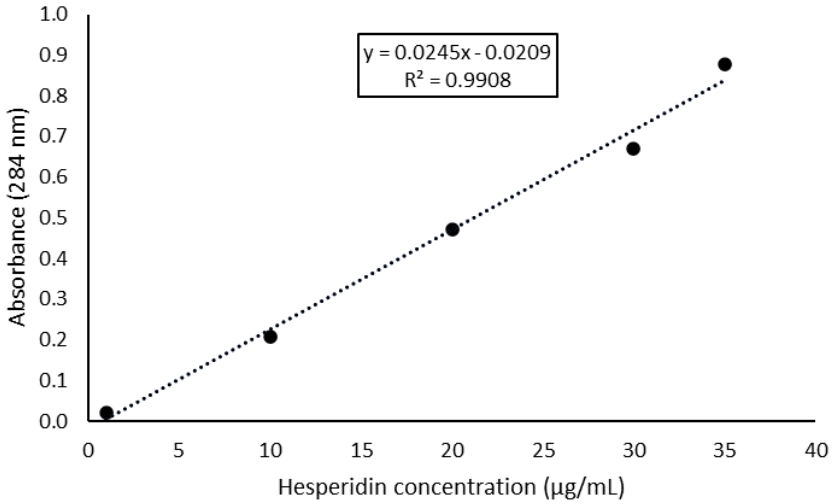


Figure S2 - Calibration curve used to determine the amount of hesperidin released from the nanoparticles, obtained with different hesperidin concentrations ranging from 1 to 35 µg/mL prepared in 10 mM PBS at pH 6.6.

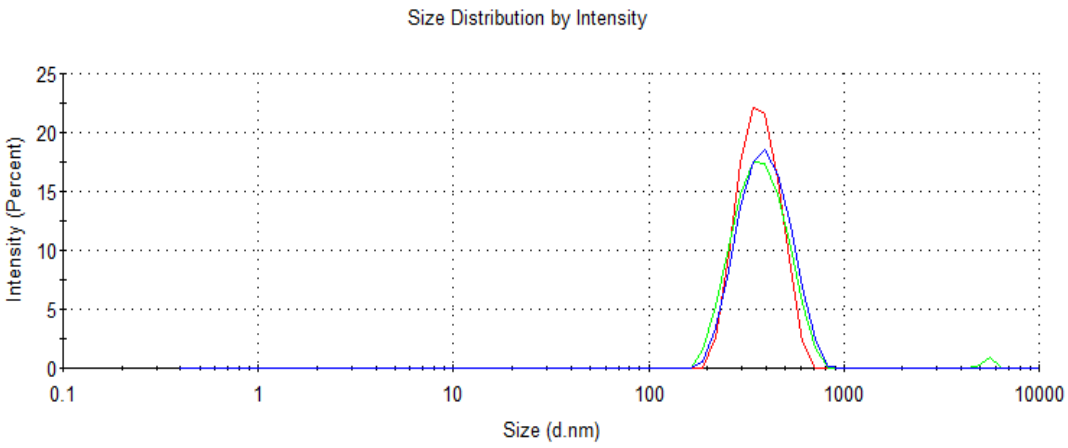


Figure S3 – Size distribution by the intensity of PecNPs (n=3).

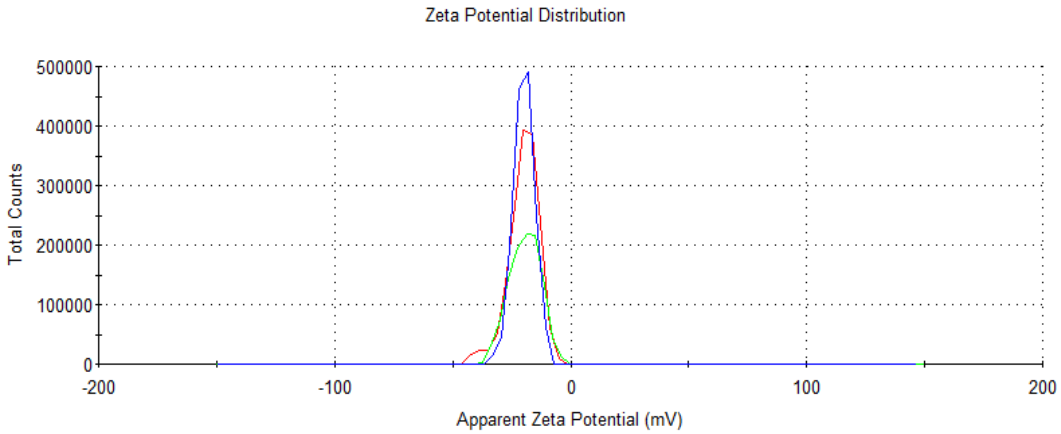


Figure S4 - Zeta Potential distribution of PecNPs (n=3).

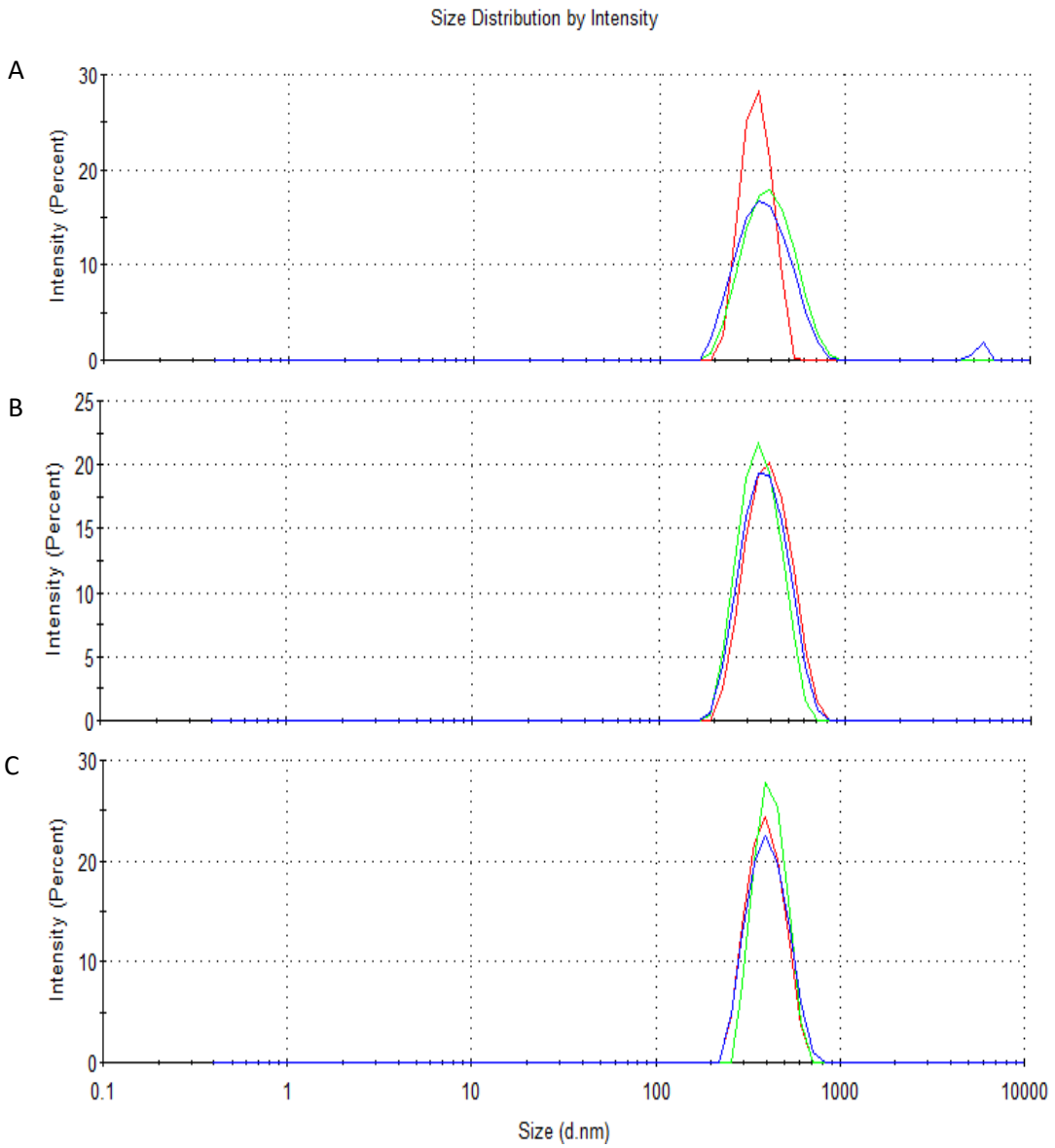


Figure S5 - Size distribution by the intensity of HES-PecNPs at a hesperidin concentration of 0.1 mg/mL (A), 0.3 mg/mL (B) and 0.5 mg/mL (C) (n=3).

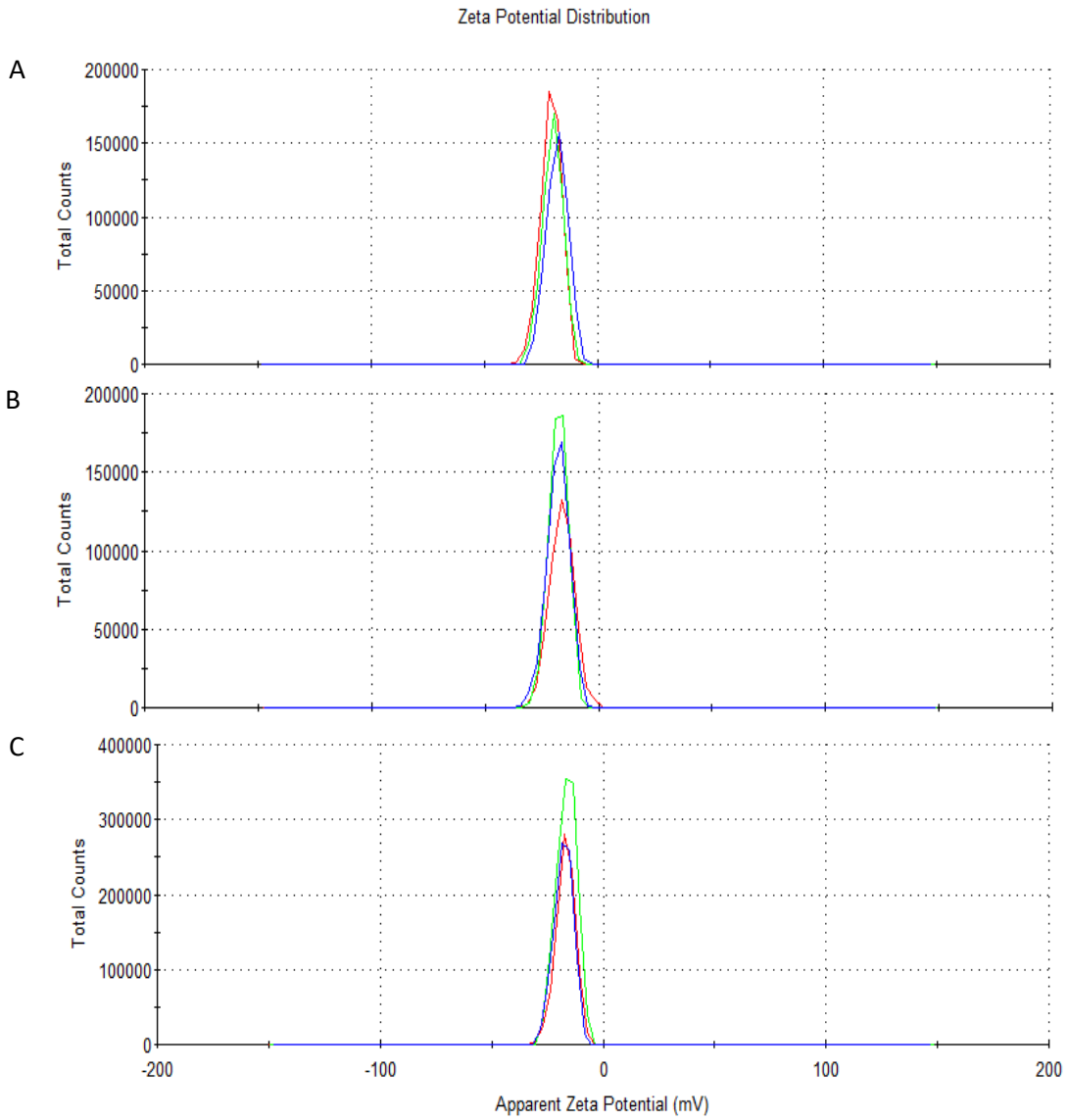


Figure S6 - Zeta potential distribution of HES-PecNPs at a hesperidin concentration of 0.1 mg/mL (A), 0.3 mg/mL (B) and 0.5 mg/mL (C) (n=3).


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