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Thesis submitted for the degree of

Doctor of Philosophy

Freeze-dried emulsions and bigels with plant extract-loaded microparticles as an eco-friendly prototype of novel materials designed with a view to sustainable water management

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Liofilizowane emulsje i biżele zawierające mikrocząstki z ekstraktem roślinnym jako ekologiczny prototyp nowych materiałów zaprojektowanych z uwzględnieniem zrównoważonej gospodarki wodnej

I would like to express my heartfelt gratitude to all those
who supported me in completing this work:

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LIST OF CONTENTS

ACRONYMS

ALG – sodium alginate
Am – *Achillea millefolium*
B – beeswax
BG – bigel
CUPRAC – CUPric Reducing Antioxidant Capacity
DPPH RSA – 2,2′-diphenyl-1-picrylhydrazyl Radical Scavenging Assay
DW – dry material weight
E – emulsion
EC – ethylcellulose
FD – freeze-dry
FRAP – Ferric Reducing Antioxidant Power
G – glycerin
GAE – gallic acid equivalents
Gh – *Glechoma hederacea*
LC – loading capacity
M – mannitol
MC – moisture content
MPs – microparticles
Pe – *Potentilla erecta*
PG – propylene glycol
Pl – *Plantago lanceolata*
QE – quercetin equivalents
S – sorbitol
SBO – sea buckthorn oil
SEM – scanning electron microscope
Sn – *Sambucus nigra*
SO – sunflower oil
T – trehalose
Tc – *Tilia cordata*
TEWL – transepidermal water loss
TFC – Total Flavonoids Content
TPC – Total Polyphenols Content
TPP – pentasodium tripolyphosphate
TPTZ – 2,4,6-tripyridyl-s-triazine
Vo – *Veronica officinalis*
Vt – *Viola tricolor*
WPI – whey protein isolate

ABSTRACT

Although water is a dominant ingredient in many cosmetic and personal care formulations, it has no direct cosmetic effect on the skin. Instead, it mainly serves as a base component and solvent for other ingredients. Emulsion is a biphasic system comprising aqueous and oily phases, which are stabilized by emulsifiers. Bigel is formed by combining hydrogel and oleogel, consisting of a water-based phase containing hydrophilic polymers and an oil phase gelled with an organogelator. The growing awareness of environmental challenges, including the global water crisis, has increased interest in developing sustainable technologies across various industries. In cosmetics, this is crucial due to their enormous production – most consumers worldwide use several cosmetic and personal care products every day. Owing to the rapidly shrinking resources of clean and accessible water, water recovery during product formulation is a responsible attitude towards climate change and the global water crisis.

The objective of this research was the design, methodology development and characterization of novel eco-friendly materials based on freeze-dried emulsions and bigels composed of natural polymers, lipids and cryoprotectants intended for cosmetic and dermatological applications. Moreover, polymer microparticles loaded with *Sambucus nigra* (elderflower) extract were incorporated into prepared materials. This extract was selected as the model active compound due to its high antioxidant content and proven beneficial effects on skin condition. Encapsulation of the extract in microparticles prior to freeze-drying aimed to protect it during processing and enable its controlled release during application to the skin. The developed material was designed to rehydrate into a functional emulsion or bigel using only a minimal amount of aqueous solvent immediately before use (during spreading on the skin), offering a new, sustainable alternative to conventional water-based cosmetic products. Accordingly, it is hypothesized that it is possible to reduce water consumption during the preparation of cosmetic materials while preserving the functional properties of a prototype based on freeze-dried emulsions and bigels containing plant extract-loaded microparticles.

Extracts from commonly found Polish medicinal plants – *Viola tricolor*, *Veronica officinalis*, *Glechoma hederacea*, *Plantago lanceolata*, *Achillea millefolium*, *Sambucus nigra*, *Tilia cordata*, and *Potentilla erecta* – were obtained using Soxhlet extraction with water and ethanol. These extracts were characterized according to total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity using CUPRAC, FRAP, and DPPH assays.

Selected extracts were encapsulated into chitosan microparticles, and their loading capacity and *in vitro* release profiles were studied. The highest level of TPC, TFC and antioxidant activity was noted in *Sambucus nigra* aqueous extract.

The microparticles were then introduced into emulsions and bigels composed of biopolymers (sodium alginate, whey protein isolate, ethylcellulose), cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, trehalose), lipids (sunflower oil, sea buckthorn oil, beeswax), and an emulsifier (Span-80). The homogenization time and speed were evaluated to optimize the size distribution of the oily phase droplets. The prepared freeze-dried emulsions and bigels were characterized by scanning electron microscopy (SEM), mechanical properties, residual moisture content, porosity and density measurements, degradation properties, swelling ratio, and biophysical skin parameter analysis, including skin hydration, color and barrier quality – transepidermal water loss (TEWL).

The results of this research contribute to:

- the development in the field of cosmetic chemistry and materials science – the methodology development of a new cosmetic form, i.e., freeze-dried emulsions and bigels (dissolving in a minimal amount of an aqueous solvent) containing microparticles with enclosed plant extract (released during the material application),
- the development of solutions for health, especially for people allergic to preservatives – reduced water content in the material can reduce the material's susceptibility to microbial growth, potentially reducing the amount of added preservatives,
- environmental protection – reducing water usage due to the potential to reuse water sublimed during freeze-drying and reducing the plastic needed for packaging of developed materials (they are in dry form and have a lower mass than traditional emulsions).

STRESZCZENIE

Woda stanowi główny składnik większości kosmetyków i produktów do pielęgnacji ciała. Jednakże nie wywiera trwałego, bezpośredniego działania kosmetycznego na skórę – jest wyłącznie składnikiem bazowym i rozpuszczalnikiem innych substancji. Emulsja to układ dwufazowy składający się z fazy wodnej i olejowej, stabilizowany za pomocą emulgatorów. Biżel powstaje poprzez połączenie hydrożelu i oleożelu, które odpowiednio zawierają fazę wodną z polimerami hydrofilowymi oraz fazę olejową żelowaną organożelatorem. Rosnąca świadomość wyzwań środowiskowych, w tym globalnego kryzysu wodnego, przyczyniła się do wzrostu zainteresowania rozwojem zrównoważonych technologii w różnych gałęziach przemysłu. W przypadku kosmetyków jest to kluczowe z uwagi na ich ogromną produkcję – większość konsumentów na całym świecie korzysta każdego dnia co najmniej z kilku produktów kosmetycznych i higieny osobistej. W obliczu szybko kurczących się zasobów czystej i łatwo dostępnej wody, odzysk wody w trakcie formułowania materiałów jest odpowiedzialnym podejściem do zmian klimatycznych i globalnego kryzysu wodnego.

Celem niniejszej pracy było zaprojektowanie, opracowanie metodologii oraz charakterystyka nowatorskich, przyjaznych środowisku materiałów bazujących na liofilizacji emulsji i biżeli, składających się z naturalnych polimerów, substancji olejowych oraz krioprotektantów, przeznaczonych do zastosowań kosmetycznych i dermatologicznych. Dodatkowo, do przygotowanych materiałów wprowadzono polimerowe mikrocząstki zawierające ekstrakt z kwiatów bzu czarnego. Ekstrakt ten został wybrany jako modelowy związek aktywny ze względu na wysoką zawartość antyoksydantów oraz udokumentowany korzystny wpływ na kondycję skóry. Enkapsulacja ekstraktu w mikrocząstkach przed procesem liofilizacji miała na celu jego ochronę oraz umożliwienie kontrolowanego uwalniania podczas aplikacji na skórę. Opracowany materiał został zaprojektowany tak, aby powracał do emulsji lub biżelu przy użyciu minimalnej ilości wodnego rozpuszczalnika bezpośrednio przed zastosowaniem (podczas rozprowadzania na skórze), co stanowi nową, zrównoważoną alternatywę dla konwencjonalnych produktów kosmetycznych. W związku z tym postawiono hipotezę, że możliwe jest ograniczenie zużycia wody podczas przygotowania materiałów kosmetycznych przy jednoczesnym zachowaniu funkcjonalnych właściwości prototypu opartego na liofilizowanej emulsji i biżelu z mikrocząstkami zawierającymi ekstrakt roślinny.

Ekstrakty z powszechnie występujących polskich roślin leczniczych – fiołka trójbarwnego, przetacznika lekarskiego, bluszczyka kurdybanka, babki lancetowatej, krwawnika pospolitego, bzu czarnego, lipy drobnolistnej oraz pięciornika kurzego ziela – otrzymano metodą ekstrakcji Soxhleta z zastosowaniem wody oraz etanolu. Ekstrakty te scharakteryzowano pod względem całkowitej zawartości polifenoli (TPC) i flawonoidów (TFC) oraz aktywności antyoksydacyjnej przy użyciu metod CUPRAC, FRAP oraz DPPH. Wybrane ekstrakty zostały zamknięte w mikrocząstkach chitozanowych, a następnie oceniono ich ładowność oraz profil uwalniania *in vitro*. Najwyższą zawartość TPC, TFC oraz aktywność antyoksydacyjną wykazano dla wodnego ekstraktu z kwiatów bzu czarnego.

Otrzymane mikrocząstki wprowadzono następnie do emulsji i biżeli opartych na biopolimerach (alginian sodu, izolat białka serwatkowego, etyloceluloza), krioprotektantach (gliceryna, glikol propylenowy, sorbitol, mannitol, trehaloza), lipidach (olej słonecznikowy, olej z rokitnika, wosk pszczeli) oraz emulgatorze (Span-80). Oceniono czas i prędkość homogenizacji w celu optymalizacji rozkładu wielkości cząstek fazy olejowej. Przygotowane liofilizowane emulsje i biżele scharakteryzowano za pomocą skaningowej mikroskopii elektronowej (SEM), badań właściwości mechanicznych, oznaczenia zawartości wilgoci, porowatości, gęstości, degradacji, współczynnika pęcznienia oraz analizy parametrów biofizycznych skóry, obejmującą nawilżenie, barwę skóry oraz jakość bariery skórnej ocenianą na podstawie pomiaru wskaźnika transepidermalnej utraty wody (TEWL).

Uzyskane wyniki badań przyczyniają się do:

- postępu w dziedzinie chemii kosmetycznej i inżynierii materiałowej – opracowania nowej formy kosmetycznej, tj. liofilizowanych emulsji i biżeli (rozpuszczających się w minimalnej ilości wodnego rozpuszczalnika) zawierających mikrocząstki z zamkniętym ekstraktem roślinnym (uwalnianym podczas aplikacji materiału),
- opracowania rozwiązania prozdrowotnego, szczególnie dla osób uczulonych na konserwanty – zmniejszona zawartość wody w materiale może ograniczyć jego podatność na wzrost drobnoustrojów, a tym samym potencjalnie zmniejszyć ilość dodawanych konserwantów,
- ochrony środowiska – ograniczenie zużycia wody dzięki możliwości odzyskiwania wody sublimowanej podczas liofilizacji oraz zmniejszenie ilości plastiku potrzebnego do pakowania opracowanych materiałów (są one w formie suchej i mają niższą masę niż tradycyjne emulsje).

LIST OF PUBLICATIONS INCLUDED IN THE DOCTORAL DISSERTATION

1. [P1] original paper

W. Walendziak, N. R. Villegas, T. E. Douglas, J. Kozlowska, *Phytochemical studies of plant extracts enclosed in chitosan microparticles and the effect of phytoformulations on skin condition*, European Polymer Journal, 2025, 233, 113968.

IF_{2025} = 6.3

MNiSW = 100

2. [P2] original paper

W. Walendziak, T. E. Douglas, J. Kozlowska, *Design, Optimization, and Characterization of Freeze-Dried Emulsions Based on Sodium Alginate and Whey Protein Isolate Intended for Cosmetic and Dermatological Applications*, ACS Omega, 2025, 10, 24932−24949.

IF_{2025} = 4.4

MNiSW = 70

3. [P3] original paper

W. Walendziak, T. E. Douglas, J. Kozlowska, *Physicochemical Properties of Freeze–Dried Bigel-Based Materials Composed of Sodium Alginate/Whey Protein Isolate Hydrogel and Ethylcellulose/Sunflower Oil Oleogel*, Biomacromolecules, 2025, 26, 2344-2355.

IF_{2025} = 5.4

MNiSW = 140

4. [P4] material for publication

W. Walendziak, T. E. Douglas, J. Kozlowska, *Development of Freeze-Dried Emulsions and Bigels with Sambucus Nigra-Loaded Chitosan Microparticles to Improve Biophysical Skin Hydration and Barrier Integrity Parameters*

INTRODUCTION

1. Skin

Human skin, as the body's largest organ, is a multilayered structure consisting of epidermis, dermis and subcutaneous tissue (Fig. 1). The outermost epidermal layer is *stratum corneum*, which is organized in a 'brick-and-mortar' arrangement, where keratin-rich corneocytes represent the 'bricks' and intercellular lipids form the 'mortar' [1].

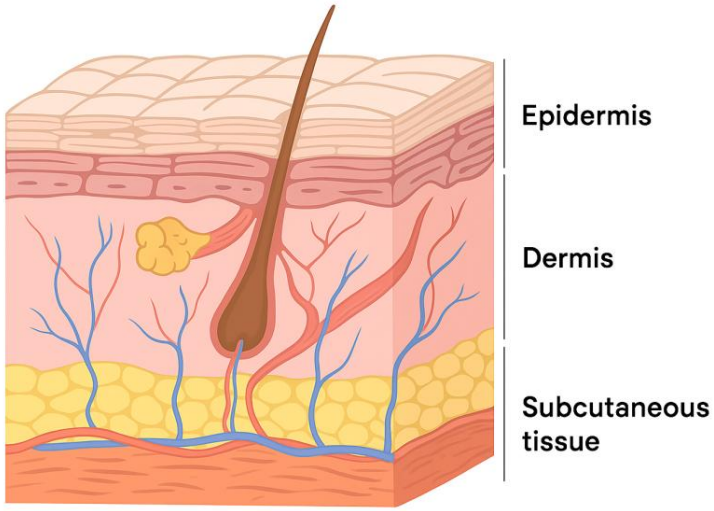


Figure 1. The structure of the skin.

The skin's structure is responsible for its essential protective, secretory, regulatory, and sensory functions [2]. As a key component of skin physiology, the epidermal barrier limits excessive transepidermal water loss (TEWL) and protects against environmental factors, pathogens and microorganisms.

The epidermis constitutes a selectively permeable barrier, thereby restricting the penetration of most exogenous substances. Nonetheless, three principal pathways of permeation have been described: diffusion across the *stratum corneum* via the transcellular or intercellular route, and penetration through cutaneous appendages, including sweat ducts, hair follicles, and their associated sebaceous glands (Fig. 2) [3,4]. In practice, most compounds are believed to traverse the skin through a combination of these mechanisms. The transcellular route represents a polar pathway that involves sequential passage through corneocytes, whereas the intercellular route provides a continuous diffusion trajectory within the lipid matrix of the

stratum corneum. Along this intercellular pathway, molecular transport may proceed through the hydrophobic lipid core or along the polar head groups of the lipid bilayers. In addition, penetration enhancers, such as glycerin and propylene glycol, can facilitate the transport of active ingredients across the *stratum corneum* by modifying the lipid organization or improving skin hydration [5]. Therefore, maintaining an appropriate skin hydration level and barrier integrity is a fundamental goal of many dermatological and cosmetic formulations.

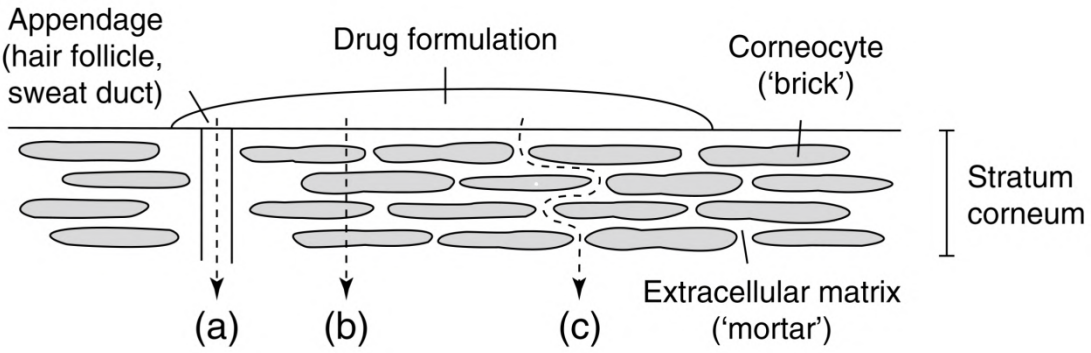


Figure 2. Diagram of the epidermis structure as the 'brick and mortar' model and permeation pathways: (a) the appendageal route; (b) the transcellular route; (c) the intercellular route [6].

This highly ordered skin structure is critical for maintaining skin homeostasis but also strongly restricts the penetration of hydrophilic and high-molecular-weight compounds. Such limitations pose significant challenges for the effective dermal delivery of cosmetic active substances. In recent years, encapsulation technologies have emerged as promising strategies to overcome these barriers, improving active substances' stability, biocompatibility, and permeability while enhancing their overall cosmetic efficacy upon topical application [7,8].

2. Global water crisis

A distinct ecological trend in cosmetic chemistry is currently evident, encompassing product formulation [9,10] and packaging strategies [11,12]. Research predominantly emphasizes biodegradable materials and natural raw substances, whereas the intensifying global water crisis remains largely neglected. Cosmetic production is enormous: according to Cosmetics Europe, the European cosmetics market in 2024 was valued at €104 billion, making Europe the third largest market for cosmetic products globally. Most consumers worldwide use at

least several cosmetic and personal care products daily. The vast number of cosmetics produced draws attention to the enormous water consumption involved. Unfortunately, clean and accessible water resources are shrinking rapidly (Fig. 3a). This is due to the world population growth with an increasing demand for water for hygiene, agriculture, food preparation, services, and industrial production. According to the World Wildlife Fund (WWF), these needs are not always met: two-thirds of the world's population may experience water shortages in the following years. The World Economic Forum also listed water scarcity in the Global Risks Report as a global environmental risk in terms of potential impact and likelihood over the next decade [13].

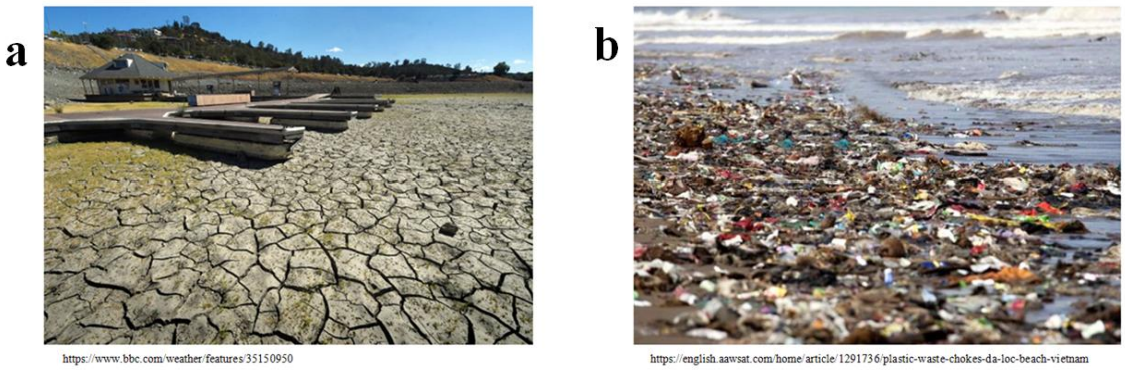


Figure 3. (a) Boats dock on a dried-up lake in California, USA, as the state entered its fourth year of drought in 2015. **(b)** A seashore littered with plastic waste – beach of Da Loc in Vietnam.

Another urgent environmental issue is plastic pollution owing to the rapidly increasing production of plastic. Every year, almost 400 million tons of plastic are produced worldwide, much of which may become pollutants. As the dominant generator of plastic waste, the packaging is responsible for almost half of the global total due to the short in-use lifetime of plastic packaging [14]. This includes cosmetics packaging, which is often "double" – for example, facial creams are usually packed in a plastic jar and a box of foil-covered paper. This method of packaging cosmetic products generates vast amounts of unnecessary plastic waste. Due to plastics' resistance to degradation, most will persist in the environment for centuries and may be transported far from their source. Accumulation of plastic in the environment threatens wildlife, their habitats, and the human population [15,16]. Plastic pollution affects land, waterways, and oceans, as shown in Figure 3b.

3. Waterless cosmetics

In response to these challenges, developing water-reduced or water-free cosmetic forms has gained significant attention [17,18]. Such approaches include powders, concentrates, or solid formulations such as shampoo and lotion bars. These strategies not only diminish water usage but also contribute to reducing the amount of plastic packaging required. Within this context, the freeze-drying of emulsions and bigels offers a novel solution, enabling the creation of lightweight, dry materials that rehydrate into functional cosmetic systems with the addition of a minimal amount of water immediately before use.

4. Emulsions vs. bigels

Emulsions remain among the most widely used cosmetic forms, composed of two mutually immiscible liquids – the internal (the dispersed phase) is finely dispersed in the external (the continuous phase) with the help of surface-active agents (Fig. 4). There are several distinguished types of emulsions. The oil phase dispersed in the aqueous phase constitutes an oil-in-water emulsion (O/W), whereas a water-in-oil (W/O) system occurs when the oil is the continuous phase and the water is the dispersed phase. Multiple emulsions are also possible, including a water-in-oil-in-water (W/O/W) and an oil-in-water-in-oil (O/W/O) emulsion whose smaller droplets are dispersed in larger ones [19]. Essential components of emulsions are emulsifiers that lower the surface tension between two phases and stabilize the system by increasing its kinetic stability. These compounds are made of the hydrophilic part (‘head’) and hydrophobic part (‘tail’), which are appropriately placed around the internal phase droplets. Emulsions found an application in cosmetics and pharmaceutics [20], medicine [21] and food industries [22], among others.

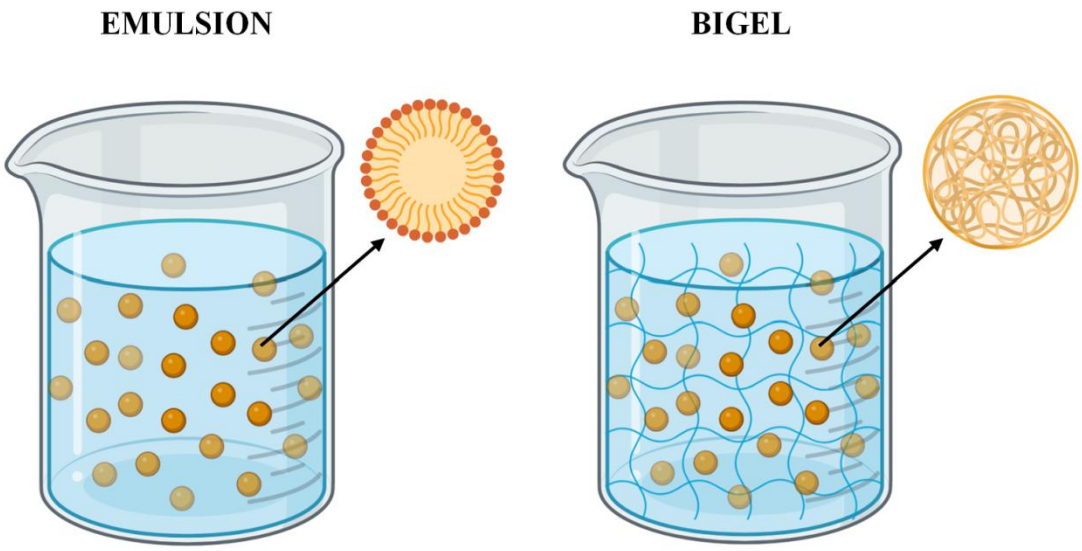


Figure 4. The structure of O/W emulsion and oleogel-in-hydrogel bigel.

Bigel, however, is a more complex system composed of a hydrogel, based on hydrophilic polymers, and an oleogel – oil gelled with an organogelator (Fig. 4) [23]. Hydrogels are three-dimensional networks of water-attracting polymer chains capable of retaining significant amounts of water [24]. They have been extensively studied in wound care, cosmetics, biomedicine, pharmaceuticals, drug delivery, and tissue engineering. Oleogels, on the other hand, are typically formed either by gelating polymer-based organogelators or through the self-assembly and non-covalent interactions of low-molecular-weight organogelators such as fatty acids, waxes, fatty alcohols, lecithin, and cyclodextrins [25]. When these two immiscible gels are combined under specific temperature conditions and high shear mixing, bigels are formed. The formation of bigels primarily depends on physical interactions between the two gelled phases, with hydrogen bonding playing a significant role in maintaining their structural integrity [26]. The physicochemical characteristics of bigels are influenced by various factors, including the composition of the aqueous and oil-based phases (using appropriate hydrophilic and lipophilic/amphiphilic polymers), the concentration of gelling agents, and the mixing ratio of hydrogel to oleogel [27,28].

Bigels present distinct advantages over conventional semisolid formulations, including enhanced physical stability compared to single-component gels, ease of application and uniform spreadability on the skin, as well as the ability to effectively improve *stratum corneum* hydration [29,30]. Emulsion systems are particularly valued for enhancing

therapeutic effects on the skin. However, emulsions are thermodynamically unstable and prone to microbial growth due to the high water content. Their decreasing stability through the storage time determines the shelf life of emulsions. These drawbacks may be overcome by freeze-drying, a dehydration method based on water removal by sublimation under reduced temperature and low pressure. It results in anhydrous or almost anhydrous, light and porous products. However, the freeze-drying process can impose mechanical stress that may compromise the stability of these systems. Cryoprotectants are compounds that prevent irreversible aggregation and coalescence of internal phase droplets during freezing [31]. Various polyols and sugars, including propylene glycol, glycerol, sorbitol, mannitol, and trehalose, function as effective cryoprotectants.

These novel formulations integrate the advantages of emulsions or bigels with those of freeze-dried systems, combining improved physicochemical stability, extended shelf life, and minimized microbial proliferation due to the very low water activity inherent to the dry state. The development of such dry forms represents a promising strategy to reduce water consumption during both production and application, addressing sustainability challenges in cosmetic chemistry. Additionally, the reduced mass and volume of freeze-dried materials facilitate transport, storage, and handling, while simultaneously enabling a significant reduction in plastic packaging, thereby contributing to lower environmental impact and plastic waste generation. Beyond sustainability, this approach enhances user convenience, as the lightweight, portable formulations are suitable for travel and on-the-go use. Overall, freeze-dried emulsions and bigels present a versatile platform for eco-conscious product development, allowing formulators to design innovative cosmetics that align with both technological performance and environmental responsibility.

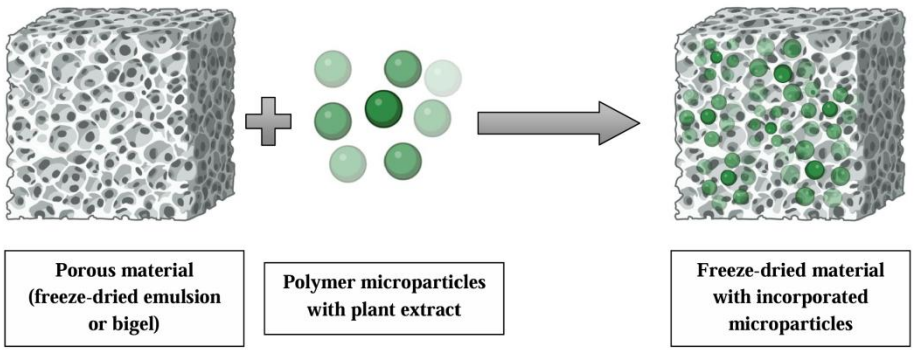


Figure 5. The scheme of incorporation of microparticles into a freeze-dried emulsion.

Water that has sublimed during freeze-drying of emulsion can be reused, which guarantees reasonable water management and the development of water-saving technology to obtain new and eco-friendly materials for cosmetic applications. The outcomes of this thesis address the need for continuous innovation in cosmetic chemistry and new possible solutions for current global problems, such as the global water crisis and plastic pollution. Moreover, these materials may be modified by the addition of microparticles loaded with active substances (Fig. 5). Incorporation of microparticles into freeze-dried emulsions and bigels offers a promising approach to designing multifunctional materials with enhanced skin compatibility and prolonged bioactivity.

5. Microencapsulation

Microencapsulation has gained recognition as a valuable tool for improving the stability and efficacy of bioactive compounds. Microencapsulation is used to obtain spherical particles with diameters varying from 1 to 1000 μm. The most widespread microparticles are microspheres and microcapsules (Fig. 6). The substantial difference in their morphology is the distribution of the active substance. Microcapsules exhibit a membrane-wall structure with an aqueous or oily core containing the bioactive substance, while microspheres have a matrix system where the bioactive substance is dispersed throughout the particles. Variations of these structures include capsules with multiple cores or with multiple shells [32].

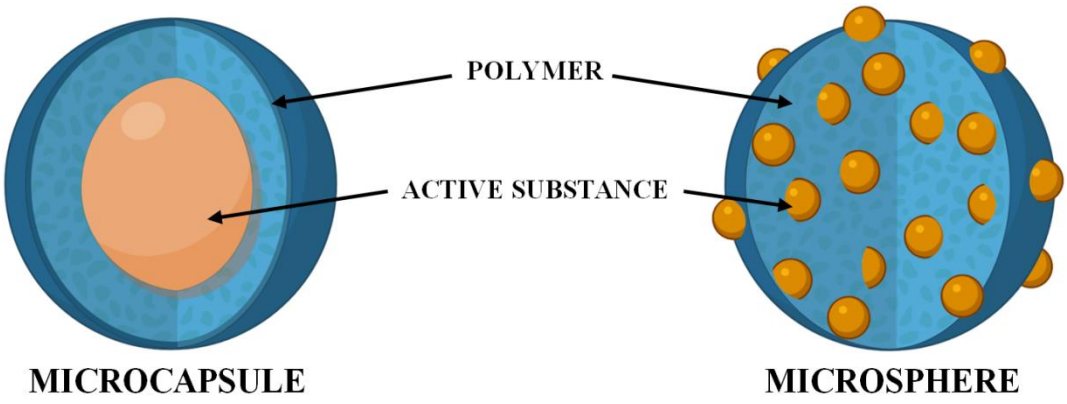


Figure 6. The structure of microparticles: microcapsule and microsphere.

Many different materials can be used as encapsulating matrices: proteins (gelatin, albumin, whey proteins, caseins, silk fibroin), polysaccharides (dextrin, starch, gum arabic, chitosan, sodium alginate, pectin, carrageenan, cellulose), fats and waxes (hydrogenated vegetable oils, beeswax, lecithin), biopolymers, copolymers or synthetic polymers (acrylonitrile, polybutadiene, poly(lactide-co-glycolide), polylactide) [33].

The main advantages of particulate systems are the isolation and protection of active substances dispersed in the core from external factors and undesired reactions (e.g., oxidation or deactivation), simultaneously increasing and maintaining these substances' stability. Further reasons for encapsulation are modification of the physicochemical properties, separation of incompatible materials, and masking of organoleptic properties like color, taste and odor of substances [34]. In addition, encapsulation allows the modification of the release of active compounds with three main mechanisms of delivering active substances from particles, namely (I) mechanical rupture of the capsule wall; (II) dissolution or melting of the wall and (III) diffusion through the wall [35]. Due to these benefits, microencapsulation plays a crucial role in pharmaceuticals, medicine, cosmetics, material engineering and food sciences, significantly improving the functionality and effectiveness of bioactive substances across diverse applications.

Encapsulation technologies have a wide range of applications in enclosing various types of solids and liquids, such as drugs, extracts, vitamins, perfumes, proteins, dyes, and bacterial cells. This is due to their undoubted advantages discussed above and the increasing number of encapsulation techniques, such as extrusion, single/double emulsion method, coacervation, spray drying, interfacial polymerization, and solvent extraction/evaporation. Extrusion is considered a particularly advantageous technique, as it enables the formation of both microspheres and microcapsules using an encapsulator, with the process being finely controlled by parameters such as vibration frequency, amplitude, nozzle diameter, and flow rate (Fig. 7).

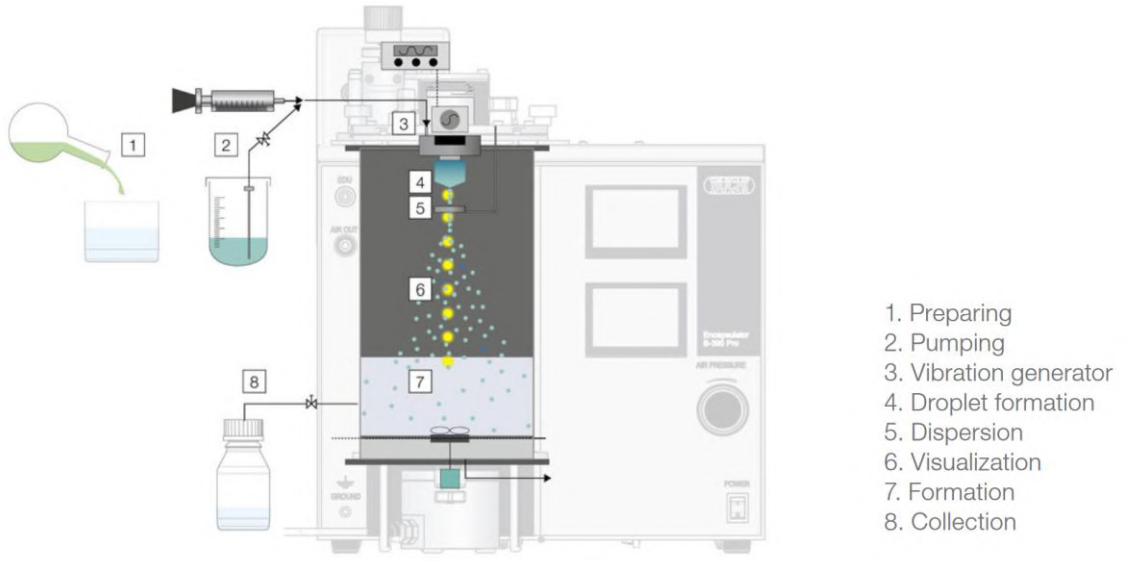


Figure 7. Encapsulator operational diagram [36].

6. Plant extracts

Novel materials for cosmetic applications often contain different active substances, including plant extracts. Cosmetic manufacturers are continuously engaged in the search for novel and exotic raw materials, frequently overlooking the proven efficacy of substances that have been well established and extensively tested over many years for their beneficial effects on skin condition. These raw materials include the following polish herbs: wild pansy (*Viola tricolor*), heath speedwell (*Veronica officinalis*), ground-ivy (*Glechoma hederacea*), ribwort plantain (*Plantago lanceolata*); flowers: yarrow (*Achillea millefolium*), elderflower (*Sambucus nigra*), small-leaved lime (*Tilia cordata*); rhizome: tormentil (*Potentilla erecta*) (Fig. 8). The selection of plant species was based on the following criteria: (I) their documented use in traditional medicine for the treatment of skin disorders, (II) their phytochemical composition, and (III) the limited data availability concerning their potential synergistic activity. Incorporating such extracts into innovative cosmetic forms supports dermatological efficacy and consumer preference for natural active ingredients.

Figure 8. Plants used in order to prepare extracts.

Medicinal plant extracts are a rich source of biologically active compounds, particularly polyphenols and flavonoids with antioxidant and anti-inflammatory properties. Elderberry (*Sambucus nigra*) is recognized for its exceptionally rich phytochemical profile. Elder flowers, berries, bark, and their preparations have already been used for generations in folk medicine in many countries. *Sambucus nigra* is a large shrub or small tree growing to 6 meters, native to Europe. In Poland, it is widespread in the lowlands and lower mountain zones. It grows in forests, bushes, parks and near houses. The ivory-white flowers with a strong odor are borne in large, flat corymbs with a 10-25 cm diameter in late spring to mid-summer. The individual flowers have five petals of 5-6 mm in diameter. For medicinal purposes, blooming inflorescences are collected at the beginning of flowering and dried in shaded and airy places.

For generations, elderflower preparations have been employed in folk medicine worldwide for their beneficial effects on the skin. Besides the healing effect on skin disorders, as well as soothing and regenerating the skin properties, elderflowers exhibit a wide range of biological activities, such as antioxidation, antibacterial, antiparasitic, neuroprotective, anti-inflammatory, anticancer, antiviral, and antidiabetic properties [37]. This is due to its complex composition since it contains a whole range of phenolic compounds, such as flavonoids (e.g., rutin, quercetin, isoquercetin, isorhamnetin, kaempferol) and phenolic acids (e.g., chlorogenic and caffeic acids) (Fig. 9). Hence, it has anti-inflammatory properties and stimulates the skin renewal process [38,39]. Elderflower extract also comprises organic acids (e.g., valeric and ferulic) and smaller amounts of tannins and essential oils.

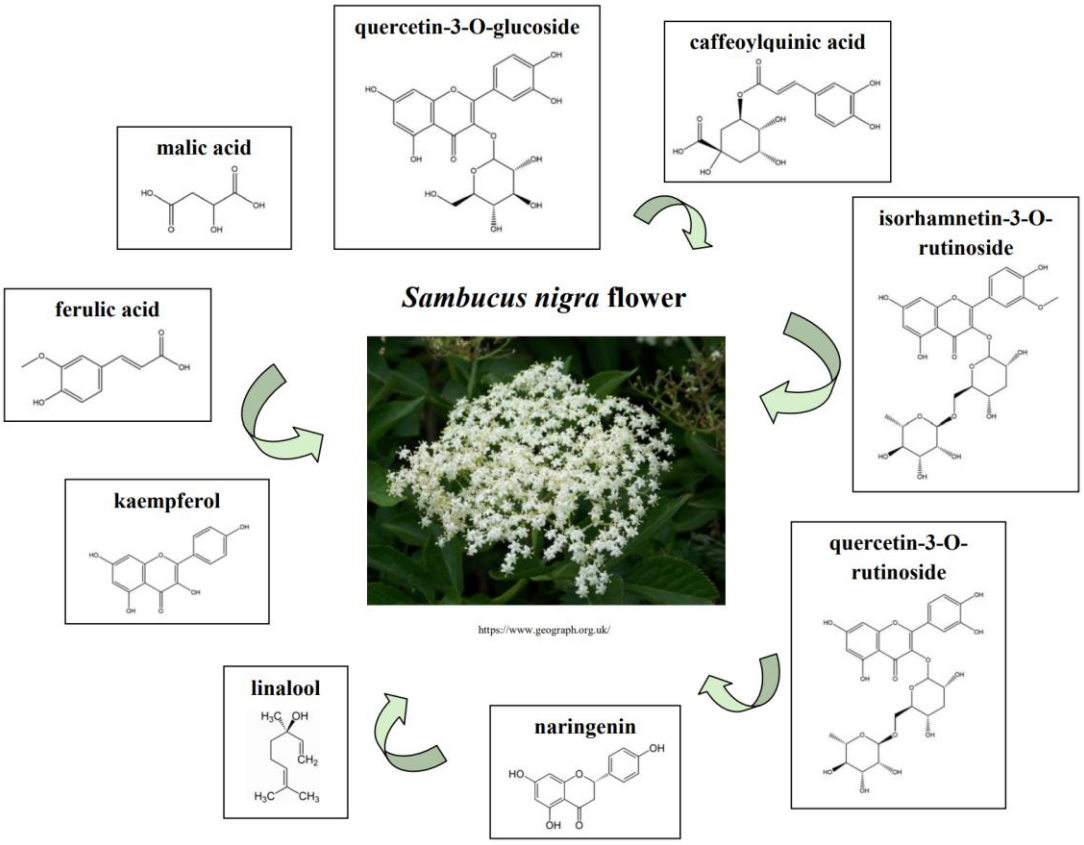


Figure 9. Principal constituents of *Sambucus nigra* flower extract showing biological activity.

7. Polymers

The functionality of these novel formulations is strongly influenced by the polymers used as structuring agents. Natural and semi-synthetic polymers, such as sodium alginate, whey protein isolate, and ethylcellulose, play a crucial role in defining the physicochemical and mechanical properties of emulsions and bigels. Their gelling, emulsifying, and stabilizing capacities allow the preparation of freeze-dried systems with tunable porosity, mechanical properties, and degradation profiles. Such properties are essential for ensuring both product performance and controlled delivery of active substances in cosmetic and dermatological applications. Meanwhile, chitosan-based microparticles are particularly advantageous due to their biocompatibility, biodegradability, and intrinsic antimicrobial properties. These polymers are inexpensive and widely available, making them cost-effective options for developing freeze-dried materials and microparticle-based formulations.

WPI is generated as a by-product of bovine milk during industrial cheese production. The primary protein fractions of ruminant milk consist of caseins and whey proteins. Most lipids, carbohydrates, and lactose are eliminated during purification, yielding a protein-rich fraction with a purity exceeding 90%. The dominant constituents of WPI are β-lactoglobulin and α-lactalbumin; however, additional bioactive proteins such as glycomacropeptides, immunoglobulins, bovine serum albumin, lactoferrin, lysozyme, and prosthetic peptones are also present [40]. The amino acid composition and three-dimensional structures of WPI constituents provide both hydrophobic and hydrophilic regions. Hydrophobic areas, formed by nonpolar residues such as leucine, valine, and phenylalanine, interact with oil droplets. In contrast, hydrophilic regions containing polar or charged residues like serine, glutamine, and lysine engage with the surrounding water. This dual character helps stabilize emulsions and bigels by preventing droplet coalescence and aggregation.

Sodium alginate, a naturally derived anionic polysaccharide, comprises α-L-guluronic and β-D-mannuronic acid residues connected through glycosidic linkages. Its structure consists of homopolymeric sequences of single residues, as well as random or alternating arrangements of both types [41]. This polymer is primarily sourced from the cell walls of marine brown algae (*Phaeophyceae*) [42]. The hydrophilic alginate carboxyl and hydroxyl groups enable strong water binding and swelling, contributing to forming a flexible and hydrated hydrogel network. The polyanionic nature of sodium alginate enables electrostatic interactions with positively charged proteins, such as WPI, further reinforcing the hydrogel network.

Ethylcellulose (EC), a cellulose derivative, has been extensively studied as a polymeric organogelator due to its capacity to structure liquid oils directly [43]. It is particularly valued for its thermal stability and ability to form transparent gels, making it suitable for a wide range of applications, including drug delivery and food formulations. As a linear polysaccharide, EC is characterized by a glass transition temperature of approximately 140°C, a property of key importance for its processing and functional performance. This relatively high glass transition temperature enhances the thermal resistance and mechanical stability of EC-based gels, thereby improving the robustness of hybrid systems such as bigels, where structural integrity under varying storage and application conditions is essential.

Chitosan is a natural cationic polysaccharide obtained by the partial deacetylation of chitin, which is abundantly present in the exoskeletons of crustaceans, insects, and the cell walls of fungi. Structurally, it comprises β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine

units. The degree of deacetylation and molecular weight strongly influence its physicochemical properties, such as solubility, viscosity, and film-forming ability. Due to its antimicrobial activity, biocompatibility, and biodegradability, chitosan has attracted significant attention in biomedical, pharmaceutical, and cosmetic applications [44,45]. The solubility of chitosan under acidic conditions allows for pH-responsive release of encapsulated compounds, a feature particularly advantageous for targeted delivery, given the slightly acidic environment of the skin [46].

Collectively, the integration of biopolymer-based freeze-dried emulsions and bigels with microparticle-encapsulated plant extracts provides a sustainable and innovative approach to modern cosmetic science. These materials offer reduced water consumption, improved stability, and multifunctional performance, aligning with both ecological and consumer-driven demands.

THE AIM OF THE STUDY

The integration of microencapsulation with sponge-like matrices was anticipated to yield an innovative, environmentally friendly material. The resulting formulations represent a novel class of products with the potential to support sustainable water management. Furthermore, these materials could serve as a platform for the development of multifunctional cosmetic and dermatological preparations aimed at skin conditioning and care.

It is **hypothesized** that water consumption during obtaining cosmetic forms can be reduced, combined with maintaining the functional properties (the material will be designed to dissolve in a minimal amount of aqueous solvent back to emulsion or bigel immediately before its application to the skin) of the prototype based on freeze-dried emulsion and bigel with plant extract-loaded microparticles. This project includes **the main task**: the development of a methodology for preparing a new cosmetic form that requires reduced water usage, based on freeze-drying of emulsions and bigels prepared from a mixture of natural polymers and oily substances with incorporated polymeric microparticles containing plant extract from *Sambucus nigra* flowers.

Therefore, **the specific objectives** of the work were as follows:

- Extraction and phytochemical studies of active compounds from Polish plants and their encapsulation into chitosan microparticles.
- Development of a preparation methodology for freeze-dried emulsions based on biopolymers, cryoprotectants, lipids, emulsifier and physicochemical characterization of obtained materials.
- Optimization of the methodology for obtaining materials based on freeze-dried bigels and physicochemical characterization of these materials.
- Modification of freeze-dried emulsion and bigel with the addition of chitosan microparticles loaded with *Sambucus nigra* flower extract.

STAGES OF IMPLEMENTING A DOCTORAL THESIS

The implementation of this thesis included three main **milestones**: (I) obtaining chitosan microparticles containing *Sambucus nigra* extract, (II) obtaining freeze-dried emulsions and bigels that allow the recovery of water used for their fabrication and are characterized by functional physicochemical properties, (III) modification of the obtained materials by the addition of prepared microparticles.

The work plan included the **specific objectives** of the research:

- Obtaining extracts from the following Polish herbs: wild pansy (*Viola tricolor*), heath speedwell (*Veronica officinalis*), ground-ivy (*Glechoma hederacea*), ribwort plantain (*Plantago lanceolata*); flowers: yarrow (*Achillea millefolium*), elderberry (*Sambucus nigra*), small-leaved lime (*Tilia cordata*); rhizome: tormentil (*Potentilla erecta*) using Soxhlet apparatus with water and ethanol as solvents **[P1]**.
- Phytochemical studies (Total Polyphenol Content, Total Flavonoid Content) and evaluation of antioxidant activity (CUPRAC, FRAP and DPPH RSA) of prepared extracts individually and in combinations of these extracts **[P1]**.
- Encapsulation of selected plant extracts into chitosan microparticles via the extrusion method using an encapsulator **[P1]**.
- Examination of the morphology and size of microparticles, evaluating the loading capacity and *in vitro* release profile of extracts loaded into microspheres **[P1]**.
- Formulation of herbal emulsions and hydrogels, assessment of skin color, skin surface hydration, and skin barrier quality (TEWL) after their application on the probands' skin **[P1]**.
- Obtaining freeze-dried emulsions based on biopolymers: sodium alginate and whey protein isolate; cryoprotectants: glycerin, propylene glycol, sorbitol, mannitol and trehalose; oils: sunflower oil and sea buckthorn oil; beeswax and emulsifier (Span-80) **[P2]**.
- Investigation of time and speed of homogenization of emulsions in order to determine the narrowest size distribution of the dispersed phase droplets in the emulsion continuous phase **[P2]**.
- Characterization of freeze-dried emulsions via SEM, mechanical properties, residual moisture content, porosity, and density measurements **[P2]**.

- Obtaining freeze-dried bigels composed of hydrogel (sodium alginate, whey protein isolate, glycerin) and oleogel (sunflower oil, ethylcellulose, Span 80) blended at different hydrogel/oleogel ratios **[P3]**.
- Characterization of freeze-dried bigels via SEM, degradation properties, mechanical properties, swelling properties, residual moisture content, porosity, and density measurements **[P3]**.
- Incorporation of chitosan microparticles loaded with *Sambucus nigra* flower extract into freeze-dried emulsion and bigel comprising biopolymers: sodium alginate, WPI, ethylcellulose; cryoprotectant: mannitol; lipids: sea buckthorn oil, beeswax; emulsifier: Span-80 **[P4]**.
- Characterization of prepared materials: SEM, mechanical properties, residual moisture content, porosity, and density measurements **[P4]**.
- Analysis of biophysical skin parameters, including skin color, hydration and barrier quality (TEWL) in order to evaluate topically applied formulations' efficacy and physiological impact, ensuring optimal permeability of active substances while minimizing adverse effects **[P4]**.

DESCRIPTION OF PERFORMED RESEARCH

This series is based on three original scientific articles and material collected for the subsequent, fourth publication. Those works are the result of research conducted at the Faculty of Chemistry at the Nicolaus Copernicus University and the School of Engineering at Lancaster University.

PUBLICATION 1 [P1]

This article presents the results of research that led to the formation of chitosan microparticles loaded with extracts prepared from Polish herbs, flowers, and rhizome (Fig. 10).

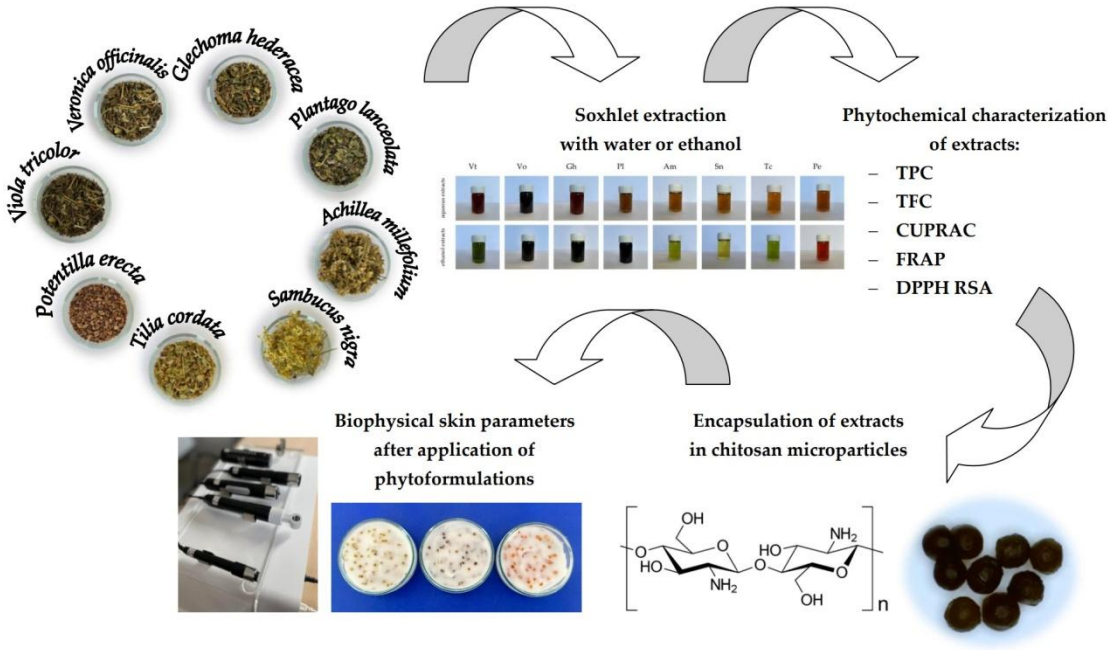


Figure 10. The summary of performed research: preparation and phytochemical characterization of plant extracts and their encapsulation in chitosan microparticles, followed by assessment of biophysical skin parameters after application of phytoformulations containing obtained extracts and extract-loaded microparticles.

In the first stage of this research, eight Polish herbal plants showing documented healing effects, including the herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; the flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and the rhizome: *Potentilla erecta,* were selected in order to prepare extracts using a Soxhlet

apparatus with water and ethanol as solvents. The obtained extracts were characterized according to the content of polyphenols (TPC), flavonoids (TFC) and antioxidant activity (CUPRAC, FRAP and DPPH RSA). Their phytochemical profiles significantly depended on the type of plant and the extraction medium. For *Plantago lanceolata* and *Potentilla erecta*, ethanol was the preferred solvent, whereas water was applied to the other plants. The extraction efficiency of the studied plants reflected the polarity of their dominant compounds. *Plantago lanceolata* and *Potentilla erecta* were better extracted with ethanol, which favors the extraction of phenylethanoid glycosides, tannins, and triterpenes, since water may not efficiently penetrate plant tissues and release bound compounds. In contrast, the remaining species, including *Viola tricolor*, *Veronica officinalis*, *Glechoma hederacea*, *Achillea millefolium*, *Sambucus nigra*, and *Tilia cordata*, yielded higher levels of bioactives in water due to their abundance of hydrophilic constituents such as flavonoid glycosides (e.g., rutin, isoquercitrin, luteolin glycosides) and phenolic acids (e.g., chlorogenic, caffeic, rosmarinic, ferulic). These differences confirm that solvent choice should be tailored to the phytochemical profile of each plant.

Phytochemical composition and antioxidant activity of all obtained extracts ranged:

- TPC: from 0.7 to 9.4 g GAE/100 g DW,
- TFC: from 1.5 g to 240 mg QE/100 g DW,
- CUPRAC: from 0.9 to 7.3 g/100g DW,
- FRAP: from 1.9 to 8.3 g/100 g DW,
- DPPH RSA: from 8.6 to 78.7%.

The highest levels of extracted active compounds were exhibited by aqueous extract from *Sambucus nigra*, *Veronica officinalis* prepared with water and ethanol, and *Potentilla erecta* ethanol extract, except for the flavonoid content, which was the lowest of all extracts. The low flavonoid content in this extract might be due to the naturally low levels of flavonoid glycosides in the rhizome, with tannins and phenolic acids being the dominant compounds.

Extracts with mixed plants showing different active compound content were also prepared in order to search for synergistic effects. Interestingly, multicomponent plant extracts, each individually characterized by high phytochemical content, resulted in new extracts with only moderate TPC, TFC, and antioxidant activity. This suggests that the plant constituents may exhibit an additive effect. However, these findings also indicate that, in some cases, using a

single carefully selected extract may be more effective than combining multiple sources, as it can provide more predictable and optimized functional properties. Hence, in the next steps of this research, aqueous extracts from (I) *Sambucus nigra*; (II) *Viola tricolor* + *Veronica officinalis*; and (III) *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* were used.

Prepared extracts were encapsulated in chitosan-based microparticles formed via ionic gelation, a mild method for encapsulating bioactives. In acidic conditions, chitosan amino groups ($–NH_2$) become protonated ($–NH_3^+$) and form ionic interactions with the negatively charged phosphate groups of TPP, forming a stabilized network and spherical microparticles. Hydroxyl groups (–OH) of chitosan's glucosamine units may further strengthen the structure through hydrogen bonding. Plant extracts containing phenolic compounds, flavonoids, and organic acids entrapped within the matrix may interact with chitosan and partially compete with TPP for amino groups, potentially affecting microparticle structure and stability. Developed microparticles exhibited repeatable size, smooth surface and spherical shape, important to minimizing the possibility of skin irritation. The loading capacity of extracts in microparticles was established as 71% for Sn extract, 55% for Vt + Vo extract, and 14% for Gh + Pl + Am + Tc + Pe extract. Although the microparticles were designed to release the extracts mechanically while spreading to the skin, the *in vitro* release profile was also examined. Approximately 70-90% of encapsulated extracts were released within the first 5 hours with a relatively high release rate. The remaining extracts were released up to three days with a sustained release rate.

In the next stage of this publication, dermatological formulations in the form of creams and hydrogels containing free extracts and extract-loaded microparticles were obtained. The hydrogel was primarily structured by xanthan gum with the addition of glycerin, propylene glycol, panthenol, and allantoin. Meanwhile, oil-in-water (O/W) emulsions were formed by glyceryl stearate and ceteareth-20, which served as non-ionic emulsifiers. Cetearyl alcohol, caprylic/capric triglycerides and isopropyl palmitate functioned as a rheology modifier. Octyldodecanol contributed to emulsion stability. The aqueous phase, containing humectants such as glycerin, propylene glycol, panthenol, and allantoin, ensured moisture retention.

Phytoformulations, as well as control samples without herbal preparations, were applied to the proband's forearm skin and instrumentally assessed with the biophysical skin parameters (skin color, skin surface hydration and skin barrier quality) using Courage + Khazaka probes.

The application of the obtained preparations did not cause damage, irritation, or skin erythema. They also showed a temporary occlusive effect, preventing the excessive evaporation of the outermost skin layer moisture, as well as prolonged skin hydration properties. These properties are the result of the rich composition of plant extracts. Multiple hydroxyl groups of polyphenols and flavonoids can form a hydrogen bond with water, retaining water in the epidermis. Other formulation constituents, such as emollients and humectants, supported the extracts' skin-conditioning features.

Concluding, extracts from eight medicinal plants and their blends were successfully prepared, characterized and enclosed in chitosan microparticles. Phytoformulations containing extracts and extract-loaded microparticles were instrumentally established to have skin-conditioning properties.

PUBLICATION 2 [P2]

This publication demonstrated the optimization of methodology to obtain freeze-dried emulsions based on biopolymers, cryoprotectants, oils, beeswax and emulsifier. Emulsions were obtained with three oily-to-aqueous mixing ratios (5/95, 10/90, 15/85). Different concentrations of WPI (1% or 3%), different types and concentrations of cryoprotectants: glycerin, propylene glycol, sorbitol, mannitol and trehalose (1% or 3%), different types of oils (sunflower or sea buckthorn) and different concentrations of beeswax (1% or 3%) were investigated.

Firstly, different times (1 min, 3 min, 5 min) and speeds (15,000 rpm, 20,000 rpm) of emulsion homogenization were explored in order to determine the narrowest size distribution of the oily droplets in the emulsion continuous phase. Homogenization of 3 min at 20,000 rpm resulted in the smallest span of 2.62, whereas 1 min and 15,000 rpm parameters led to a 28.33 span. Higher rotation speed of the homogenizer fabricated emulsions with smaller oily droplet sizes (from 2.02 μm to 7.53 μm). Therefore, homogenization parameters were set at 3 min and 20,000 rpm since they resulted in the most desirable results. Different oily-to-water phase ratios (5/95, 10/90 and 15/85) and concentration of WPI were also investigated, and the 10/90 mixing ratio with 3% of WPI was selected as the optimal amount of oily phase, due to the lowest span and mean oil droplet size. All obtained samples (with different types and concentrations of cryoprotectants, oil and beeswax) were analyzed by droplet size distributions of emulsions, which varied depending on the composition of formulations. Higher oily droplet sizes and their broader distribution were noted in materials composed of higher concentrations of cryoprotectants, which might have been connected with their influence on viscosity and interfacial properties of emulsions. The addition of beeswax also resulted in a significant rise in the mean droplet size owing to the higher density and viscosity of the oily phase.

The prepared FD materials were soft and spongy, but some compositions tended to be slightly more brittle and rigid or with linearly arranged pores (Fig. 11). Samples formulated with sunflower oil appeared whitish, while those containing sea buckthorn oil exhibited a bright orange hue, reflecting the intense natural color of the oil. The materials displayed irregular interconnected macropores, which were influenced by the nucleation of ice grains within the polymer network. Macropores then replaced these ice grains during sublimation. In order to avoid the aggregation of the oily phase during the freezing of samples, cryoprotectants

preventing ice crystallization were added, including glycerin, propylene glycol, sorbitol, mannitol and trehalose.

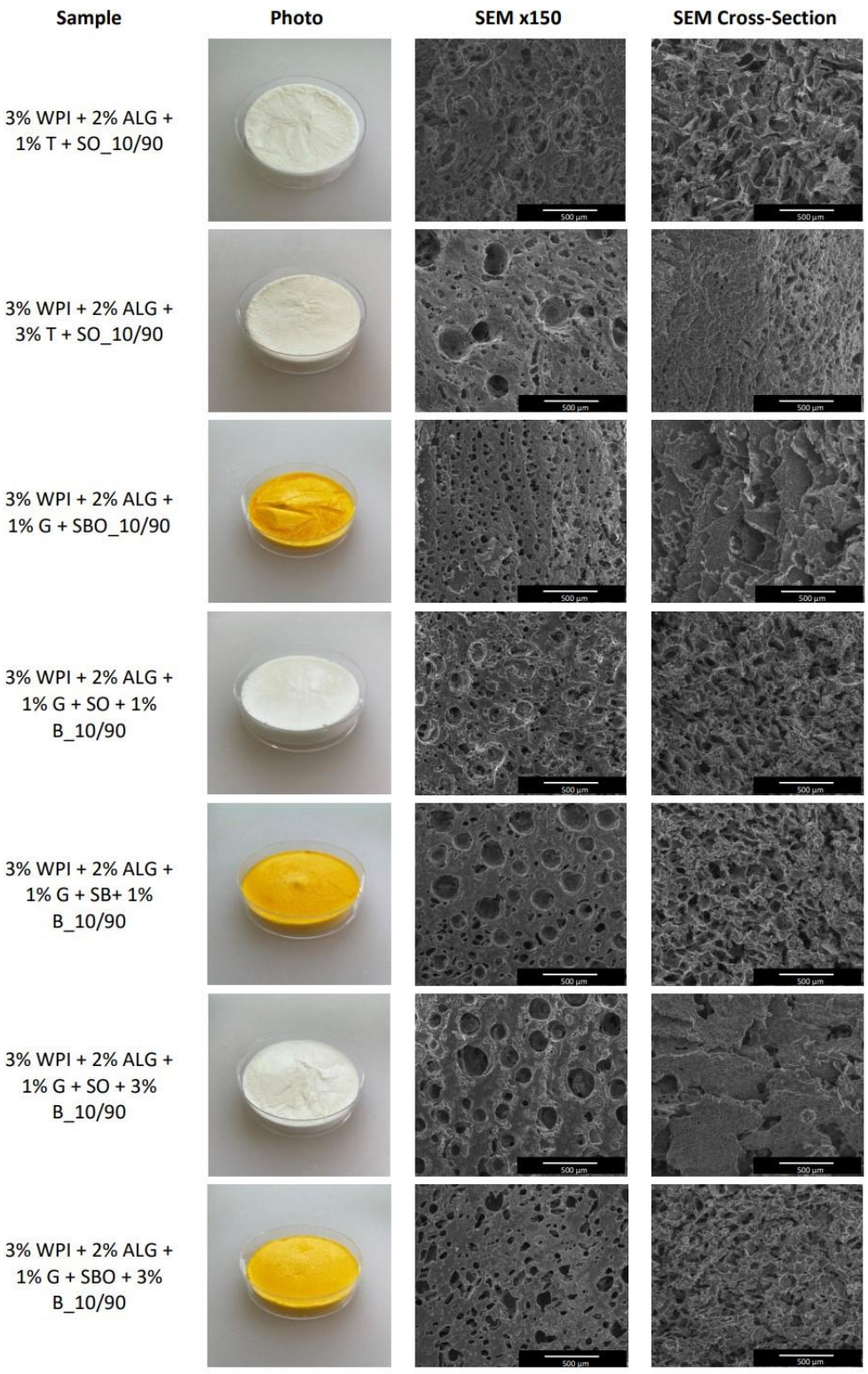


Figure 11. Pictures of obtained materials (the diameter of the container is 60 mm) and SEM images of their structures at a magnification of ×150 (scale bar = 500 μm) and cross-sections at a magnification of ×150 (scale bar = 500 μm).

In the next stage of this research, the physicochemical properties of the obtained freeze-dried emulsions were investigated. They significantly depended on all the variables in emulsion formulations and were as follows: porosity ranged from 59 to 95%, density varied from 116 mg/ml to 308 mg/ml, residual moisture content was from 2.3% to 10.9%, and mechanical properties differed from 240 kPa to 1.7 MPa.

The porosity of a material strongly influences its ability to absorb and retain water during rehydration, supporting a rapid return to its emulsion form upon use. In contrast, density affects the product's overall weight and has implications for packaging and transportation requirements, contributing to sustainability objectives. The type of cryoprotectant did not significantly affect porosity. However, a 3% addition of glycerin and propylene glycol increased sample density, likely because these small, flexible molecules integrate more easily into the matrix. Porosity results also suggest that cryoprotectants were uniformly dispersed throughout the aqueous phase, enabling consistent water removal during freeze-drying. Variations in WPI content had little effect on porosity or density, whereas higher oil content and the addition of beeswax significantly decreased porosity and increased density of samples. Porous structure formation relies on ice crystals, which sublimate during freeze-drying; samples with a higher aqueous phase exhibited greater porosity and lower density due to more ice crystal formation, while the greater presence of lipids or beeswax reduced pore formation, as these components do not sublimate and create a more hydrophobic, compact structure.

Reduced moisture levels limit the potential for microbial proliferation, which may lessen or even remove the necessity for preservatives — a clear advantage in formulations designed for sensitive skin. The highest values of residual moisture content were noticed for samples with higher aqueous phase ratios and lower concentration of WPI, as well as with 3% of glycerin and propylene glycol, owing to their high capacity for hydrogen bonding, which prevented complete removal of moisture during the sublimation process, which also led to higher density of these samples. Moreover, differences in cryoprotectant hygroscopicity affect residual moisture after freeze-drying, with sorbitol, mannitol, and trehalose retaining less moisture than highly hygroscopic glycerin and propylene glycol.

Mechanical properties are key determinants of product handling, storage stability, and end-user performance. Freeze-dried emulsions with enhanced mechanical strength resist deformation and maintain structural integrity during transport, while retaining sufficient

softness for efficient rehydration and easy application. In this study, compressive maximum force ranged from 4 N to 28 N, and Young's modulus ranged from 240 kPa to 1.7 MPa (Table 1), with higher Young's modulus indicating greater resistance to deformation under stress. Stiffer matrices better preserve their shape under compression and are less prone to structural changes when subjected to external forces.

Table 1. Mechanical properties of prepared freeze-dried emulsion matrices based on biopolymers: sodium alginate and whey protein isolate; cryoprotectants: glycerin, propylene glycol, sorbitol, mannitol and trehalose; oils: sunflower oils and sea buckthorn oil; beeswax and emulsifier (Span-80), as well as different oily to aqueous phases ratios (5/95, 10/90 and 15/85) during compression.

Sample	Young's Modulus (kPa)	Compressive Maximum Force (N)
1% WPI + 2% ALG + 1% G + SO_5/95	239.5 ± 18.3	4.02 ± 0.24
1% WPI + 2% ALG + 1% G + SO_10/90	598.1 ± 62.9	9.04 ± 0.25
1% WPI + 2% ALG + 1% G + SO_15/85	350.1 ± 57.6	6.69 ± 1.19
3% WPI + 2% ALG + 1% G + SO_5/95	1296.3 ± 168.3	12.85 ± 2.29
3% WPI + 2% ALG + 1% G + SO_10/90	671.2 ± 87.5	11.90 ± 0.88
3% WPI + 2% ALG + 1% G + SO_15/85	331.2 ± 37.3	8.76 ± 0.93
3% WPI + 2% ALG + SO_10/90	509.5 ± 28.4	10.91 ± 0.73
3% WPI + 2% ALG + 3% G + SO_10/90	326.0 ± 38.8	8.22 ± 0.72
3% WPI + 2% ALG + 1% PG + SO_10/90	1699.4 ± 197.4	19.46 ± 0.32
3% WPI + 2% ALG + 3% PG + SO_10/90	589.5 ± 80.1	18.70 ± 0.74
3% WPI + 2% ALG + 1% S + SO_10/90	1122.7 ± 122.4	21.67 ± 1.37
3% WPI + 2% ALG + 3% S + SO_10/90	349.2 ± 17.0	17.18 ± 3.51
3% WPI + 2% ALG + 1% M + SO_10/90	1446.3 ± 137.2	19.74 ± 2.41
3% WPI + 2% ALG + 3% M + SO_10/90	1154.5 ± 143.9	19.95 ± 2.88
3% WPI + 2% ALG + 1% T + SO_10/90	1545.4 ± 95.5	14.96 ± 0.66
3% WPI + 2% ALG + 3% T + SO_10/90	1359.6 ± 207.2	28.20 ± 1.09
3% WPI + 2% ALG + 1% G + SBO_10/90	705.9 ± 84.1	11.86 ± 0.54
3% WPI + 2% ALG + 1% G + SO + 1% B_10/90	906.4 ± 63.5	10.67 ± 0.70
3% WPI + 2% ALG + 1% G + SBO + 1% B_10/90	844.6 ± 76.2	9.44 ± 0.48
3% WPI + 2% ALG + 1% G + SO + 3% B_10/90	1098.6 ± 102.8	13.76 ± 0.55
3% WPI + 2% ALG + 1% G + SBO + 3% B_10/90	960.8 ± 145.1	11.69 ± 0.68

Variations in WPI concentration significantly affected mechanical resistance, particularly in samples with a 5/95 oil-to-aqueous phase ratio. Mechanical strength increased with WPI concentration up to a point, as higher levels promote a denser, interconnected network via hydrogen bonding and van der Waals interactions, enhancing stiffness. Stiffness was also higher in samples with a 10/90 mixing ratio compared to 15/85, and in formulations

containing beeswax or 1% cryoprotectants. The oil-to-water ratio influenced deformation resistance, as higher oil content can act as a plasticizer and lubricant between protein molecules, reducing stress resistance and disrupting network formation, resulting in a more flexible structure. Similarly, higher cryoprotectant content decreased Young's modulus by enhancing matrix flexibility and weakening intermolecular interactions between protein and polysaccharide molecules. However, mannitol and trehalose generally exerted a weaker plasticizing effect than glycerin and sorbitol, particularly at higher concentrations, which promotes tighter polymer alignment, reduced intermolecular spacing during freezing, and an increased Young's modulus.

Analysis of the biopolymer-based freeze-dried emulsions revealed that their physicochemical characteristics promote enhanced stability and mitigate microbial growth. Additionally, these formulations contribute to environmental sustainability by reducing both water usage and demand for plastic packaging. Altogether, the results highlight the potential of freeze-dried biopolymer emulsions as customizable and eco-friendly options for applications within the cosmetic and dermatological industries.

PUBLICATION 3 [P3]

Preparation and determination of physicochemical properties of materials based on bigels composed of WPI/sodium alginate hydrogel and ethylcellulose/sunflower oil oleogel were the subject of this paper.

Bigels were prepared by blending oleogel (10% or 15% of EC, Span 80, sunflower oil) and hydrogel (1% or 3% of WPI, sodium alginate, glycerin) in 5/95, 10/90 and 15/85 oleogel/hydrogel mixing ratios. After freeze-drying, bigels gained a porous matrix without phase separation areas, though morphology varied with oleogel content. Lower oleogel ratios produced rough, wrinkled pore walls with honeycomb-like ice crystal imprints, while higher ratios yielded smoother pore surfaces.

The physicochemical characteristics of the freeze-dried bigels – including moisture content, density, mechanical, swelling and degradation properties – were significantly influenced by polymers content and the oleogel-to-hydrogel mixing ratio. In contrast, porosity values (45 – 58%) were generally unaffected by formulation variables, except for a notably lower porosity (35%) observed in the sample containing 10% EC in oleogel and 3% WPI in hydrogel at a 10/90 ratio. Mechanical performance, expressed through Young's modulus, ranged between 1.25 and 3.7 MPa. The highest values were obtained for the formulation containing 15% EC in the oleogel and 1% WPI in the hydrogel at a 5/95 mixing ratio. Mechanical properties reflected the interplay between hydrogel elasticity and oleogel rigidity. Increased EC content imparts greater stiffness because EC forms a dense, rigid network in the oleogel that resists deformation, resulting in a higher Young's modulus.

Degradation was assessed at skin-relevant pH, as FD bigels were formulated without cross-linking to enable redispersion into bigels immediately prior to topical use. Higher oleogel content resulted in a denser, more compact matrix (increased density from ~100 to 200 mg/ml) and extended degradation (from 6 h up to 7 days) (Fig. 12). This is due to the hydrophobic nature of the oleogel, which limits water uptake and protects the matrix from hydrolytic breakdown. EC in the oleogel forms a stable, nonpolar network that enhances structural integrity over time. In contrast, hydrogel-rich samples, containing hydrophilic components such as WPI and sodium alginate, absorbed more water, swelled, and allowed greater medium penetration, accelerating degradation. Hydrolysis and proteolysis, which break down polysaccharides into monomers and proteins into peptides or amino acids, drove

these processes. Formulations with higher WPI content degraded more rapidly due to enhanced water absorption, protein unfolding, and solvent accessibility, while higher EC and oleogel content slowed degradation by forming a hydrophobic barrier. The degradation trends corresponded with swelling behavior, showing that higher medium uptake – driven by polymer content and the oleogel/hydrogel ratio – promotes faster structural breakdown.

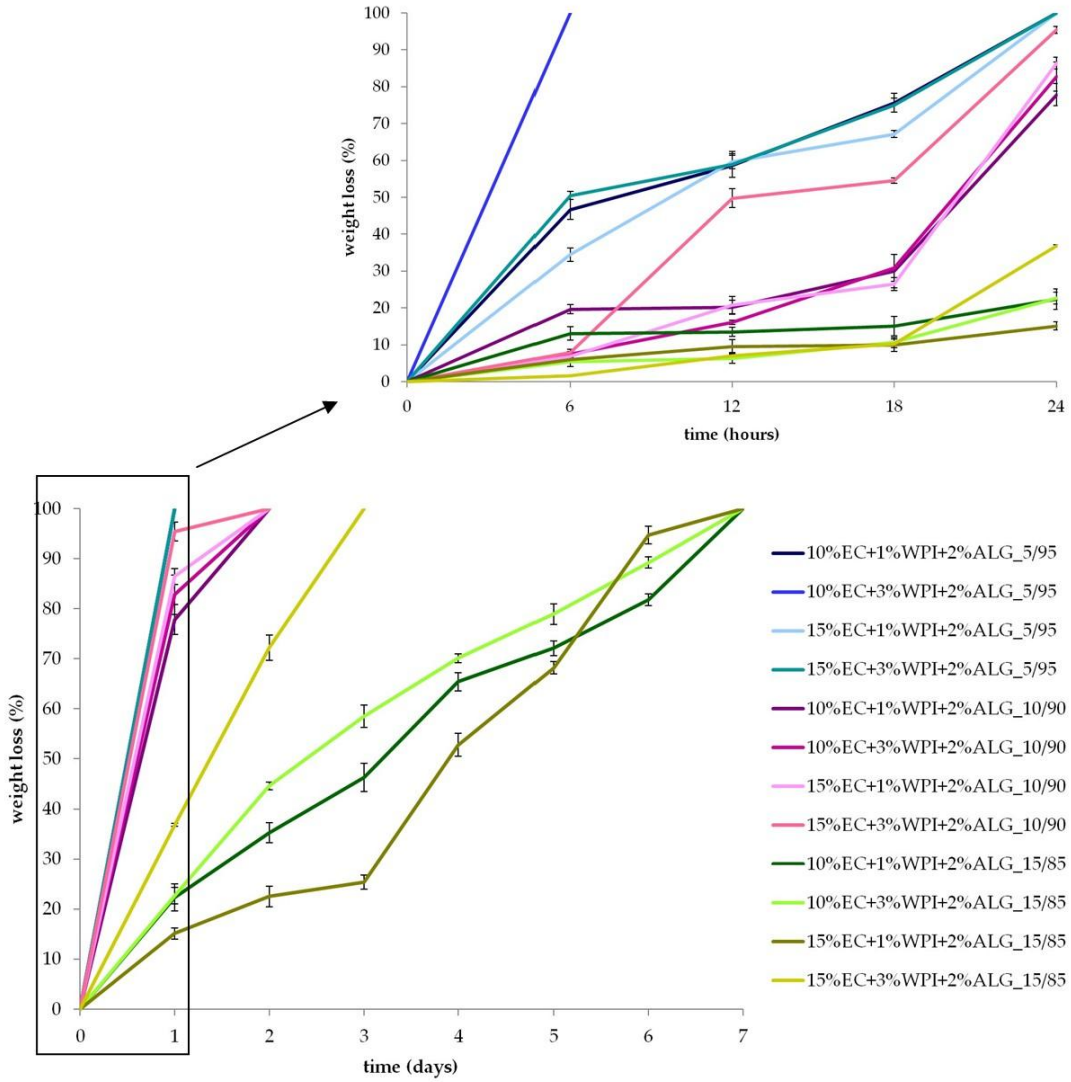


Figure 12. The values of weight loss during degradation measurements of freeze-dried bigels.

Lower oleogel ratios combined with higher EC concentrations increased swelling capacity (50–255%) and moisture content (5–20%). Higher EC content formed a denser oleogel network that could trap residual water, whereas higher WPI reduced moisture by promoting protein-protein interactions that enhanced water removal during freeze-drying. Swelling was driven by the hydrophilic polymer network, with sodium alginate and WPI promoting water

uptake, while the hydrophobic oleogel fraction limited water penetration and reduced swelling (Fig. 13). The extent of swelling was strongly influenced by the functional groups of WPI and sodium alginate, with hydrophilic groups (–OH, –COOH, $–NH_2$) facilitating water binding and network expansion.

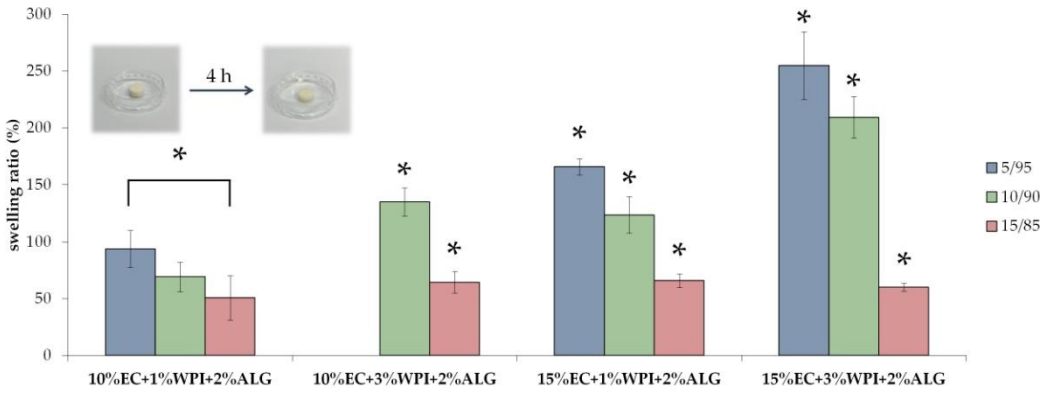


Figure 13. The swelling ratio of freeze-dried bigels. The pictures present an exemplary sample (15% EC + 1% WPI + 2% ALG) before and after 4 h of incubation in PBS buffer. ANOVA-one way with Tukey's pairwise (Cl = 95%) was performed to compare the results statistically. Significant differences among one group of materials with different oleogel/hydrogel mixing ratios were marked on the graph with (*).

The overall performance of freeze-dried bigels may be governed by hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic associations between the hydrogel (WPI/sodium alginate/glycerin) and oleogel (EC/sunflower oil/Span 80) phases. In the hydrogel, WPI stabilized the network via protein-protein interactions, disulfide bridges, hydrogen bonding, and electrostatic interactions with sodium alginate. Glycerin acted as a plasticizer, preventing excessive protein aggregation and improving flexibility. The oleogel network was formed by EC through polymer chain entanglements and van der Waals forces.

These results emphasize the potential of freeze-dried bigels as versatile and adaptable biomaterials extending beyond dermatological and cosmetic use. Their biphasic structure enables the simultaneous incorporation of hydrophilic and lipophilic active compounds while providing stability and favorable characteristics for topical application. Such properties render them promising candidates for advanced skincare products, wound healing therapies, and transdermal drug delivery.

MATERIAL FOR PUBLICATION 4 [P4]

This research includes the modification of freeze-dried emulsion and bigel with the addition of chitosan microparticles containing *Sambucus nigra* flower extract. Structure, physicochemical properties and the influence on biophysical skin parameters were investigated (Fig. 14).

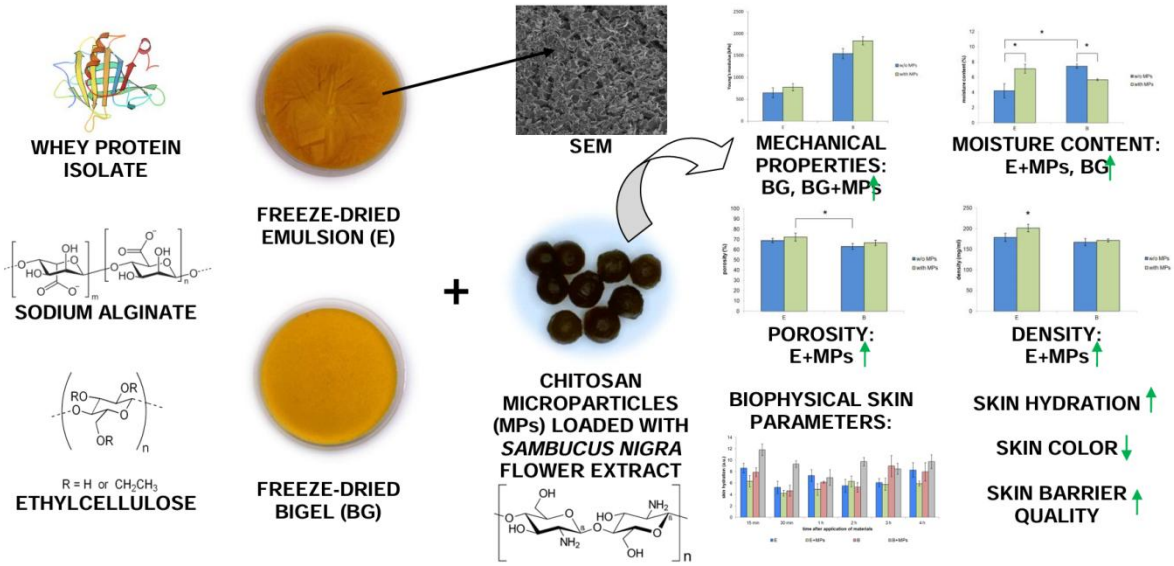


Figure 14. Freeze-dried emulsions and bigels with elderflower extract-loaded microparticles, and their characterization via SEM, mechanical properties, residual moisture content, porosity, density, and analysis of biophysical skin parameters.

Materials and methods

Materials and methods used in previous research were applied in order to obtain aqueous *Sambucus nigra* flower extract and encapsulate it within chitosan microparticles, as well as to obtain emulsions (E) and bigels (BG). The aqueous phase consisted of 3% (w/w) of WPI, 2% (w/w) of sodium alginate (ALG), and 1% (w/w) of mannitol. Meanwhile, the emulsion oily phase comprised sea buckthorn oil, 3% (w/w) of beeswax and 1% (w/w) of Span 80. Moreover, 10% (w/w) of ethylcellulose (EC) was added to form an oleogel. Aqueous and oily phases were mixed and homogenized for 3 min at 20,000 rpm. Microparticles at 5% (w/w) concentration based on the total mass of emulsion or bigel were added before casting the solution on the glass plates. Subsequently, formulations were frozen (−20°C) and freeze-dried (−55°C, 5 Pa, 24 h).

Prepared materials were characterized via SEM, mechanical properties, residual moisture content, porosity, density, and analysis of biophysical skin parameters, including skin color, hydration and barrier quality (TEWL).

Appearance and Structure of Materials

Porous three-dimensional matrices were successfully obtained through freeze-drying of emulsions and bigels (Fig. 15). Microparticles were not distinctly visible in SEM images due to their relatively large size. Nevertheless, their presence was confirmed through broader material imaging, where their dark color from elderflower extract was noticeable and marked with arrows (Fig. 15). The structural characteristics of the freeze-dried emulsions and bigels differed significantly. While both retained a vivid orange hue due to the presence of sea buckthorn oil, their textural properties and structure varied. The freeze-dried emulsions exhibited a more irregular and linear network, whereas bigels displayed a more homogenous and uniform structure. Despite these differences, all obtained matrices shared a soft and spongy texture. SEM analysis revealed the intricate architecture of these materials, characterized by an interconnected network of irregular micropores. This porous structure suggests potential applications in fields such as drug delivery and biomaterials, where controlled porosity and structural integrity are crucial factors.

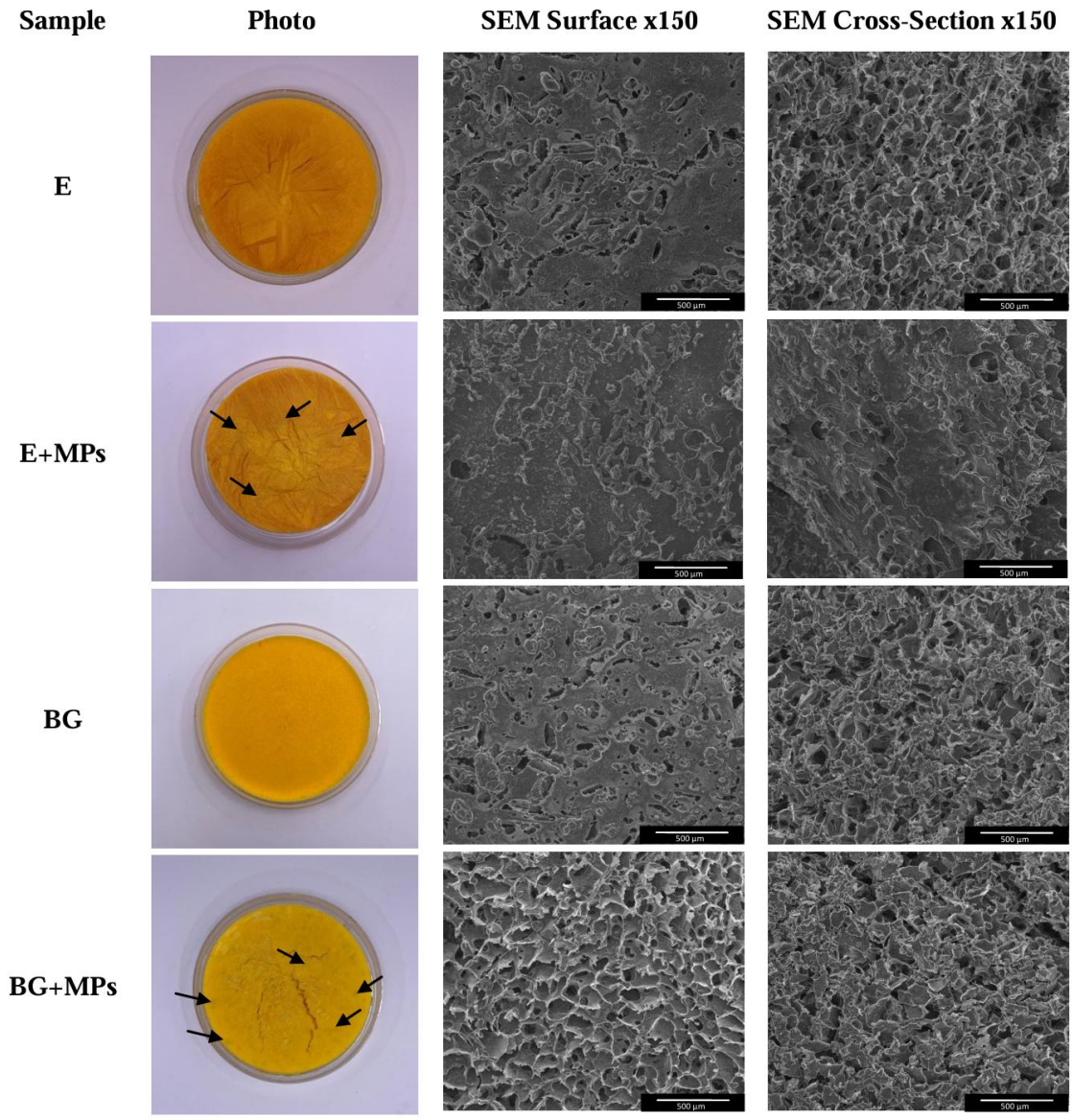


Figure 15. Pictures of obtained freeze-dried materials (the diameter of the container is 60 mm) and SEM images of their surface and cross-section structure in magnification ×150 (scale bar = 500 µm).

Mechanical Properties

The study revealed that Young's modulus ranged from approximately 647-777 kPa for emulsions to 1541-1837 kPa for bigels, while the maximum compressive force varied from 9.7 N in emulsions to 12.9 N in bigels (Table 2). These findings indicate that freeze-dried bigels exhibit significantly greater rigidity and resistance to deformation under applied force compared to emulsions.

Table 2. Mechanical properties of prepared freeze-dried emulsions and bigels with and without the addition of elderflower-loaded chitosan microparticles during compression. Different superscript letters indicate statistically significant differences ($p \leq 0.05$).

Sample	Young's Modulus (kPa)	Compressive Maximum Force (N)
E	646.5 ± 112.9^{c}	9.7 ± 0.2^{b}
E+MPs	776.7 ± 81.3^{c}	10.4 ± 1.3^{b}
BG	1541.3 ± 117.9^{b}	12.6 ± 0.4^{a}
BG+MPs	1836.6 ± 93.4^{a}	12.9 ± 0.7^{a}

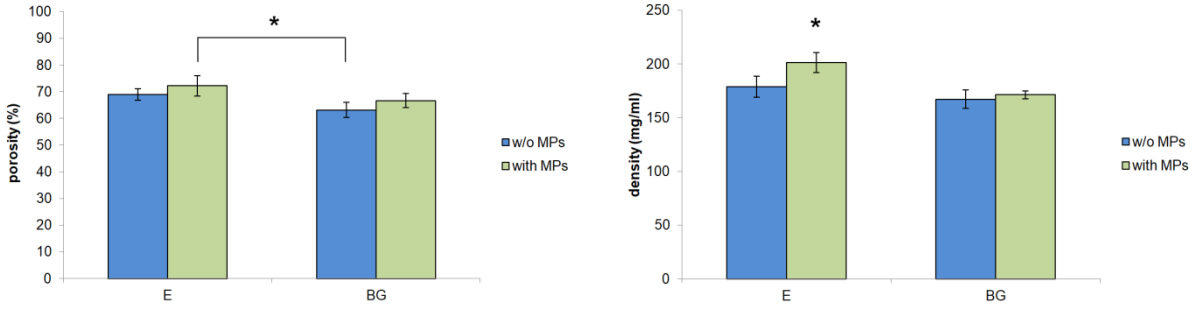
Furthermore, their composition influenced the structural integrity and mechanical strength of freeze-dried emulsions and bigels. Notably, the incorporation of chitosan microparticles led to an increase in both Young's modulus and the maximum compressive force, suggesting enhanced mechanical stability. This modification may be particularly beneficial for applications requiring materials with improved resistance to mechanical stress. The observed differences in mechanical behavior between emulsions and bigels highlight the importance of formulation strategies in tailoring material properties for specific functional applications.

The combination of biopolymers (WPI, ALG) and lipids (beeswax, sea buckthorn oil) in both bigel and emulsion resulted in strong internal interactions and entangled networks. However, the presence of ethylcellulose in bigels further contributed to mechanical stiffness. The increase in mechanical properties of bigels compared to emulsions may be primarily due to their two structured phases, namely hydrogel and oleogel. This biphasic system created a more interconnected and reinforced network, enhancing rigidity and resistance to deformation. In contrast, emulsions consisting of a single continuous phase with dispersed droplets were more deformable under compression.

Additionally, the incorporation of chitosan microparticles into FD materials might have further strengthened the network by increasing intermolecular interactions, leading to an even greater Young's modulus and compressive force.

Porosity and Density Measurements

The porosity and density of FD materials based on biopolymers (WPI, ALG and EC), cryoprotectant (mannitol), lipids (sea buckthorn oil and beeswax) and emulsifier (Span 80) were evaluated through the liquid displacement method using isopropanol. This analysis revealed that FD emulsions exhibited porosity ranging from 69% to 72%, whereas bigels displayed a slightly lower porosity varying from 63% to 67% (Fig. 16a). The slightly reduced porosity observed in bigels may be attributed to their biphasic structure with the presence of both hydrogel and oleogel phases. The structure of bigels, reinforced by strong interactions between biopolymers and lipids, likely resulted in reduced pore formation during freeze-drying, compared to emulsions.



(**a**) (**b**)

Figure 16. Porosity **(a)** and density **(b)** of prepared freeze-dried emulsions (E) and bigels (BG) with and without the addition of elderflower-loaded chitosan microparticles (MPs). Significant differences ($p \leq 0.05$) between samples were marked on the graph with (*).

Bigels exhibited slightly lower density (167 mg/ml to 171 mg/ml) than emulsions (179 mg/ml to 210 mg/ml) (Fig. 16b). This suggests that bigels, while structurally more rigid, may retain a different internal architecture or phase distribution that affected mass distribution and compaction. The incorporation of extract-loaded chitosan microparticles into the emulsion increased its density. This may be due to the microparticles acting as structural fillers that modify the pore network while increasing mass per unit volume [47,48].

Residual Moisture Content

Results of residual moisture content after freeze-drying of emulsions and bigels were investigated as the percentage of weight loss during drying samples to a constant weight (Fig.

17). Moisture content varied across formulations, with the FD emulsion containing 4.2%, while the addition of microparticles increased it to 7.1%. Bigels had a higher residual moisture content of 7.5%, but their value decreased to 5.7% when combined with microparticles. These variations may be attributed to differences in water-binding capacity, phase organization, and microparticle interactions. The higher moisture content in bigels could result from their two structured phases, which retain water more effectively. However, the unexpected reduction in moisture for bigels with microparticles suggests that chitosan may facilitate more efficient water removal during freeze-drying. These variations in moisture content highlight the influence of formulation and additives on the water-holding capacity of FD materials, which can impact their stability and performance in various applications.

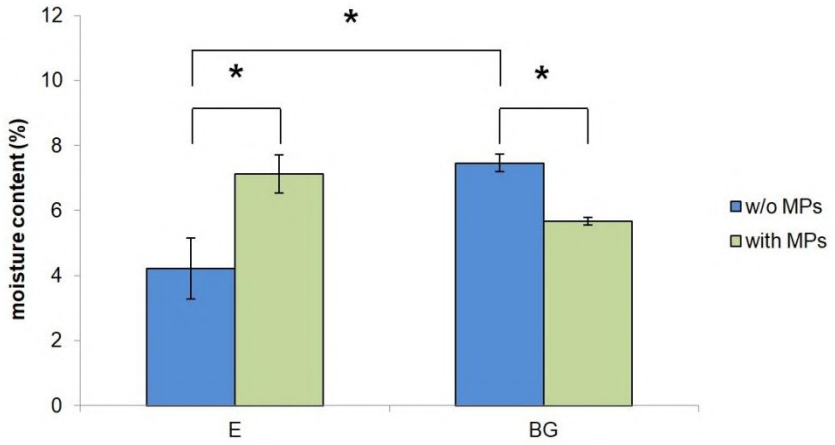


Figure 17. The moisture content of prepared freeze-dried emulsions (E) and bigels (BG) with and without the addition of elderflower-loaded chitosan microparticles (MPs). Significant differences ($p \leq 0.05$) between samples are marked on the graph with (*).

Biophysical Skin Parameters Measurements

Biophysical skin parameters, including skin surface hydration, skin barrier quality (TELW) and skin color, were assessed with the use of Courage+Khazaka probes. The obtained freeze-dried emulsions and bigels, with and without the addition of elderflower-loaded chitosan microparticles, were topically applied to the probands' skin and reconstructed back to emulsion or bigel with water during the spreading of materials on the skin. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Nicolaus Copernicus University in Toruń (KB 67/2021).

The *stratum corneum's* hydration level indirectly indicates overall skin moisture. A corneometer measures hydration at a very shallow depth, focusing solely on the outermost skin layer to prevent interference from deeper skin layers. Since the device relies on electrical capacitance, the presence of substances like salts or residues from applied products on the skin surface has little impact on the accuracy of the readings.

According to the obtained results (Fig. 18), it is evident that the topical treatment with all tested materials led to an increase in the hydration of the *stratum corneum*. The highest rise in water content in the outermost skin layer was observed after applying bigel modified with extract-loaded microparticles, while the emulsion containing microparticles demonstrated the weakest skin-hydration effect. Fifteen minutes after treatment, the skin hydration levels in areas treated with the emulsion and emulsion with microparticles were 8.6 a.u. and 6.3 a.u., respectively. In comparison, bigel and bigel with microparticles resulted in hydration levels of 7.9 a.u. and 11.8 a.u., respectively. After an additional 15 minutes, the hydration levels decreased to a range of 4.2–5.2 a.u., with the highest value of 9.3 a.u. recorded for bigel with microparticles. One and two hours after application, the hydration values ranged from 4.9 to 7.3 a.u.and from 5.3 to 9.7 a.u., respectively. After three hours, skin areas treated with bigels exhibited higher hydration levels (8.5–9.0 a.u.) compared to emulsions (5.8–6.1 a.u.). At the final measurement point, the *stratum corneum* hydration levels ranged from 8.0 to 9.7 a.u. for areas treated with emulsion and bigels, while the skin treated with the emulsion containing extract-loaded microparticles showed a lower value of 5.9 a.u.

The enhanced skin hydration observed after applying the freeze-dried emulsions and bigels can be attributed to the synergistic effect of their carefully selected components. Sodium alginate and WPI form a protective film on the skin, enhancing moisture retention [49,50]. Furthermore, the composition of WPI is similar to that of the natural nourishing factor (NMF) naturally present in the *stratum corneum*. They are both rich in amino acids like serine, glycine, and proline, which can enhance skin hydration by supplying building blocks for NMF and improving moisture retention. Therefore, WPI may support the skin's natural hydration process by rebuilding the NMF. Mannitol acts as a cryoprotectant, preserving the biopolymer matrix's structural integrity and thus may improve its rehydration capacity [51], while sea buckthorn oil and beeswax indirectly moisturize the skin [52,53]. Chitosan microparticles loaded with *Sambucus nigra* flower extract might enable sustained release of flavonoids and phenolic compounds, which have antioxidant and moisturizing properties [54]. The superior hydration effect of bigel modified with microparticles may result from the

structured network of the biopolymer-lipid matrix, which enhances water retention and prolongs the moisturizing effect.

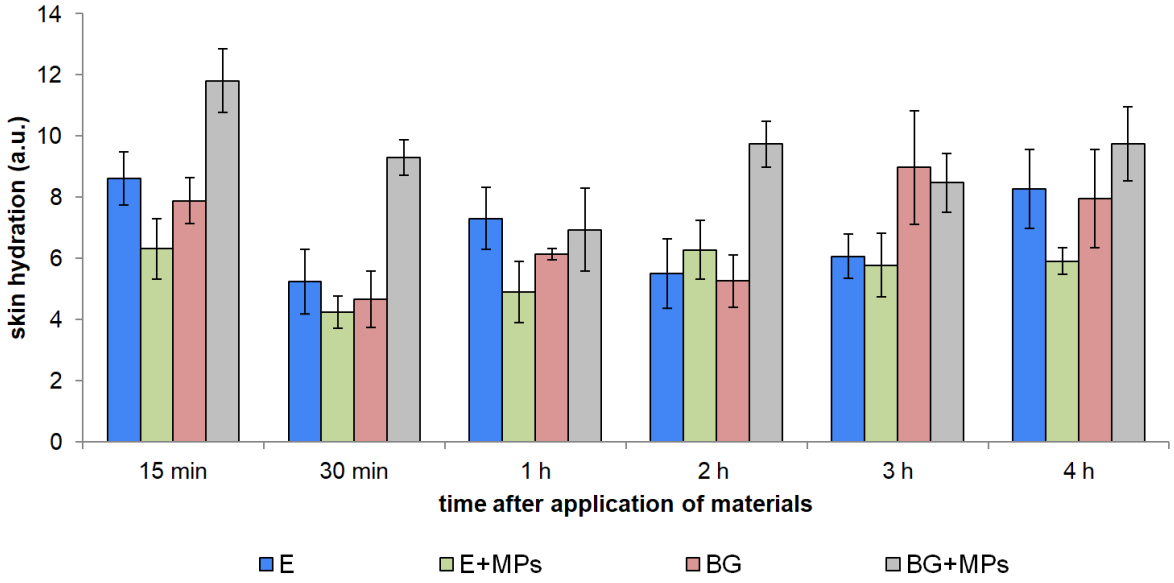


Figure 18. Corneometric skin measurements after topical application of prepared freeze-dried emulsions (E) and bigels (BG) with and without the addition of elderflower-loaded chitosan microparticles (MPs). The results show differences in corneometer indications between the treated and untreated control areas at each measured interval.

The evaporation of water from the skin is a natural aspect of the body's metabolism. TEWL serves as an indicator of the skin's barrier integrity, as any disruption in this function leads to an increase in water loss. Thus, measuring TEWL is essential for determining the effectiveness of topical products.

Tewametric measurements indicate that the application of all prepared samples led to a reduction in TEWL, thereby enhancing the epidermal permeability barrier function and improving overall skin barrier integrity (Fig. 19). TEWL values significantly decreased from 11.3–12.4 g/h/m² to 10.1–12.0 g/h/m², suggesting that the formulations strengthened the skin's protective barrier. This improvement can be attributed to the combined action of biopolymers and lipids, which form a protective film on the skin surface, reducing transepidermal water loss. Additionally, the moisturizing properties of *Sambucus nigra* flower extract-loaded chitosan microparticles, along with the occlusive effect of beeswax and sea buckthorn oil, contribute to improved water retention and barrier repair, resulting in enhanced skin hydration and reduced TEWL. Furthermore, the composition of sea buckthorn oil is

similar to that of the skin's natural lipids, particularly essential fatty acids like omega-3, omega-6, omega-7, and omega-9, as well as lipid-soluble vitamins [55,56]. These lipids can integrate into the skin's lipids, supporting hydration and barrier repair, which is shown as reduced TEWL.

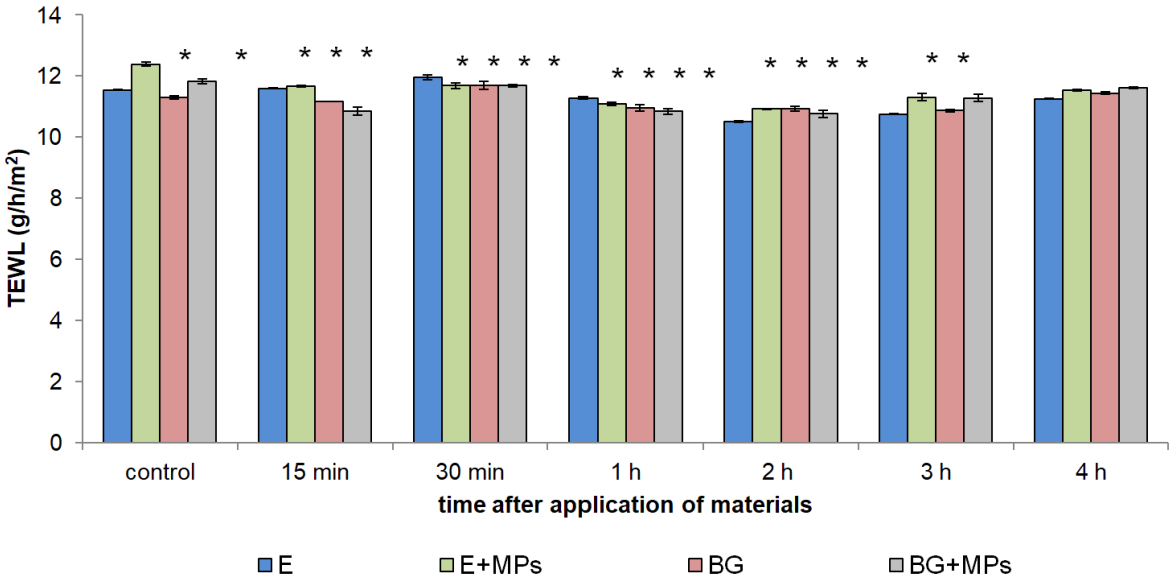


Figure 19. Tewametric measurements of skin before (control) and after topical application of prepared freeze-dried emulsions (E) and bigels (BG) with and without the addition of elderflower-loaded chitosan microparticles (MPs). * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

The L*a*b* color space coordinates are used to represent skin color measurements. The L* value indicates skin brightness; the a* value represents the position on the red-green axis, and it is used to assess skin redness, microcirculation, and erythema, while the b* value corresponds to the blue-yellow axis (skin pigmentation) [57].

Based on colorimeter indications (Fig. 20), all of the formulated samples led to a reduction in skin redness, with values decreasing from 7.25–7.68 a.u. before the application of the materials to 4.28–6.62 a.u. following the application of freeze-dried emulsions and bigels, both with and without the addition of chitosan microparticles containing *Sambucus nigra* flower extract. This reduction in redness can be attributed to sea buckthorn, which has been shown to exhibit decreasing melanin content and skin erythema properties [58]. Moreover, biopolymers (sodium alginate, WPI, ethylcellulose), lipids (sea buckthorn oil, beeswax), and cryoprotectant (mannitol) likely played a key role in soothing the skin, reducing

inflammation, and improving skin tone. These components formed a protective barrier that helped calm the skin, diminish erythema, and promote a uniform appearance. In addition, the soothing and anti-inflammatory effects of the herbal extract and chitosan might have helped to calm the skin and reduce erythema, leading to a more even skin tone. The active compounds in the formulations, such as flavonoids and phenolic compounds, may have contributed to reducing the skin's redness by targeting inflammatory pathways and promoting a balanced complexion.

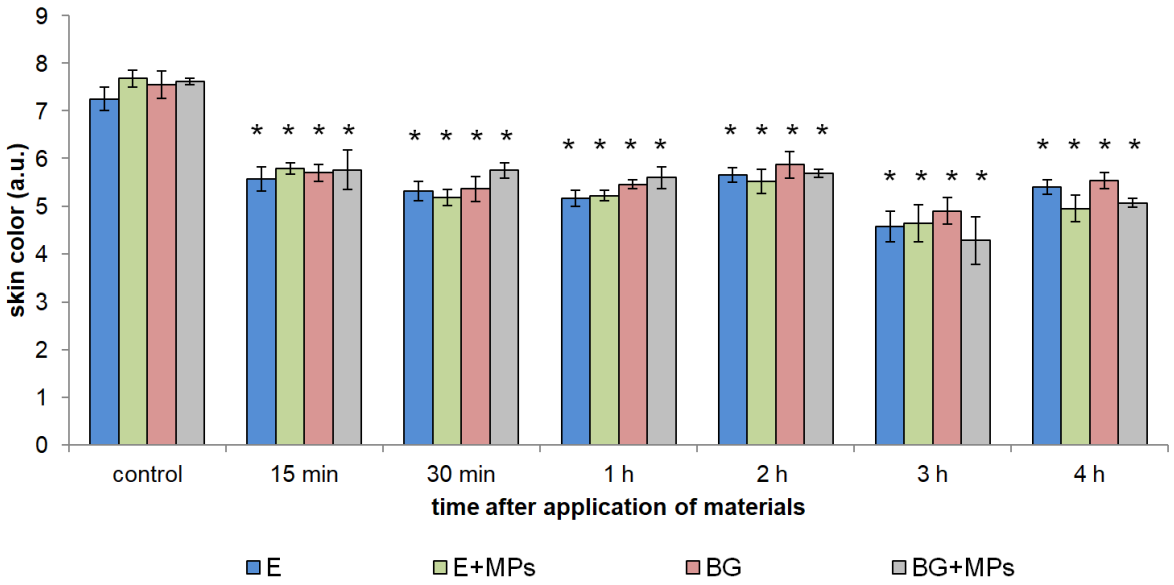


Figure 20. Colorimetric measurements of skin before (control) and after topical application of prepared freeze-dried emulsions (E) and bigels (BG) with and without the addition of elderflower-loaded chitosan microparticles (MPs). * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

Conclusions

The study successfully developed freeze-dried emulsions and bigels modified with chitosan microparticles loaded with *Sambucus nigra* flower extract, demonstrating the enhanced cutaneous effect. The combination of biopolymers (WPI and sodium alginate) and cryoprotectant (mannitol) in the aqueous phase, lipids (sunflower oil and beeswax) and emulsifier (Span 80) in the oily phase of the emulsion and additionally ethylcellulose in the bigel created a stable, porous structure after freeze-drying. FD bigel exhibited higher values of Young's modulus and residual moisture content, whereas FD emulsion presented higher porosity and density. Eco-friendly, three-dimensional materials with a porous structure

containing polymeric microparticles with encapsulated plant extract (flowers of *Sambucus nigra)* were designed to dissolve in a minimal amount of aqueous solvent back to emulsion or bigel immediately before its application to the skin. Active substances from elderflower extract showing antioxidant properties and a positive effect on the skin condition were protected in chitosan microparticles during the freeze-drying process and released at the moment of the cosmetic application to the skin (during spreading on the skin). The application of fabricated materials significantly increased the hydration of the *stratum corneum* and decreased skin redness and TEWL, indicating improved skin barrier quality and moistened and soothed skin due to the materials' components. Therefore, the combination of microencapsulation and sponge-like materials resulted in the development of an innovative, eco-friendly material form that is effective and suitable for cosmetic and dermatological applications.

CONCLUSIONS AND FINAL REMARKS

The studies that were conducted resulted in the preparation of three original research articles and material for the subsequent publication. The main conclusions arising from these works are outlined below. Collectively, these studies demonstrate the scientific novelty and practical relevance of the findings, highlighting their contribution to the development of biopolymer-based freeze-dried systems for cosmetic and dermatological applications.

1. Plant extracts from selected Polish herbs, flowers, and rhizomes can be efficiently obtained using Soxhlet extraction with water and ethanol as solvents, providing bioactive compounds with measurable polyphenol and flavonoid content **[P1].**
2. Phytochemical profiling and antioxidant assays (CUPRAC, FRAP, DPPH RSA) demonstrated that individual extracts and their combinations exhibit synergistic or additive antioxidant activity, highlighting their potential as natural bioactive ingredients in topical formulations **[P1]**.
3. Encapsulation of plant extracts into chitosan microparticles via extrusion allowed for effective loading and controlled release, with particle morphology and size enhancing their suitability for skin applications **[P1]**.
4. Herbal emulsions and hydrogels formulated with selected extracts improved skin hydration, barrier function (TEWL), and skin color parameters upon topical application, demonstrating their efficacy and safety for dermatological use **[P1]**.
5. Freeze-dried emulsions based on biopolymers (sodium alginate, whey protein isolate), cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, trehalose), oils (sunflower oil, sea buckthorn oil), beeswax and emulsifier (Span-80) were successfully prepared and optimized **[P2, P4]**.
6. Optimization of emulsion homogenization parameters, including time and speed, allowed the preparation of emulsions with the narrowest droplet size distribution, which is crucial for stability, uniformity, and reproducible delivery of active compounds **[P2]**.
7. Freeze-dried emulsions exhibited well-preserved porous structures, tunable mechanical properties, promising porosity and density, and predictable residual moisture content, making them suitable as versatile carrier systems **[P2, P4]**.

8. Mixing hydrogel (sodium alginate, whey protein isolate, glycerin, mannitol) and oleogel (ethylcellulose, sunflower oil, sea buckthorn oil, beeswax) resulted in the formulation of bigels suitable for freeze-drying **[P3, P4]**.
9. Hydrogel/oleogel ratios and polymer concentrations strongly influenced swelling, degradation, porosity, density, moisture content and mechanical behavior of freeze-dried bigels, allowing precise tailoring of material properties for specific topical applications **[P3]**.
10. Incorporation of chitosan microparticles loaded with *Sambucus nigra* extract into freeze-dried emulsions and bigels maintained structural integrity and enhanced delivery of active substances, without compromising material stability, confirming the feasibility of combining multiple delivery systems within a single formulation **[P4]**.
11. Topical application of prepared composites demonstrated controlled and safe interaction with the skin, optimizing the bioavailability of active substances while minimizing adverse effects, supporting their potential for advanced skincare and transdermal drug delivery systems **[P1, P4]**.

ACADEMIC ACHIEVEMENTS

EDUCATION

2020 – 2025 Nicolaus Copernicus University in Toruń
Academia Copernicana Doctoral School
Freeze-dried emulsions and bigels with plant extract-loaded microparticles as an eco-friendly prototype of novel materials designed with a view to sustainable water management
Supervisors: J. Kozłowska (NCU),
T. Douglas (Lancaster University, UK)

2018 – 2020 Nicolaus Copernicus University in Toruń
Faculty of Chemistry, Department of Chemistry of Biomaterials and Cosmetics
Cosmetic Chemistry
Master of Science
Emulsion films and 3D matrices for cosmetic applications
Supervisor: J. Kozłowska

2015 – 2018 Nicolaus Copernicus University in Toruń
Faculty of Chemistry, Department of Chemistry of Biomaterials and Cosmetics
Cosmetic Chemistry
Bachelor of Science
The use of microparticles from natural polymers for cosmetic applications
Supervisor: J. Kozłowska

PUBLICATIONS

1. W. Walendziak, N. R. Villegas, T. E. Douglas, J. Kozlowska, *Phytochemical studies of plant extracts enclosed in chitosan microparticles and the effect of phytoformulations on skin condition*, European Polymer Journal, 2025, 233, 113968. (IF_{2025} = 6.3; MNiSW = 100)
2. W. Walendziak, T. E. Douglas, J. Kozlowska, *Design, Optimization, and Characterization of Freeze-Dried Emulsions Based on Sodium Alginate and Whey Protein Isolate Intended for Cosmetic and Dermatological Applications*, ACS Omega, 2025, 10, 24932−24949. (IF_{2025} = 4.4; MNiSW = 70)
3. W. Walendziak, T. E. Douglas, J. Kozlowska, *Physicochemical Properties of Freeze–Dried Bigel-Based Materials Composed of Sodium Alginate/Whey Protein Isolate Hydrogel and Ethylcellulose/Sunflower Oil Oleogel*, Biomacromolecules, 2025, 26, 2344-2355. (IF_{2025} = 5.4; MNiSW = 140)
4. N. Stachowiak-Trojanowska, W. Walendziak, T. E. Douglas, J. Kozlowska. *Whey Protein Isolate as a Substrate to Design Calendula officinalis Flower Extract Controlled-*

Release Materials. International Journal of Molecular Sciences, 2024, 25(10), 5325. (IF_{2024} = 4.9; IF_{2025} = 4.9; MNiSW = 140)

5. W. Prus-Walendziak, J. Kozlowska, *Lyophilized Emulsions in the Form of 3D Porous Matrices as a Novel Material for Topical Application,* Materials, 2021, 14(4), 950. (IF_{2021} = 3.7; IF_{2025} = 3.2; MNiSW = 140)
6. W. Prus-Walendziak, J. Kozlowska, *Design of Sodium Alginate/Gelatin-Based Emulsion Film Fused with Polylactide Microparticles Charged with Plant Extract,* Materials, 2021, 14(4), 745. (IF_{2021} = 3.7; IF_{2025} = 3.2; MNiSW = 140)
7. J. Kozlowska, W. Prus-Walendziak, N. Stachowiak, A. Bajek, L. Kazmierski, B. Tylkowski, *Modification of Collagen/Gelatin/Hydroxyethyl Cellulose-Based Materials by Addition of Herbal Extract-Loaded Microspheres Made from Gellan Gum and Xanthan Gum,* Materials, 2020, 13(16), 3507. (IF_{2020} = 3.6; IF_{2025} = 3.2; MNiSW = 140)
8. J. Kozlowska, B. Tylkowski, N. Stachowiak, W. Prus-Walendziak, *Controlling the Skin Barrier Quality through the Application of Polymeric Films Containing Microspheres with Encapsulated Plant Extract,* Processes, 2020, 8(5), 530. (IF_{2020} = 2.8; IF_{2025} = 2.8; MNiSW = 70)
9. J. Kozlowska, W. Prus, N. Stachowiak, *Microparticles based on natural and synthetic polymers for cosmetic applications*, International Journal of Biological Macromolecules, 2019, 129, 952-956. (IF_{2019} = 5.2; IF_{2025} = 8.5; MNiSW = 100)
10. J. Kozlowska, N. Stachowiak, W. Prus, *Stability studies of collagen-based microspheres with Calendula officinalis flower extract,* Polymer Degradation and Stability, 2019, 163, 214-219. (IF_{2019} = 4.0; IF_{2025} = 7.4; MNiSW = 100)
11. W. Prus, J. Kozlowska, *The influence of new polymeric microbeads in peeling products on skin condition,* Molecular Crystals and Liquid Crystals, 2018, 671(1), 140-147. (IF_{2018} = 0.6; IF_{2025} = 0.7; MNiSW = 15)
12. W. Prus, J. Kozlowska, *The influence of collagen from various sources on skin parameters*, Engineering of Biomaterials, 2018, 21(146), 14-17.

SUMMARIC IF_{2025} = 50; MNiSW = 1155; *h*-index = 5

CHAPTER IN A MONOGRAPH

1. J. Kozlowska, A. Bajek, N. Stachowiak, W. Prus-Walendziak, B. Tylkowski, *Application of microencapsulation in medical and pharmaceutical industry,* In: Microencapsulation 2nd Edition; editors: B. Tylkowski, M. Giamberini, S. Fernandez Prieto, ISBN 978-3-11-064176-9, De Gruyter, Berlin/Boston, 2020

PATENTS

1. J. Kozlowska, W. Prus*, Preparat kosmetyczny do oczyszczania i złuszczania naskórka (Cosmetic preparation for cleaning and exfoliation of the epidermis),* PL236187, 15.01.2019
2. J. Kozlowska, W. Prus, *Sposób wytwarzania kompozycji kosmetycznej do złuszczania komórek naskórka (Cosmetic composition for exfoliating epidermal cells and the method of its production)*, PL239755, 24.05.2018

RESEARCH PROJECTS

1. Financing attendance at a foreign conference (Royal Australian Chemical Institute National Congress 2022 „Chemistry: Catalysing solutions to global challenges", Brisbane, Australia, 03-08.07.2022) in the Project PROM - The international exchange of Ph.D. students and academic staff at the Nicolaus Copernicus University in Toruń
2. Financing attendance at a foreign conference (10th International Conference on Mechanochemistry and Mechanical Alloying 2022 (INCOME 2022), Cagliari, Italy, 06-10.06.2022) in the Grants4NCUStudents under the „Excellence Initiative – Research University" programme
3. Principal Investigator of the Young Scientists Grant awarded by the Dean of the Faculty of Chemistry of the Nicolaus Copernicus University: *Methodology optimization for obtaining modern, functional materials based on freeze-dried bigels for cosmetic and dermatological applications*, 05-11.2022
4. Principal Investigator of the Young Scientists Grant awarded by the Dean of the Faculty of Chemistry of the Nicolaus Copernicus University: *New, ecological solutions in the design of cosmetic preparations*, 05-11.2021
5. Principal Investigator of the Grants4NCUStudents research project under the „Excellence Initiative - Research University„ programme: *The use of polymer microparticles in new cosmetic products for skin changed during anticancer treatment.* Supervisor: PhD Justyna Kozlowska, 05.2020-06.2021
6. Member of the Nicolaus Copernicus University Priority Research Team *IPM Team (Interdisciplinary Innovation in Personalized Medicine Team)*, 04.2019-09.2025
7. Co-investigator in the National Science Centre project, Sonata, (No. UMO-2016/21/D/ST8/01705): *New materials containing microparticles incorporated into a polymer matrix for medical, pharmaceutical and cosmetic applications*. Principal Investigator: PhD Justyna Kozlowska, 04.2017-01.2022

INTERNATIONAL INTERNSHIPS

1. Internships in a research laboratory: *Optimization of the fabrication method and characterization of polymeric films based on whey protein isolate. Fabrication of hydrogels (whey protein isolate), oleogels (rapeseed oil, ethyl cellulose and Span 80) and bigels,* Supervisor: PhD Timothy E.L. Douglas, School of Engineering, Lancaster University, UK, 22.11-12.12.2021
2. Erasmus+ internship: *Experimental investigation of novel composite membranes prepared from biodegradable polymers with polyphenols and flavonoids,* Supervisor: PhD Bartosz Tylkowski, CTQC – Chemistry Technology Centre of Catalonia, Tarragona, Spain, 14.01-14.03.2019

NATIONAL INTERNSHIPS

1. Laboratorium Naturella Sp. z o.o. (a biotechnology research and implementation company that offers cleaning and personal care products). Internships in the quality control and R&D department. Bydgoszcz, Poland, 02.07-28.09.2018
2. Bell Sp. z o.o. (manufacturer and distributor of color cosmetics). Internships in the quality control and R&D department. Warsaw, Poland, 03-28.07.2017

SCIENCE CONFERENCES

Oral presentations

1. XVIII Kopernikańskie Seminarium Doktoranckie (XVIII Copernican Doctoral Seminar), *Freeze-dried emulsion and bigel systems: novel eco-conscious materials for cosmetic applications*, Toruń, Poland, 26-27.06.2025
2. VI Interdyscyplinarna Konferencja Nano(&)BioMateriały – od teorii do aplikacji (6th Interdisciplinary Conference Nano(&)BioMaterials – from theory to application), *Modyfikacja materiałów na bazie liofilizowanych emulsji i biżeli dodatkiem mikrocząstek zawierających ekstrakt z kwiatów bzu czarnego (Modification of materials based on freeze-dried emulsions and bigels with the addition of microparticles containing elderflower extract),* Toruń, Poland, 12-14.06.2024
3. UK-Poland-Ukraine Bioinspired Materials Conference, *Characteristics of spongy polymeric bigels containing whey protein isolate, sodium alginate and ethyl cellulose*, online, 29-30.11.2022
4. UK-Russia Conference Advanced Biomaterials to combat cancer, *Chitosan microparticles containing plant extracts intended for skin changed during anticancer treatment*, Lancaster, UK, 08-10.12.2021
5. E-Zjazd Wiosenny Sekcji Studenckiej Polskiego Towarzystwa Chemicznego (e-Spring Congress of the SSPTChem 2021), *Polimerowe mikrocząstki jako nośniki ekstraktów roślinnych o właściwościach przeciwutleniających (Polymer microparticles as carriers of plant extracts with antioxidative properties),* online, 27-29.05.2021
6. UK-Poland Conference on Bioinspired Materials, *Microcapsules with herbal preparations for dermatological applications*, online, 23-24.11.2020
7. Kopernikańskie E-Seminarium Doktoranckie (Copernican Doctoral E-Seminar), *Polimerowe materiały kompozytowe zawierające mikrocząstki z ekstraktem roślinnym (Polymeric composite materials containing microparticles with plant extract)*, Toruń, Poland, 07.09.2020
8. III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg (III National Symposium on Bioorganic, Organic and Biomaterials Chemistry BioOrg 2019), *Charakterystyka filmów emulsyjnych na bazie alginianu sodu i żelatyny (Chraracteristic of emulsion films based on sodium alginate and gelatin),* Poznań, Poland, 07.12.2019
9. 1st International Conference Functional and Engineering Materials – FEM 2019, *The effect of the addition of microparticles on the properties of emulsion films*, Łódź, Poland, 16-18.10.2019
10. XIII Kopernikańskie Seminarium Doktoranckie (XIII Copernican Doctoral Seminar), *Innowacyjny preparat kosmetyczny do złuszczania martwych komórek naskórka (Innovative cosmetics product to exfoliate dead skin cells),* Bachotek, Poland, 16-18.06.2019
11. IV Interdyscyplinarna Konferencja Nano(&)BioMateriały – od teorii do aplikacji (4th Interdisciplinary Conference Nano(&)BioMaterials – from theory to application), *Filmy emulsyjne modyfikowane mikrocząstkami do zastosowań kosmetycznych (Emulsion films modified with microparticles to cosmetic applications),* Toruń, Poland, 06-07.06.2019

12. International Symposium on Encapsulation Technologies, *Microparticles in cosmetics*, Tarragona, Spain, 22-24.10.2018
13. 27th Annual International Conference of the Polish Society for Biomaterials „Biomaterials in Medicine and Veterinary Medicine", *The influence of collagen from various sources on skin parameters*, Rytro, Poland, 11-14.10.2018
14. The 1st International Conference „Chemistry for Beauty and Health", *Comparison of the influence of polyethylene and new polymeric microbeads in peeling products on skin condition*, Toruń, Poland, 13-16.06.2018
15. The 11th International Conference „Electronic Processes in Organic and Inorganic Materials", *The influence of new polymeric microbeads in peeling products on skin condition,* Ivano-Frankivsk, Ukraine, 21-25.05.2018
16. II Ogólnopolskie Sympozjum Nauk Przyrodniczo-Rolniczych (II National Symposium on Natural and Agricultural Sciences), *Optymalizacja rozmiaru mikrokapsułek z alginianu sodu zastosowanych w recepturze nowego peelingu do celów kosmetycznych (Optimization of the size of microcapsules from sodium alginate used in a new peeling formula for cosmetic purposes)*, Poznań, Poland, 07-08.04.2018

Co-author of oral presentations

1. 7th International Caucasian Symposium on Polymers & Advanced Materials ICSP & AM 7, *Sodium alginate/gelatin-based materials fused with polylactide microparticles as tools to improve the activity of active substance to be administered through the skin*, J. Kozlowska, W. Prus-Walendziak, Tbilisi, Georgia, 27-30.07.2021
2. Nowoczesna implantologia: dylematy i nadzieje: III Ogólnopolska Konferencja Naukowa Implanty 2021 (Modern implantology: dilemmas and hopes: 3rd National Scientific Conference Implants 2021)*, Mikrocząstki z gumy gellan jako nośniki substancji aktywnych w polimerowych skafoldach (Gellan gum microparticles as carriers of active substances in polymer scaffolds),* J. Kozłowska, W. Prus-Walendziak, N. Stachowiak, A. Bajek, Ł. Kaźmierski, Gdańsk, Poland, 18.06.2021
3. American Association for Advances in Functional Materials (AAAFM) UCLA 2019, *Modification of sodium alginate/starch films by addition of microspheres*, J. Kozlowska, W. Prus, N. Stachowiak, Los Angeles, USA, 19-22.08.2019
4. Nowoczesna implantologia: dylematy i nadzieje: II Ogólnopolska Konferencja Naukowa Implanty 2019 (Modern implantology: dilemmas and hopes: 2nd National Scientific Conference Implants 2019)*, Funkcjonalne materiały zawierające mikrocząstki w polimerowych matrycach do kontrolowanego uwalniania substancji aktywnych (Functional materials containing microparticles in polymer matrices for controlled release of active substances*), J. Kozłowska, N. Stachowiak, W. Prus-Walendziak, A. Muszyńska, Gdańsk, Poland, 28-29.06.2019

Poster presentations

1. XVI Kopernikańskie Seminarium Doktoranckie (XVI Copernican Doctoral Seminar), *Wpływ czasu i prędkości homogenizacji na rozkład wielkości cząstek emulsji (The influence of homogenization time and speed on the emulsion particle size distribution),* Toruń, Poland, 29-30.06.2023

2. The 8th EuChemS Chemistry Congress, *Physicochemical properties of porous bigel-based materials composed of sodium alginate/whey protein isolate hydrogel and sunflower oil oleogel*, Lisbon, Portugal, 28.08.-01.09.2022
3. Royal Australian Chemical Institute (RACI) 2022 National Congress „Chemistry: Catalysing solutions to global challenges", *Casting light on the mechanical properties of materials based on freeze-dried bigels*, Brisbane, Australia, 03-08.07.2022
4. XV Kopernikańskie Seminarium Doktoranckie (XV Copernican Doctoral Seminar), *Biżele – nowoczesne materiały na bazie hydrożelu i oleożelu: badanie właściwości mechanicznych i stopnia pęcznienia (Bigels – modern materials based on hydrogel and oleogel: study of mechanical properties and swelling degree)*, Toruń, Poland, 20-22.06.2022
5. 10th International Conference on Mechanochemistry and Mechanical Alloying 2022 (INCOME 2022), *Sunflower oil and whey protein isolate-based bigels – mechanical properties*, Cagliari, Italy, 06-10.06.2022
6. Silesian Meetings on Polymer Materials POLYMAT2022, *Phytochemical studies and evaluation of the antioxidant activity of extracts prepared from different plants and extraction solvents*, Zabrze, Poland, 17.03.2022
7. XIV Kopernikańskie Seminarium Doktoranckie (XIV Copernican Doctoral Seminar), *Ektrakcja związków biologicznie czynnych z surowców roślinnych do zastosowań w produktach do pielęgnacji skóry pacjentów onkologicznych (Extraction of biologically active compounds from plant materials for use in skin care products for cancer patients)*, Toruń, Poland, 20-22.09.2021
8. ChemBiotIC Chemistry & Biotechnology International Conference, *Examination of skin barrier quality, hydration and colour after the application of freeze-dried emulsions*, Wrocław, Poland, 24-25.06.2021
9. III Ogólnopolska Konferencja Implanty 2021 – koncepcja a realia we współczesnej implantologii (3rd National Conference Implants 2021 – concept and reality in modern implantology), *Badanie struktury porowatych materiałów na bazie liofilizowanych emulsji (Examination of the structure of porous materials based on lyophilized emulsions)*, Gdańsk, Poland, 18.06.2021
10. IX Kopernikańskie Sympozjum Studentów Nauk Przyrodniczych (IX Copernican Symposium of Students of Natural Sciences), *Biofizyczne parametry skóry po aplikacji filmów emulsyjnych na skórę (Biophysical skin parameters after the application of emulsion films to the skin)*, Toruń, Poland, 18-20.09.2020
11. 27th Annual International Conference of the Polish Society for Biomaterials „Biomaterials in Medicine and Veterinary Medicine", *The influence of collagen from various sources on skin parameters*, Rytro, Poland, 11-14.10.2018
12. I Krajowa Konferencja Naukowa „Chemia dla Urody i Zdrowia" (The 1st National Conference „Chemistry for Beauty and Health"), *Opracowanie receptury nowego peelingu do celów kosmetycznych (Development of a new peeling formula for cosmetic purposes)*, Toruń, Poland, 08-10.06.2017

Co-author of poster presentations

1. 12th World Biomaterials Congress (WBC 2024), *Whey protein isolate-based microspheres and films used in controlled release,* J. Kozlowska, N. Stachowiak-Trojanowska, W. Prus-Walendziak, T. Douglas, Daegu, South Korea, 26-31.05.2024
2. 32nd Annual Conference of the European Society for Biomaterials (ESB 2022), *Sodium alginate/inulin microparticles containing Calendula officinalis flower extract for dermatologic applications*, J. Kozlowska, W. Prus-Walendziak, N. Stachowiak, J. Skopińska-Wiśniewska, B. Kaczmarek-Szczepańska, Bordeaux, France, 04-08.09.2022
3. 31st Annual International Conference of the Polish Society for Biomaterials „Biomaterials in Medicine and Veterinary Medicine", *The preparation and characterization of microparticles based on whey protein isolate*, J. Kozłowska, E. Golińska, K. Istal, B. Diarra, W. Prus-Walendziak, N. Stachowiak, Rytro, Poland, 13-16.10.2022
4. 7th International Caucasian Symposium on Polymers & Advanced Materials ICSP & AM 7, *Polymeric matrices based on gelatin and isolated whey protein with pot marigold extract for topical application*, J. Kozlowska, W. Prus-Walendziak, N. Stachowiak, T. Douglas, Tbilisi, Georgia, 27-30.07.2021
5. 28th Annual International Conference of the Polish Society for Biomaterials „Biomaterials in Medicine and Veterinary Medicine", *The preparation and characterization of microparticles based on whey protein isolate*, N. Stachowiak, W. Prus-Walendziak, T. Douglas, J. Kozlowska, Rytro, Poland, 10-13.10.2019

AWARDS

- The Best Graduate at the Faculty of Chemistry at the Nicolaus Copernicus University in the academic year 2019/2020
- The Best Student at the Faculty of Chemistry at the Nicolaus Copernicus University in the academic year 2018/2019

- Gold Medal award for *Plant-Powered Freeze-Dried Emulsions and Bigels for EcoSmart Cosmetics*, Bangkok International Intellectual Property, Invention, Innovation and Technology Exposition: IPITEx during Thailand Inventors' Day 2025, Bangkok, Thailand, 02-06.02.2025

- Award of the Regional Branch of the Polish Association of Chemical Engineers SITPChem for the best Master's thesis in the academic year 2019/2020 titled *Emulsion films and 3D matrices for cosmetic applications*
- Award of the Torun Branch of the Polish Chemical Society for the best Bachelor's thesis in the academic year 2017/2018, titled *The use of microparticles from natural polymers for cosmetic applications*

- 2nd degree award from the NCU Rector for the group's scientific records in the year 2024
- 2nd degree award from the NCU Rector for the group's scientific records in the year 2021
- 2nd degree award from the NCU Rector for the group's scientific records in the year 2019

- Third award for the best oral presentation in the Chemical Sciences Section, XVIII Copernican Doctoral Seminar, *Freeze-dried emulsion and bigel systems: novel eco-conscious materials for cosmetic applications*, Toruń, Poland, 26-27.06.2025
- Second award for the best oral presentation in the Biological and Medical Sciences Section, XIII Copernican Doctoral Seminar, *Innovative cosmetics product to exfoliate dead skin cells*, Bachotek, Poland, 16-18.06.2019
- Award for the best student oral presentation, International Symposium on Encapsulation Technologies, *Microparticles in cosmetics*, Tarragona, Spain, 22-24.10.2018
- Award for the best oral and poster presentation (Outstanding Rapid Fire and Poster Presentation), 27th Annual International Conference of the Polish Society for Biomaterials „Biomaterials in Medicine and Veterinary Medicine", *The influence of collagen from various sources on skin parameters*, Rytro, Poland, 11-14.10.2018
- Award for the best oral presentation, the 11th International Conference „Electronic Processes in Organic and Inorganic Materials", *The influence of new polymeric microbeads in peeling products on skin condition*, Ivano-Frankivsk, Ukraine, 21-25.05.2018
- Award for the best poster presentation, the 1st National Conference „Chemistry for Beauty and Health", *Development of a new peeling formula for cosmetic purposes*, Torun, Poland, 08-10.06.2017

SCHOLARSHIPS

- Scholarship of the Minister of Science and Higher Education for outstanding achievements (2018/2019, 2019/2020)
- Scholarship of the Mayor of the City of Torun (2018/2019, 2019/2020)
- Rector's Scholarship for the best students (2016/2017, 2017/2018, 2018/2019, 2019/2020)

TRAINING COURSES/CERTIFICATES

Training courses:

- Participation in the „*studies with Mentor*" program under the supervision of Dr Justyna Kozłowska (academic years 2017/2018, 2018/2019, 2019/2020)
- Participation in the training course KLUCZ UMK (academic year 2017/2018)
- Participation in the internship program AS-KIER UMK (07-09.2018)

Certificates:

- Rules for placing chemical consumer products for everyday use on the market and labelling requirements. Cosmetic raw materials – required documentation, 2023
- REACH and CLP in the cosmetic industry – obligations of Nicolaus Copernicus University when placing cosmetic raw materials on the market, 2023
- Safety Assessment of Cosmetics in the EU, Brussels, Belgium, 2021
- Nature Masterclasses: Scientific Writing and Publishing, 2021
- English TELC B2, 2020

- The latest solutions in the field of surface topography research with optical methods, 2019
- Support for scientific apparatus with elements of the quality system ISO9001 and PN-EN 17025, 2018
- Research expertise in the ISO 9001:2015 and PN-EN ISO 17025:2005 systems and product registration in the REACH system, 2018
- Internal auditor in a research laboratory according to PN-EN ISO 17025:2005 and PN-EN ISO 9001:2015, 2018
- Training on soft skills: teamwork, public speeches, interpersonal communications, 01 – 02.2018

ORGANIZING ACTIVITY

- Member of the Organizing Committee of the national scientific conference XV Copernican Doctoral Seminar (2022) and XVI Copernican Doctoral Seminar (2023), Toruń, Poland
- Member of the Organizing Committee of the international scientific conference „UK-Russia Conference: Advanced Biomaterials to Combat Cancer", Lancaster, UK (2021)
- Assistance in organizing the 1st International Conference on „Chemistry for Beauty and Health", Toruń, Poland (2018)

SCIENCE POPULARIZATION ACTIVITIES

- Conducting popular science workshops *Chemiczne żelki* during the Toruń Night of Scientists, Toruń, Poland, 30.09.2022
- Conducting popular science workshops *Lecimy w kulki* during the 20th Toruń Festival of Science and Art, Toruń, Poland, 24.04.2022
- An expert in the field of cosmetic products during the workshop *I, woman,* Modernity Centre „Mill of Knowledge", Toruń, Poland, 05.03.2020

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87, Page 20 87 (2019) 20. https://doi.org/10.3390/SCIPHARM87030020.
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Phytochemical studies of plant extracts enclosed in chitosan microparticles and the effect of phytoformulations on skin condition

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ABSTRACT

The cosmetic industry constantly competes in search of new and exotic raw materials, often forgetting the strength in the action of well-known and tested for many years. The main aim of this study was to formulate dermatological preparations containing plant extracts-loaded microparticles and to investigate the effects of their topical application on skin conditions. Extracts were prepared using the Soxhlet apparatus with water and ethanol as solvents with the following common Polish herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome: *Potentilla erecta*. They were characterised by the content of polyphenols, flavonoids and antioxidant capacity (CUPRAC, FRAP and DPPH RSA). Subsequently, extracts were enclosed in chitosan microparticles with studied loading capacity and *in vitro* release profile. The highest level of TPC, TFC and antioxidant activity was noted in aqueous extract from *Sambucus nigra*. Biophysical skin parameters were instrumentally assessed after application to the probands skin of obtained herbal creams and hydrogels. Preliminary studies of the application of phytoformulations revealed that the skin was not irritated, and the skin's barrier permeability was maintained. Moreover, we observed a significant increase in skin hydration. Better short-term hydration properties showed cream containing microparticles with a loaded extract from *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* and hydrogel with free extracts from *Sambucus nigra* and *Viola tricolor* + *Veronica officinalis*. Therefore, prepared herbal dermatological preparations are suitable for skin conditioning.

1. Introduction

There is a growing demand and continuous search for new phytochemicals in the cosmetics market due to consumers' rising expectations worldwide for green and natural products. However, numerous plants commonly occurring in Poland begin to be somewhat forgotten due to the "pursuit" of new, exotic plant materials. Herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*, flowers of *Achillea millefolium, Sambucus nigra, Tilia cordata*, and rhizome of *Potentilla erecta* have been recognised in Polish traditional folk medicine and herbalism for their beneficial effects on the skin. They have been reported to possess regenerating, soothing, antioxidant, anti-inflammatory, antibacterial, immunosuppressive, and antiproliferative properties [1–7]. These pharmacological activities are attributed to their phytoconstituents, mainly phenolic compounds, a group of small molecules with at least one phenol unit in their structures. They are secondary metabolites synthesised in the shikimic acid of plants and pentose phosphate through phenylpropanoid pathways. Phenolic compounds can be divided into subgroups: phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbens, and curcuminoids. The main flavonoids found in *Viola tricolor* are violanthin, rutin, anthocyanidin [8] and quercetin glycosides [9]. Moreover, numerous studies also reported the presence of cyclotides [1,10] and bisabolol [11]. Dominant compounds among phenolic and sterolic acids content of *Veronica officinalis* were determined as quercitrin, p-coumaric acid, ferulic acid, luteolin, hispidulin and β-sitosterol [12]. *Glechoma hederacea* comprises phenolic acids (chlorogenic, rosmarinic, caffeic, and ferulic acids) and flavonoid O-glycosides (rutin and genistin) [13,14]. Major constituents of *Plantago lanceolata* belong to polyphenols, tannins, flavonoids, alkaloids, terpenoids and iridoid glycosides, such as p-hydroxybenzoic,

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vanillic, gallic and chlorogenic acids, apigenin, luteolin and luteolin-7-O-glucoside [15,16]. *Achillea millefolium* has a high content of flavonoids (mainly luteolin O-acetylhexoside and apigenin O-acetylhexoside), organic acids (including malic, oxalic and quinic acids), tocopherols (γ-, α- and β-isoforms) and phenolic acids (mainly cis and *trans*-3,5-O-dicaffeoylquinic acids) [17]. *Sambucus nigra* flowers are a rich source of flavonoids, particularly rutin, quercetin and kaempferol, as well as phenolic acids, such as caffeoylquinic acid [18,19]. Vanillic acid was the dominant phenolic acid, whereas rutoside and (−)-epicatechin (tannin precursor) were major flavonoids found in *Tilia cordata* flowers [20]. *Potentilla erecta* rhizome contains tannins (pyrogallol), ellagitannins (including agrimoniin and pedunculagin), phenolic acids (coumaric, sinapic, caffeic, and gallic acids and their derivatives), flavonoids (kaempferol and quercetin and their derivatives), as well as triterpene saponins [3,21].

The human skin, as the largest organ covering the body, plays an important immunity role in protecting the body against pathogens and excessive water loss. This is assured due to the skin structure comprising three main layers: the epidermis, the dermis, and the hypodermis. The outermost layer of the epidermis – *stratum corneum* – comprises several layers of corneocytes embedded in a lipid matrix, forming a structure similar to "brick and mortar" with keratin-rich corneocytes as "bricks" and intercellular lipids as "mortar". Being a barrier from pathogens, the *stratum corneum* also prevents the penetration of cosmetic active substances to deeper skin layers. However, recent advances in encapsulation technology have significantly improved not only the chemical stability of active substances but also their biocompatibility, skin permeability, and skin cosmetic effects when applied topically [22,23].

Microencapsulation is a technique by which solid, liquid or gaseous active substances can be enclosed within a matrix [24,25]. Microparticles are synthesised, differing from each other in their respective diameters, which range from 1 to 1000 μm, and in their great diversity of spherical shapes, symmetrical or not. Microencapsulation may be achieved by various techniques [26,27] or materials [28]. On the other hand, substances can be microencapsulated for numerous purposes: to keep the material confined for a certain period, to allow its gradual and controlled diffusion or to launch its release under certain conditions [29,30]. Nowadays, it is a new technology that has been employed in the cosmetics industry as well as in the pharmaceutical, agrochemical and food industries, textiles, being used in drugs, extracts, vitamins, perfumes, oils, proteins, dyes, and bacterial cells, among others [31–40].

This research aimed to develop phytoformulations containing microparticles loaded with plant extracts and to instrumentally assess skin conditions after their topical application (Fig. 1). Herbs: *Viola tricolor,*

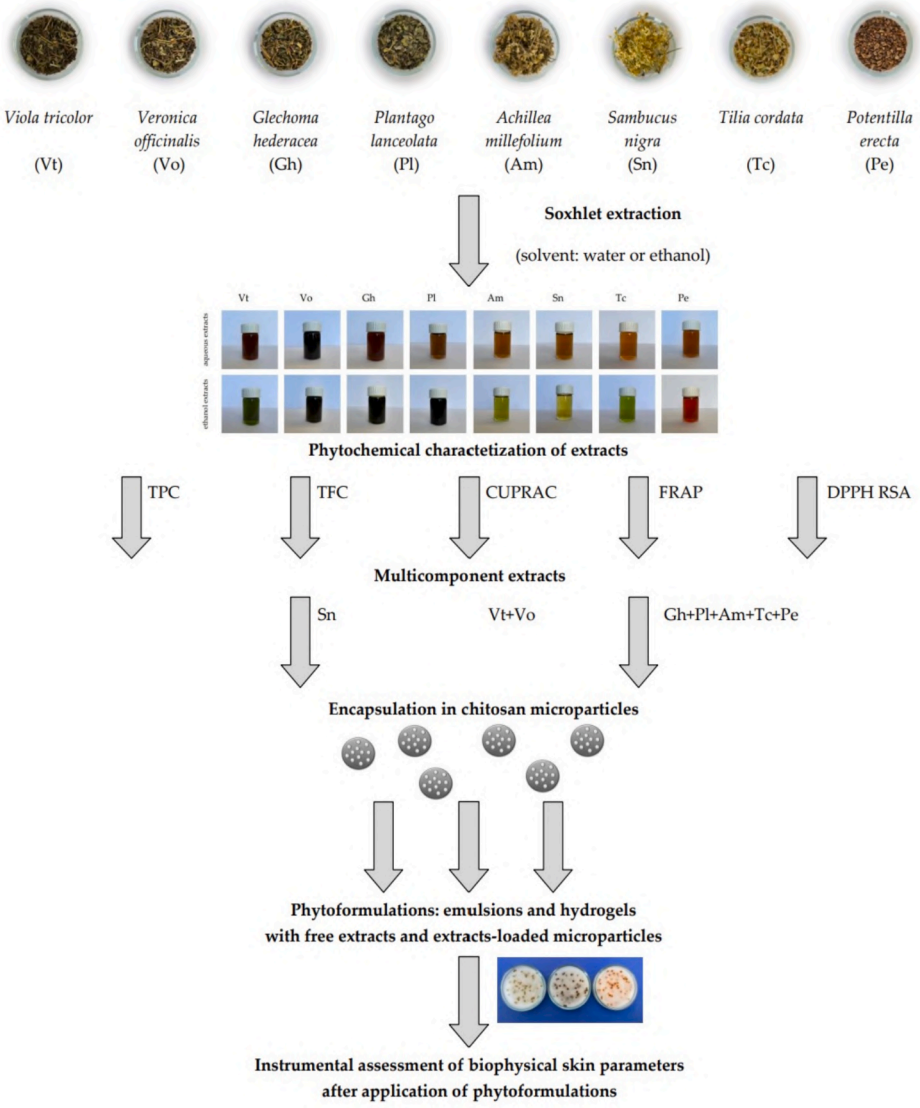


Fig. 1. The preparation scheme of extracts, their phytochemical analysis and encapsulation in chitosan microparticles, as well as the preparation of phytoformulations.

Veronica officinalis, Glechoma hederacea, Plantago lanceolata; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome: *Potentilla erecta* were extracted using the Soxhlet apparatus with water and ethanol as solvents. In order to formulate a preparation best suited for conditioning the skin, the plant species were selected based on several factors: (I) use in traditional medicine to treat skin disorders, (II) phytochemical composition, and (III) insufficient data on synergistic activity. We characterised their phytochemical profile by the content of polyphenols, flavonoids and antioxidant capacity (CUPRAC, FRAP and DPPH RSA) individually and in combinations of these extracts. Subsequently, selected extracts were enclosed in chitosan microparticles. The loading capacity and *in vitro* release profile of loaded extracts were examined. Preliminary studies of formulated herbal emulsions and hydrogels were performed on the probands' skin to analyse skin colour, skin surface hydration, and skin barrier quality (manifested as trans-epidermal water loss—TEWL).

2. Materials and methods

2.1. Materials

Dry plant raw materials (herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers of *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome of *Potentilla erecta*), tocopherol, *Vitis vinifera* seed oil and *Prunus domestica* seed oil were purchased from herbal wholesaler Nanga (Zlotow, Poland). Folin– Ciocalteu reagent, gallic acid, DPPH (2,2′-diphenyl-1-picrylhydrazyl, free radical), xanthan gum and chitosan (ultra low molecular weight, MW: 20,000 (avg.)) were acquired from Sigma-Aldrich (Poznan, Poland). Sodium carbonate, ethyl alcohol, and paraffinum liquidum were purchased from Stanlab (Lublin, Poland). Sodium phosphate, disodium phosphate, glycerin, and propylene glycol were acquired from Chempur (Piekary Slaskie, Poland). Quercetin, aluminium chloride anhydrous, copper (II) chloride dihydrate, ammonium acetate, neocuproine hemihydrate, iron (III) chloride hexahydrate, 2,4,6-tripyridyl-S thiazine (TPTZ), Trolox® and pentasodium tripolyphosphate (TPP), allantoin, panthenol were supplied by Pol-Aura (Dywity, Poland). Hydrochloric acid, acetic acid, sodium acetate anhydrous and methyl alcohol were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Cetearyl alcohol, caprylic/capric triglicerydes, octyldodecanol, ceteareth-20, isopropyl palmitate, and glyceryl stearate were purchased from CHEMCO (Sobowidz, Poland).

2.2. Extracts preparation

The plant extraction was conducted using the Soxhlet apparatus. 10 g of each dried plant raw material was extracted using 200 ml water or ethanol as solvents for 3 h. The extracts from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta* were obtained (Table 1). After characterisation of extract through Total Polyphenols and Flavonoids Content as well as antioxidant activity, aqueous extracts from mixed raw materials were prepared: (I) herbs of *Viola tricolor* + *Veronica officinalis*; (II) herbs of *Glechoma hederacea* + *Plantago lanceolata* + flowers of *Achillea* millefolium + *Tilia cordata* + rhizome of *Potentilla erecta*.

2.3. Extracts characterisation

2.3.1. Total polyphenols content (TPC)

Total Polyphenols Content was determined spectrophotometrically using the Folin-Ciocalteu method [41]. Extracts were diluted ten times using extraction solvent (water or ethanol), and then 20 μl of each sample was taken and added to 1.58 ml of distilled water and 100 μl of Folin-Ciocalteu reagent. Subsequently, 300 μl of saturated Na_2CO_3 solution was added to the mixture after 4 min and incubated for 40 min at 37 °C until a characteristic blue colour occurred. The absorbance was measured at a wavelength of 725 nm using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Three measurements were made for each type of extract and calculated based on gallic acid using the standard curve equation in the concentration range of 0–0.50 mg/ml (R = 0.9997). TPC was then expressed as grams of gallic acid equivalents (GAE) per 100 g of dry material weight (DW).

Table 1
Studied medicinal plants.

No	Botanical Name	Popular Name	Abbreviation	Family	Vegetal Part
1	*Viola tricolor*	heartsease, wild pansy	Vt	Violaceae	herb
2	*Veronica officinalis*	heath speedwell, common gypsyweed	Vo	Plantaginaceae	herb
3	*Glechoma hederacea*	ground-ivy	Gh	Lamiaceae	herb
4	*Plantago lanceolata*	ribwort plantain, narrow-leaf plantain	Pl	Plantaginaceae	herb
5	*Achillea millefolium*	yarrow	Am	Asteraceae	flower
6	*Sambucus nigra*	elder, elderberry	Sn	Adoxaceae	flower
7	*Tilia cordata*	small-leaved lime, small-leaved linden	Tc	Malvaceae	flower
8	*Potentilla erecta*	tormentil, septfoil	Pe	Rosaceae	rhizome

2.3.2. Total flavonoids content (TFC)

Total Flavonoids Content was evaluated spectrophotometrically using a method based on forming chelates of Al(III)-flavonoids due to many oxo and hydroxyl groups presented in flavonoids [42]. Each extract was diluted 20 times with extraction solvent (water or ethanol). 800 μl of each extract were added to 80 μl of 5 % (v/v) $AlCl_3$ and 1.12 ml of acetic acid and methanol mixture (ratio 1:19). Afterwards, prepared samples were incubated in a dark place for 30 min, and the resulting complexes were measured using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at a wavelength of 425 nm. Measurements were performed in triplicate and calculated using the calibration curve for quercetin in the 0–0.025 mg/ml concentration range (R = 0.9997). TFC was demonstrated as a milligrams of quercetin equivalents (QE) per 100 g of dry material weight (DW).

2.3.3. Antioxidant activity

CUPRAC (CUPric Reducing Antioxidant Capacity) is a method in which the ability to reduce copper (II) ions is tested [43]. Orange–yellow coloured Cu(I)-neocuproine chelate is formed due to the redox reaction between copper-neocuproine and antioxidants, which can be measured via a spectrophotometer. 20 μl of extracts diluted ten times were added to 780 μl of distilled water, 400 μl of 1 M ammonium acetate (pH = 7), 400 μl of 0.01 M $CuCl_2$ and 400 μl of 0.0075 M neocuproine. Obtained mixtures were placed in a dark place for 30 min and measured in triplicate at a wavelength of 450 nm using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Results were calculated based on the gallic acid calibration curve in the concentration range of 0–0.25 mg/ml (R = 0.9992). CUPRAC results were shown as grams per 100 g of dry material weight (DW).

FRAP (Ferric Reducing Antioxidant Power) measures the ability to reduce iron (III) ions [44]. This method is based on the spectrophotometric measurement of the reduction of the Fe (III)-TPTZ complex (iron-2,4,6-tripyridyl-S thiazine complex) to the Fe (II)-TPTZ complex under

the influence of antioxidants. Each extract was diluted ten times, taken out (20 μl) and added to 1.98 ml of a mixture of 0.3 M acetate buffer (pH = 3.6), 0.02 M FeCl3 and 0.01 M TPTZ solution in 0.04 M HCl in ratio 10:1:1, respectively. Samples were incubated in a dark place for 15 min. As a result of the reaction, the colourless reagent began to show an intense blue colour measured spectrophotometrically at a wavelength of 593 nm (UV-1800, Shimadzu, Kyoto, Japan). Three measurements were made for each type of extract and calculated based on Trolox® using the standard curve equation in the concentration range of 0–0.25 mg/ml (R = 0.9991). FRAP was expressed as gram per 100 g of dry material weight (DW).

DPPH Radical Scavenging Assay (RSA) was determined using the Brand-Williams method [45] with some modifications [46,47]. Extracts' free radical scavenging activity was analysed by adding 20 μl of each extract to 1.58 ml of ethanol and 400 μl of 300 μM DPPH. Samples were incubated in a dark place for 15 min until the discolouration occurred. A control sample served a DPPH solution in ethanol without adding extracts. After incubation, the absorbance was measured spectrophotometrically at a wavelength of 517 nm (UV-1800, Shimadzu, Kyoto, Japan). All of the measurements were replicated three times. The antiradical activity was calculated using the following formula:

$$RSA = (A_{DPPH} - A_{extract})/A_{DPPH} \cdot 100 \quad (1)$$

where A_{DPPH} is the average absorbance of the DPPH solution without adding extracts, and $A_{extract}$ is the average absorbance of the DPPH solution after the addition of tested extracts.

2.4. Microparticles preparation

Microparticles (MPs) were fabricated via the extrusion method using an encapsulator (B-395 Pro, BÜCHI Labortechnik AG, Flawil, Switzerland) [48]. In order to obtain chitosan microparticles, chitosan in a concentration of 2 % (w/w) was dissolved in a solution of 2 % (v/v) acetic acid and selected aqueous extracts in a 50:50 ratio. Based on the phytochemical profile, the following aqueous extracts were encapsulated: (I) herbs of *Viola tricolor* + *Veronica officinalis*; (II) herbs of *Glechoma hederacea* + *Plantago lanceolata* + flowers of *Achillea millefolium* + *Tilia cordata* + rhizome of *Potentilla erecta*. The mixture was transferred into the pressure bottle of an encapsulator and forced through a nozzle with a 450 μm diameter. Droplets were separated by an electrical field and shaped by cross-linking in the bath containing 8 % (w/w) pentasodium tripolyphosphate (TPP) solution. Collected microparticles were rinsed with distilled water.

2.5. Microparticles Characterization

2.5.1. Morphology and size

The appearance and sizes of the prepared microparticles were observed by the optical microscope Motic SMZ-171 BLED (Hong Kong, China).

2.5.2. Loading capacity

The loading capacity (LC) was determined by quantifying the polyphenol content using the Folin–Ciocalteu test [41]. Weighed extract-loaded microparticles were put into 2 ml of 2 % (v/v) acetic acid for 1 h, and afterwards, they were centrifuged (10,000 rpm, 10 min). The procedure of preparing samples for TPC was reused to evaluate the loading capacity of extracts in microparticles. The absorbance was measured at 725 nm using a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The analyses were performed in triplicate. The presented results were calculated based on the calibration curve for gallic acid as the standard solution and using the following equation [49]:

$$LC = \text{phenolic concentration}\,(\text{initial} - \text{supernatant})/\text{microparticle weigh} \cdot 100 \quad (2)$$

2.5.3. In vitro release profile

The microparticles containing plant extracts were weighted (in triplicate), placed in a 12-well polystyrene plate and poured with 2 ml of acetate buffer (pH = 5.4). They were incubated at 37 °C for 3 days while the solution was collected after 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 5 h, 24 h, 48 h, 72 h. The content of polyphenolic compounds was determined by the Folin–Ciocalteu test [41]. The procedure of preparing samples for the loading capacity described above was reused for the *in vitro* release study. The absorbance was measured at 725 nm using a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The release of extracts from the chitosan microparticles was conducted in triplicate and calculated based on their loading capacity results.

2.6. Dermatological preparations

Herbal dermatological preparations were formulated as an emulsion (cream) and hydrogel (Table 2). The cream consisted of two phases (oily and aqueous). The oil phase was composed of 7 % of cetearyl alcohol, 6 % of caprylic/capric triglicerydes, 5 % of paraffinum liquidum, 5 % of *Vitis vinifera* seed oil, 2 % of *Prunus domestica* seed oil, 1.5 % of octyldodecanol, 1.1 % of ceteareth-20, 1 % of isopropyl palmitate, 0.9 % of glyceryl stearate. However, the aqueous phase of the cream contained 3 % of glycerin, 2 % of propylene glycol, 1.5 % of allantoin, 1.5 % of panthenol, and water up to 100 %. In order to prepare the cream, both phases were dissolved and heated to 75–85 °C and then combined and mixed with a mechanical stirrer until the formulation cooled down. After cooling down, 0.5 % of tocopherol was added to the formulation to prevent its thermal degradation.

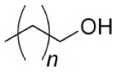
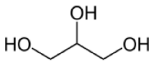
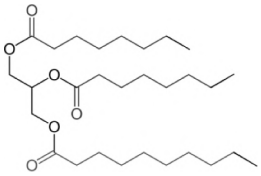
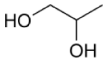
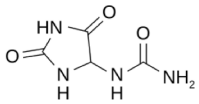
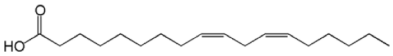
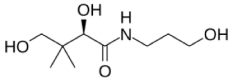
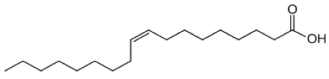
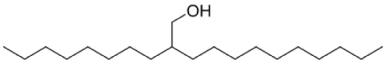
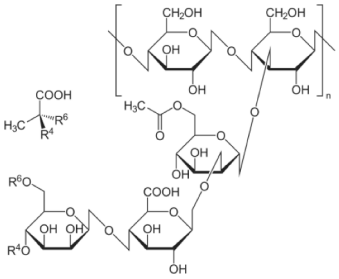
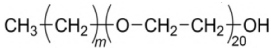
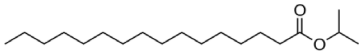
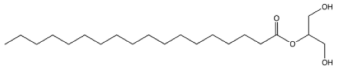
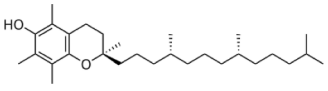
Whereas the hydrogel was composed of 3 % of glycerin, 2 % of propylene glycol, 1.5 % of allantoin, 1.5 % of panthenol, and 1 % of xanthan gum. The components of hydrogel were dissolved in water at room temperature.

Besides the base cream and hydrogel, the preparations containing 5 % of aqueous plant extracts and 5 % of extract-loaded microparticles were also obtained. They were prepared by the same method as base formulations, except the extracts were added to the mixtures at low temperatures during mechanical stirring along with tocopherol, whereas the microparticles were added to prepared formulations and gently mixed.

2.7. Biophysical skin parameters measurements

Courage + Khazaka probes using MPA software were used to perform preliminary studies of the analysis of the biophysical skin parameters after the application of prepared phytoformulations containing plant extracts [50]. Skin barrier quality (manifested as transepidermal water loss—TEWL), skin surface hydration and skin colour were examined using the tewameter (Tewameter TM 300, Courage + Khazaka, Köln, Germany), corneometer (Corneometer CM 825, Courage + Khazaka, Köln, Germany) and colourimeter (Skin-Colorimeter CL 400, Courage +

Table 2
The composition of prepared cream and hydrogel with chemical structures of added ingrediends.

Cream		Hydrogel
Oil Phase	**Aqueous Phase**	
7 % of Cetearyl Alcohol 	3 % of Glycerin 	3 % of Glycerin
6 % of Caprylic/ Capric Triglycerides 	2 % of Propylene Glycol 	2 % of Propylene Glycol
5 % of Paraffinum Liquidum 	1.5 % of Allantoin 	1.5 % of Allantoin
5 % of Vitis Vinifera Seed Oil 	1.5 % of Panthenol 	1.5 % of Panthenol
2 % of Prunus Domestica Seed Oil 	up to 100 % Aqua	1 % of Xanthan Gum 
1.5 % of Octyldodecanol 		
1.1 % of Ceteareth-20 		
1 % of Isopropyl Palmitate 		up to 100 % Aqua
0.9 % of Glyceryl Stearate 		
0.5 % of Tocopherol 		
	The addition of:	
+ 5 % of extracts +5% of microparticles	+ 5 % of extracts +5% of microparticles	

Khazaka, Köln, Germany), respectively.

Five probands with normal skin (women aged 20–29) participated in the study. Four 4 × 4 cm sections were designated on the volar forearm skin of both arms (Fig. 2). One section served as the control field, and the remaining seven places were covered with different formulations – creams and hydrogels containing plant extracts, plant extract-loaded microparticles, as well as base preparations without extracts. The measurements were performed 30 min, 1 h, 2 h, 3 h and 4 h after applying samples. This test took place in a controlled temperature (20–22 °C) and humidity (relative humidity 40–60 %).

Before the start of measurements (control) and at each time point, TEWL and skin colour were evaluated in triplicate for each proband. However, corneometric tests require simultaneous testing on the control and treated areas at each point in time. These results show the difference

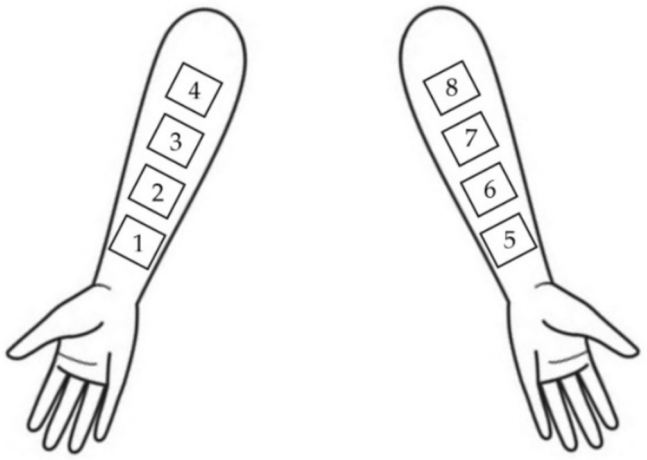


Fig. 2. Four application sites (4 x 4 cm) designed on the volar forearm of each arm (1 to 8, including one site serving as a control field for corneometric measurements).

in the corneometer indications (performed five times for each proband at each skin site) between the test field and control field at the appropriate points.

2.8. Statistical analysis

One-way ANOVA with Tukey's pairwise was performed to statistically compare results. The outcome of the extracts' phytochemical studies (TPC, TFC, CUPRAC, FRAP and DPPH RSA) were compared across all samples extracted with different solvents. The results of skin condition assays (colour and TEWL) for cosmetics were compared to those made for the control field (before the start of the study). Meanwhile, the results of skin hydration were compared to the control samples without herbal preparations. Past 4.09 (PAleontological Statistics Software, Oslo, Norway) was used for all analyses. Data are shown as the mean ± S.D. for each experiment. p-values $\leq$ 0.05 were considered significant.

3. Results and Discussion

3.1. Extracts preparation and characterisation

Fig. 3 presents extracts made from Polish herbal plants showing documented healing effects, including herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta* using Soxhlet apparatus with water and ethanol as solvents. Phytochemical characterisation of prepared extracts included the content of polyphenols (TPC), flavonoids (TFC) and antioxidant activity (CUPRAC, FRAP and DPPH) (Table 3).

Based on obtained results, we can notice that content of polyphenols, flavonoids, and antioxidant activity significantly depended on the type of used plant, as well as the type of used solvent (Table 3). Aqueous and ethanol extracts from *Viola tricolor* showed relatively low content of polyphenols, i.e. 0.70 ± 0.06 g GAE/100 g DW and 0.89 ± 0.18 g GAE/100 g DW, respectively, as well as low antioxidant activity (DPPH was 16.3 ± 4.0 % and 8.6 ± 0.1 %, respectively). However, the results were inconsistent with those noted in other studies. It can be associated with different extraction methods or solvents and calculations based on different equivalents. Jurca et al. screened three species of the *Violaceae family* (*Viola odorata* L., *Viola tricolor* L. and *Viola wittrockiana* Gams.) in order to assess their polyphenolic contents and antioxidant activities [51]. They extracted flowers with 70 % ethanol using a magnetic stirrer and sonicator, which resulted in TPC of 445 mg GAE/100 g DW, TFC of 2.69 mg QE/ml and antioxidant activity: CUPRAC 2.68 mmol Trolox/100 g, FRAP 14.7 g mmol Trolox/ml and DPPH 74 %. They suggested that anthocyanins and phenols presented in *Viola* species flowers play an essential role as antioxidants. Therefore, they can be used as a source of natural antioxidants in pharmaceutical compounds, food processing and human and food medicine. Another study performed by Araújo et al. revealed antioxidant activity, phenolic and flavonoid contents of ethanol extracts of selected edible flowers, including *Viola tricolor* [52]. Extract from *Viola tricolor* had higher polyphenols and flavonoid contents than other studied plants, namely 63.43 mg GAE/g DW and 32.84 mg catechin equivalents (CE)/g DW, respectively.

Veronica officinalis herb extracts prepared with water displayed the highest level of TPC (5.75 ± 0.24 g GAE/100 g DW) and FRAP (6.80 ± 0.43 g/100 g DW), and TFC (639.7 ± 27.4 mg QE/100 g DW) lower only from *Sambucus nigra* extract. The ethanol Vo extract also had a high content of active compounds. TPC (4.37 ± 0.23 g GAE/100 g DW), CUPRAC (3.71 ± 0.10 g/100 g DW) and FRAP (4.95 ± 0.39 g/100 g DW) had high values, lower only from ethanol extract from *Potentilla erecta* and TFC (979.8 ± 4.5 mg QE/100 g DW) lower from ethanol extract from *Plantago lanceolata*. Mocan et al. prepared ethanol extract of three *Veronica* species, including *Veronica officinalis*, in an ultrasonic bath and investigated their chemical composition, antioxidant and antimicrobial effects [12]. Their *Veronica officinalis* extract presented lower than ours TPC values of 32.37 ± 1.27 mg GAE/g DW and TFC (2.52 ± 0.16 mg QE/g DW).

Comparably low content of polyphenols and flavonoids, as well as antioxidant activity, were observed for extracts from *Glechoma hederacea* using both water and ethanol as solvents. TPC were 1.61 ± 0.12 and 1.12 ± 0.09 g GAE/100 g DW, TFC were 376.2 ± 24.8 and 848.5 ± 3.5 mg QE/100 g DW, CUPRAC were 1.74 ± 0.20 and 1.09 ± 0.07 g/100 g DW, FRAP were 2.99 ± 0.37 and 1.85 ± 0.17 g/100 g DW, DPPH RSA were 68.1 ± 5.0 % and 31.3 ± 1.5 %, respectively for aqueous and ethanol extracts. Our findings were not supported by other research performed by Belščak-Cvitanović et al. [53]. They assessed the aqueous

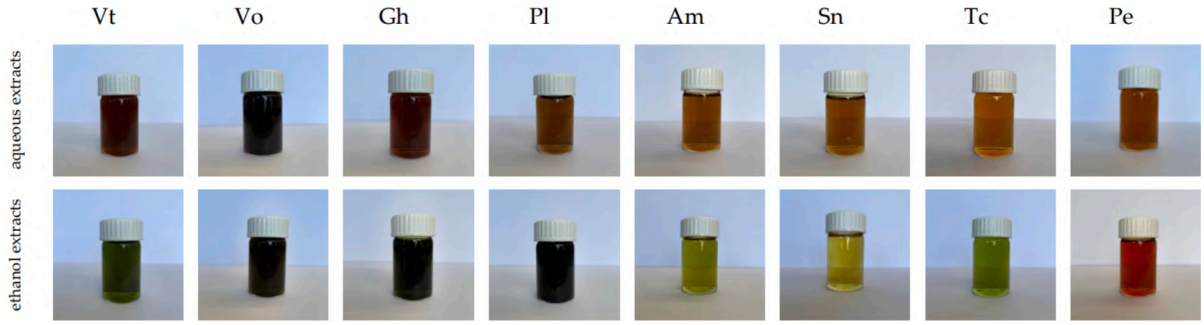


Fig. 3. Photographs of prepared aqueous and ethanol extracts from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta*.

Table 3
Results of phytochemical studies – the content of polyphenols, flavonoids and antioxidant activity (CUPRAC, FRAP, DPPH RSA) – of aqueous and ethanol extracts made from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta*, as well as their mixtures. Different superscript letters in columns presenting the results of different methods for both aqueous and ethanol extracts indicate statistically significant differences ($p \leq 0.05$).

extracts	TPC (g GAE/100 g DW)		TFC (mg QE/100 g DW)		CUPRAC (g/100 g DW)		FRAP (g/100 g DW)		DPPH RSA (%)	
	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol
Vt	0.70 ± 0.06 ^{l}	0.89 ± 0.18 ^{l}	512.2 ± 35.5 ^{i}	651.2 ± 6.7 ^{g,h}	1.17 ± 0.16^{k,l,m}	0.92 ± 0.07^{m}	2.47 ± 0.45^{f,g,h}	1.91 ± 0.08^{h}	16.3 ± 4.0^{f}	8.6 ± 0.1^{f}
Vo	5.75 ± 0.24^{b}	4.37 ± 0.23^{c}	639.7 ± 27.4 ^{g,h}	979.8 ± 4.5^{c}	5.93 ± 0.18^{b}	3.71 ± 0.10^{c}	6.80 ± 0.43^{b}	4.95 ± 0.39^{c}	73.6 ± 1.8 ^{a,b}	77.6 ± 3.2 ^{a,b}
Gh	1.61 ± 0.12 ^{j,k}	1.12 ± 0.09 ^{k,l}	376.2 ± 24.8 ^{j}	848.5 ± 3.5 ^{e}	1.74 ± 0.20^{h,i,j,k}	1.09 ± 0.07^{l,} ^{m}	2.99 ± 0.37 ^{e,f}	1.85 ± 0.17^{h}	68.1 ± 5.0^{b}	31.3 ± 1.5 ^{e}
Pl	1.33 ± 0.10 ^{k,l}	2.24 ± 0.19 ^{h,i,j}	239.2 ± 22.6 ^{k}	1456.5 ± 10.7 ^{a}	1.63 ± 0.32 ^{i,j,k,l}	2.04 ± 0.04^{g,} ^{h,i}	2.44 ± 0.23^{f,g,h}	2.57 ± 0.15^{f,g,h}	35.4 ± 3.4 ^{e}	72.2 ± 2.4 ^{a,b}
Am	3.15 ± 0.04 ^{d,e,f}	1.09 ± 0.06 ^{k,l}	409.2 ± 19.2 ^{j}	430.4 ± 10.1 ^{j}	3.24 ± 0.27^{c,d}	1.27 ± 0.10 ^{j,} ^{k,l,m}	4.45 ± 0.09^{c}	2.19 ± 0.10^{g,h}	78.7 ± 1.7 ^{a}	73.6 ± 2.0 ^{a,b}
Sn	5.35 ± 0.13^{b}	2.77 ± 0.18^{f,g,h}	1378.3 ± 34.4^{b}	916.9 ± 8.8 ^{d}	6.11 ± 0.23^{b}	2.28 ± 0.17^{f,} ^{g,h}	6.10 ± 0.26^{b}	3.04 ± 0.17 ^{e,f}	76.3 ± 3.1 ^{a,b}	75.0 ± 3.6 ^{a,b}
Tc	3.30 ± 0.03 ^{d,e,f}	2.11 ± 0.30 ^{i,j}	388.7 ± 9.7 ^{j}	597.0 ± 12.8^{h}	3.86 ± 0.24^{c}	1.46 ± 0.12 ^{i,} ^{j,k,l,m}	4.46 ± 0.07^{c}	2.81 ± 0.25^{f,g}	78.3 ± 2.4 ^{a,b}	77.3 ± 5.2 ^{a,b}
Pe	2.38 ± 0.01 ^{g,h,i}	9.41 ± 0.48 ^{a}	136.6 ± 20.7^{l}	205.0 ± 1.5^{k}	2.85 ± 0.11 ^{d,e,f}	7.31 ± 0.35 ^{a}	4.26 ± 0.21^{c,d}	8.29 ± 0.29 ^{a}	78.6 ± 4.2 ^{a}	71.0 ± 2.8 ^{a,b}
Mixed extracts										
Vt + Vo	3.58 ± 0.24 ^{d,e}	2.97 ± 0.18 ^{e,f,g}	714.1 ± 32.1^{f}	610.6 ± 38.3^{h}	2.93 ± 0.13 ^{d,e}	2.73 ± 0.19 ^{d,e,f}	3.64 ± 0.11 ^{d,e}	4.54 ± 0.40^{c}	47.5 ± 2.3 ^{d}	50.2 ± 3.1^{c,d}
Gh + Pl + Am + Tc + Pe	0.85 ± 0.01 ^{l}	3.69 ± 0.45 ^{d}	221.2 ± 12.8^{k}	675.1 ± 17.3^{f,g}	1.83 ± 0.36^{h,i,j}	2.56 ± 0.16 ^{e,f,g}	2.63 ± 0.10^{f,g,h}	4.75 ± 0.40^{c}	48.7 ± 2.5^{c,d}	57.6 ± 4.0^{c}

TPC – Total Polyphenols Content; TFC – Total Flavonoids Content; CUPRAC – CUPric Reducing Antioxidant Capacity; FRAP – Ferric Reducing Antioxidant Power; DPPH RSA – DPPH Radical Scavenging Assay.

extracts, infusions, macerates and decoctions from several Croatian plants, including *Glechoma hederacea*, against the damaging effects of oxidative stress. They noticed significant differences in polyphenols and flavonoids content related to the used extraction method. Infusions were prepared with warm water (80 °C) for a short time (10 min), macerates were performed with cold water for a more extended period (72 h), whereas decoctions were done with boiling water for 20 min. TPC of *Glechoma hederacea* were in a range of 12.45 – 31.82 mg GAE/g DW with a significantly lower value for decoction, which indicated that boiling water may led to phenolic compounds' degradation. TFC ranged from 5.98 mg GAE/g DW for decoction to 20.00 mg GAE/g DW for macerate. Furthermore, FRAP valued from 1.15 mM Fe(II) for macerate to 7.43 mM Fe(II) for extract.

Plantago lanceolata aqueous extract showed a relatively low amount of active substances, i.e. polyphenols content was 1.33 ± 0.10 g GAE/100 g DW, and flavonoids content was 239.2 ± 22.6 mg QE/100 g DW. Their content resulted in antioxidant activity detected via FRAP 2.44 ± 0.23 g/100 g DW, CUPRAC 1.63 ± 0.32 g/100 g DW and DPPH 35.4 ± 3.4 %. Moderately higher values of phytochemical analysis were observed for ethanol extracts. TPC was 2.24 ± 0.19 g GAE/100 g DW, whereas TFC had the highest value of all prepared extracts (1456.5 ± 10.7 mg QE/100 g DW). FRAP was similar to the value read for aqueous extract, i.e. 2.57 ± 0.15 g/100 g DW. However, the CUPRAC value was moderate compared to prepared extracts (2.04 ± 0.04 g/100 g DW). DPPH RSA value was similar to other extracts – 72.2 ± 2.4 %. *Plantago lanceolata* has also been studied by Lukova et al. during the simultaneous extraction of polyphenols and degradation of polysaccharides [54]. TPC after enzymatic hydrolysis was around 40 mg GAE/g DW and DPPH ~ 70 %. They also established its CUPRAC (56 μM Trolox® equivalents (TE)/g DW) and FRAP (92 μM TE/g DW). Jelena Živković et al. obtained *Plantago lanceolata* ethanol extracts using an ultrasonic bath at various temperatures (20–80 °C) for different periods (5–65 min) [55]. TPC of investigated extracts varied between 24.34 and 42.96 mg GAE/g DW. Moreover, they suggested the following conditions as optimal: extraction time of 64 min, ethanol concentration of 45 %, solid-to-solvent ratio of 1:49 and extraction temperature of 40 °C.

Aqueous extract prepared from *Achillea millefolium* presented moderate values of TPC (3.15 ± 0.04 g GAE/100 g DW), TFC (409.2 ± 19.2 mg QE/100 g DW), CUPRAC (3.24 ± 0.27 g/100 g DW), and FRAP (4.45 ± 0.09 g/100 g DW), whereas the highest value of DPPH (78.7 ± 1.7 %). Ethanol appeared to be worse solvent for extraction of *Achillea millefolium* flowers due to relatively low content of extracted polyphenols (1.09 ± 0.06 g GAE/100 g DW) and flavonoids (430.4 ± 10.1 mg QE/100 g DW), and hence antioxidant activity: CUPRAC (1.27 ± 0.10 g/100 g DW) and FRAP (2.19 ± 0.10 g/100 g DW). Conversely, DPPH of ethanol extract was high – 73.6 ± 2.0 %. Horablaga et al. highlighted the importance of sample preparation and extraction methods on the phytochemical profile of 12 common medicinal plants in Romania, including *Achillea millefolium* [56]. TPC of *Achillea millefolium* extract varied between 14.53 and 52.85 mg GAE/g DW, whereas TFC ranged from 2.50 to 4.27 mg QE/g DW. Antioxidant activity expressed as FRAP were from 6.20 to 43.96 mM Fe^{2+}/100 g. Results depended on the level of plant shredding and extraction method (conventional solvent extraction, ultrasound-assisted extraction and microwave extraction). They noted that values for TPC and antioxidant activity increased significantly with the level of plant shredding (from coarse shredding to fine gridding).

Extracts prepared from *Sambucus nigra* appeared to have a satisfactory level of bioactive compounds compared to other plant extracts. Both water and ethanol were suitable solvents for extraction since their TPC were 5.35 ± 0.13 and 2.77 ± 0.18 g GAE/100 g DW, TFC were 1378.3 ± 34.4 and 916.9 ± 8.8 mg QE/100 g DW, respectively. Antioxidant capacity measured by the CUPRAC method were 6.11 ± 0.23 and 2.28 ± 0.17 g/100 g DW, and by the FRAP method were 6.10 ± 0.26 and 3.04 ± 0.17, while by DPPA RSA were 76.3 ± 3.1 and 75.0 ± 3.6 %, respectively for aqueous and ethanol extracts. *Sambucus nigra* was also noticed by other researchers for its beneficial phytochemical characterisation. A study presented by Boroduske et al. compared the phytochemical profile of flower and berries ethanol extracts from cultivated and wild *Sambucus nigra* populations [57]. The highest average TFC of sampled wild *Sambucus nigra* was 9.57 mg rutin equivalents (RE)/g DW for berry extracts and 77.59 mg RE/g DW for flower extracts. Moreover, the highest TPC were 41.31 mg GAE/g DW for berry extracts and 451.72 mg GAE/g DW for flower extracts. Their results indicated that the

sampling site significantly affected the phytochemical profile of elderberry fruits and flowers. Another study performed by Viapiana et al. reported slightly lower values for TPC and TFC of *Sambucus nigra* flowers and barriers aqueous infusions [58]. The TPC of berries and flower infusions ranged from 19.81 to 23.90 mg GAE/g DW and from 15.23 to 35.57 mg GAE/g DW, respectively. Meanwhile, the TFC of infusions ranged from 2.60 to 4.49 mg RE/g DW in elderberry and from 5.27 to 13.19 mg RUTE/g DW in elderflower. Furthermore, they assessed the antioxidant potential of the *Sambucus nigra* infusions via DPPH and FRAP assays. It revealed that the infusions prepared from flowers had higher values of DPPH and FRAP, which correlated with higher TPC and TFC. Another study reported a TPC value of 43 mg GAE/g DW of methanol elderberry fruit extract, a TFC value of 15 mg RE/g DW, and a DPPH of 62.56 % [59].

Both aqueous and ethanol *Tilia cordata* extracts had moderate phytochemical profiles compared to other extracts, i.e. TPC were 3.30 ± 0.03 and 2.11 ± 0.30 g GAE/100 g DW, and TFC were 388.7 ± 9.7 and 597.0 ± 12.8 mg QE/100 g DW, respectively. Antioxidant activity evaluated by CUPRAC were 3.86 ± 0.24 and 1.46 ± 0.12 g/100 g DW, whereas FRAP were 4.46 ± 0.07 and 2.81 ± 0.25 g/100 g DW, respectively for aqueous and ethanol extracts. On the contrary, these extracts had high antioxidant activity measured via DPPH RSA (~77–78 %). A study presented by Kosakowska et al. compared the content of phenolic acids and flavonoids in extracts from *Tilia cordata* flowers collected from different sites in Poland [20]. The mean phenolic acid content was 0.96 g/100 g DW (it varied from 0.17 to 1.87 g/100 g DW), whereas TFC – 0.20 g/100 g DW (from 0.09 to 0.52 g/100 g DW). The phytochemical composition differed significantly depending on the site of small-lived lime populations; however, they did not observe a clear relationship between geographical localisation and the content of active compounds in flowers.

Potentilla erecta ethanol extract had the highest polyphenols content of all prepared extracts (9.41 ± 0.48 g GAE/100 g DW) and antioxidant properties (CUPRAC: 7.31 ± 0.35 g/100 g DW; FRAP: 8.29 ± 0.29 g/100 g DW). Extracts prepared using both water and ethanol as solvents had a high value of DPPH RSA (~71–79 %). Based on TPC (2.38 ± 0.01 g GAE/100 g DW), TFC (136.6 ± 20.7 mg QE/100 g DW) and CUPRAC (2.85 ± 0.11 g/100 g DW) results, we can conclude that water was worse solvent for extraction of *Potentilla erecta.* Furthermore, it contained the lowest content of flavonoids regardless of the extraction solvent type. Dróżdż et al. reported that the content of polyphenolics decreases with increasing concentration of alcohol above 60 % [60]. They prepared *Potentilla erecta* rhizome extracts using water and ethanol–water (60:40) as solvents due to the higher solubility of active compounds in an alcoholic environment. TPC was higher when extracted with 60 % ethanol (111 ± 2.8 mg GA/g) than hot water (74.2 ± 1.90 mg GA/g). Another study performed by Tomczyk et al. revealed that aqueous extract from selected *Potentilla* species, including *Potentilla erecta* herb, had high concentrations of polyphenols such as tannins and phenolic acids, as well as flavonoids (TPC 69.3 mg GAE/g DW and TFC 2.5 mg QE/g DW) [61].

According to these findings, it can be concluded that ethanol is the preferable extraction medium for flavonoids (except *Sambucus nigra* flowers). More polyphenols were extracted with water from *Veronica officinalis, Glechoma hederacea, Achillea millefolium, Sambucus nigra* and *Tilia cordata,* whereas ethanol extracted more polyphenols from *Plantago lanceolata* and *Potentilla erecta*. In the case of antioxidant activity measured by CUPRAC and FRAP methods, more preferable was water for *Veronica officinalis, Glechoma hederacea, Achillea millefolium, Sambucus nigra, Tilia cordata*, while ethanol for *Potentilla erecta.* Higher values for DPPH RSA presented water for *Viola tricolor, Veronica officinalis, Glechoma hederacea, and Achillea millefolium*, while ethanol for *Plantago lanceolata*. Therefore, ethanol turned out to be the preferable solvent for extracting active compounds from *Plantago lanceolata* and *Potentilla erecta*, whereas water – for the rest of the plants.

In this study, we also obtained extracts by mixing plants showing different content of active compounds. Mixing extracts with a high phytochemical content separately led to obtaining new extracts with moderate TPC, TFC and antioxidant activity. Based on the outcome, the obtained multicomponent extracts showed an additive effect. Therefore, in the next steps of this research, extracts from (I) the *Sambucus nigra* flowers; and mixtures from (II) herbs of *Viola tricolor* + *Veronica officinalis*; and (III) herbs *Glechoma hederacea* + *Plantago lanceolata* and flowers of *Achillea millefolium* + *Tilia cordata* and rhizome of *Potentilla erecta* were used.

Other researchers also searched for a synergistic effect of a wide range of bioactives responsible for antioxidant capacity and, hence, potential health benefits. Maleš et al. characterised the antioxidant activity of sage (Salvia officinalisL.), wild thyme (Thymus serpyllumL.) and laurel (Laurus nobilisL.) aqueous extracts and their two- and three-component mixtures [62]. They revealed that individual plant extracts possessed a high content of polyphenolic compounds. However, most two- and three-component extracts have been shown to have additive effects. Moreover, three-component mixtures contained lower TPC than two-component mixtures. Other researchers also did not find synergistic effect of ethanol and water extracts prepared from twelve plants and their combination (*Rosa damascena*, *Viola odorata*, *Matricaria chamomilla*, *Althaaea officinalis*, *Melissa officinalis*, *Elaeagnus angustifolia*, *Foeniculum vulgare*, *Pimpinella anisum*, *Saccharum officinarum*, *Prunus dulcis*, and *Zea mays*) [63]. Manham et al. noticed no additional effect of herbal blends over individual plants – their results for TPC and antioxidant activity fell in the mid-range. It is tough to find a synergistic effect of polyherbal blends. Therefore, it is not always best to have numerous extracts in products intended for skin than one carefully selected.

3.2. Microparticles Characterization

Aqueous extracts from (I) *Sambucus nigra*; and mixtures from (II) *Viola tricolor* + *Veronica officinalis*; and (III) *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* were selected in order to be enclosed in chitosan microparticles due to the lack of potential irritating properties to the skin compared to ethanol. Fig. 4 shows the resulting extract-loaded microparticles. They had repeatable size, smooth surface and spherical shape, which minimised the possibility of skin deterioration after applying them to the skin. Fabricated microparticles had a soft polymeric matrix enabling the release of enclosed extracts during their spreading to the skin (mechanical release). The preparation of polymeric microparticles was also studied by other researchers who reported obtaining smooth and spherical chitosan microparticles that can be applied in cosmetics to alleviate skin irritation [64,65].

The formation of chitosan-based microparticles relied on ionic gelation, a widely used method for encapsulating bioactive compounds due to its mild processing conditions. Chitosan is a positively charged, naturally derived polysaccharide containing amino groups ($–NH_2$), which become protonated ($–NH_3^+$) in acidic environments (such as acetic acid). TPP is a multivalent anionic cross-linker composed of phosphate groups linked by oxygen bridges. When chitosan droplets came into contact with the TPP solution, ionic interactions occurred between the protonated amino groups of chitosan and the negatively charged phosphate groups of TPP [66–68]. This led to the formation of a cross-linked network at the droplet surface, stabilizing the structure into spherical microparticles (Fig. 5). Furthermore, the hydroxyl groups (–OH) of the chitosan's glucosamine units might stabilized and enhanced the integrity of the network through hydrogen bonding with nearby chitosan chains or oxygen atoms in phosphate groups (non-bridging $–O^-$ or =O) of TPP [69]. The encapsulated bioactive compounds were thereby entrapped within the polymer matrix. Prepared plant extracts – rich in phenolic compounds, flavonoids, organic acids, and other bioactive constituents – may interact with chitosan through hydrogen bonding and electrostatic interactions [70,71]. Such interactions could partially compete with TPP for the protonated amino groups ($–NH_3^+$) of chitosan,

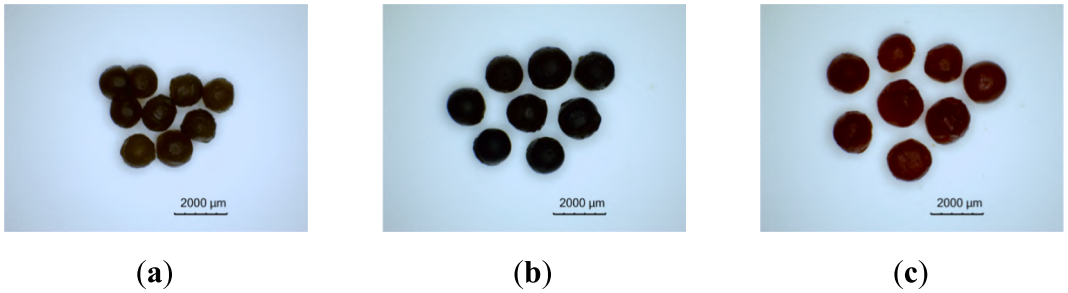


Fig. 4. Chitosan microparticles with enclosed aqueous extracts from: (**a**) *Sambucus nigra*; (**b**) *Viola tricolor* + *Veronica officinalis*; (**c**) *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta*.

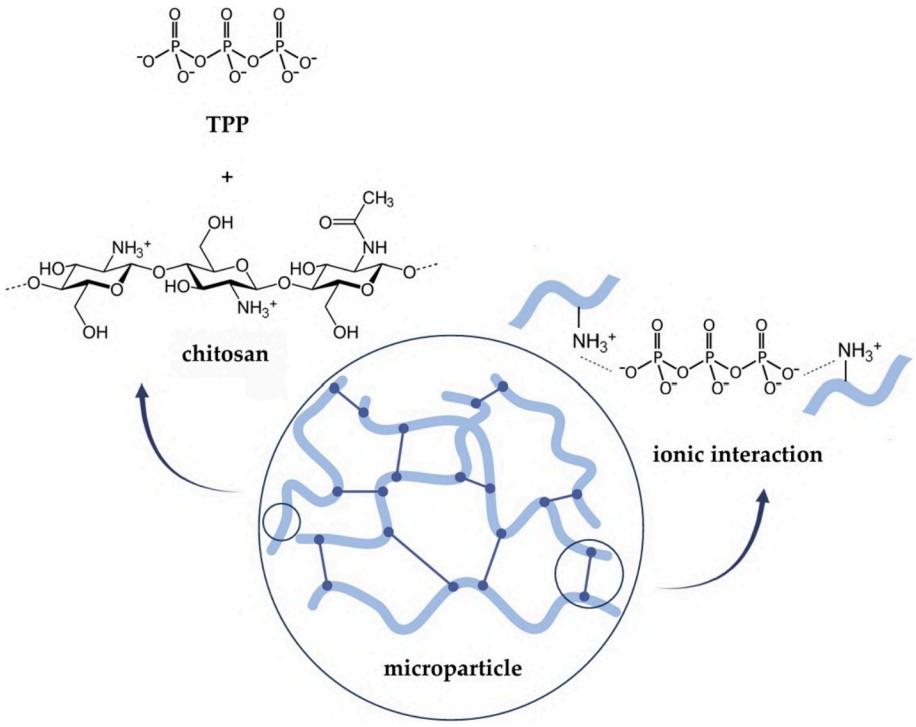


Fig. 5. Scheme illustrating the formation of microparticles through cross-linking of chitosan with TPP.

potentially altering the structure of the polymeric matrix.

We determined the loading capacity of aqueous extracts enclosed in microparticles. The highest loading capacity was noted for microparticles containing *Sambucus nigra* flower extract (71.4 ± 6.3 %). Significantly lower loading capacity was observed for microparticles with the extract from herbs of *Viola tricolor* and *Veronica officinalis* (55.4 ± 2.3 %), whereas for microparticles containing Gh + Pl + Am + Tc + Pe extract loading capacity was the lowest – 13.7 ± 0.7 %. Based on these

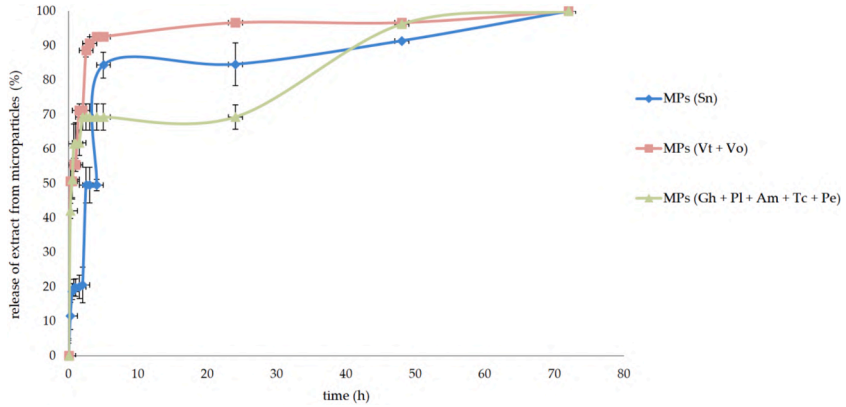


Fig. 6. *In vitro* release profile of aqueous *Sambucus nigra* (Sn); *Viola tricolor* + *Veronica officinalis* (Vt + Vo); *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* (Gh + Pl + Am + Tc + Pe) extracts from chitosan microparticles.

results, it can be concluded that plant extracts have been successfully enclosed in chitosan microparticles. The loading capacity of plant extracts in chitosan microparticles is a subject of numerous studies. Other researchers reported loading capacity on different levels: 4.9 % for red ginger oleoresin extract [49]; ~13.8–20.7 % for pomegranate peel extract [72]; ~70–90 % for extracts prepared from L. cuneifolia, L. divaricata, L. nitida, Z. punctata and T. andina [73].

In vitro release profiles of extracts from microparticles in acetate buffer (pH = 5.4, 37 °C) were also established (Fig. 6). We observed that the extracts were released from the microparticles within three days and occurred in two main stages. The first one lasted 5 h, and during that time, approximately 70–90 % of loaded plant extracts were released from the microparticles with a relatively high release rate. This initial burst release is frequently linked to the presence of active compounds from the outer layer of microparticles, which allows a quick release during the swelling of polymeric microparticles in the release medium [74]. Dissolving the extract in the acidic solution during the preparation of microparticles could also facilitate the initial burst release owing to the better accessibility of some polyphenols in the acidic release medium. In contrast, a sustained release rate characterised the second stage, where bioactive substances must diffuse through the polymeric network. The plant extracts within the chitosan matrix could stabilise formed microparticles, delaying the disintegration of the polysaccharide [75]. The intertwining of polysaccharide chains with the polyphenol molecules and, thus, stabilisation of this system has been attributed to physical interactions, as well as covalent and hydrogen bonding between both components [76–78]. The release profile was studied in static conditions; however, a mechanical mechanism would apply during the spreading of microparticles to the skin (disruption of chitosan matrix), which fastens the release rate of active substances.

These findings were in agreement with studies performed by other research groups. Moreno et al. obtained chitosan microparticles through electrospraying containing extracts from five medicinal plants (L. cuneifolia, L. divaricata, L. nitida, Z. punctata and T. andina) [73]. They also established these extracts' *in vitro* release profile (pH = 4.5, 37 °C), which had a similar release profile to ours. The rapid release of phenolic compounds occurred in the first 4–5 h, during which more than 80 % of extracts were released. Probably, it was accelerated by the hydration of chitosan microparticles in an aqueous medium. The initial burst release was followed by the constant release rate for the next 12 h. Nguyen et al. performed release study in pH 1.2, 4.5, 6.8 and 7.8 of carrageenan/chitosan/α–mangostin (xanthone derivative compound extracted from the pericarps of tropical fruit Mangosteen) microparticles [79]. The release depended on the pH of the release medium with better release in a more acidic solution, which was also found by other researchers [80]. They established that active substance was distributed on the surface and inside microparticles. Therefore, an initial burst was observed within the first 2 h of analysis, followed by a slow release rate due to the release of extracts linked with the polymer matrix. After around six hours, approximately 40–100 % of the active substance was released.

3.3. Dermatological preparations

Dermatological formulations in the form of creams and hydrogels containing free extracts and extract-loaded microparticles were obtained, as well as control samples without herbal preparations (Fig. 7). Obtained products were homogenous. Added extract influenced the colour of phytoformulations. Moreover, added microparticles were uniformly distributed through the emulsion and hydrogel. The topical spreading of phytoformulations with microparticles caused the mechanical release of enclosed extracts due to the disruption of soft polymeric matrices.

The structural network of the hydrogel was primarily established by xanthan gum – a polysaccharide composed of 1,4-β-d-glucose backbone with trisaccharide side chains (consisting of mannose–glucuronic acid–mannose) attached to every other glucose residue. Upon hydration, xanthan gum formed a stable three-dimensional matrix through the creation of double-helix structures stabilized by intermolecular interactions [81]. These helices arose from ordered regions within the polymer, where two xanthan chains aligned and twisted together, supported by hydrogen bonding between hydroxyl and carboxyl groups [82]. This helical organization might be accompanied by the physical entanglement of polymer chains and the formation of a gel network. The hydrogel structure was further reinforced by hydrogen bonds formed with water molecules and other hydrophilic components presented in the formulation, including polyols (glycerin, propylene glycol), panthenol, and allantoin [83]. Glycerin and propylene glycol functioned as humectants, enhancing water retention within the hydrogel by forming hydrogen bonds with water molecules and interacting with the hydroxyl groups of xanthan gum. Allantoin, as a humectant, could also participate in weak hydrogen bonding with hydroxyl groups present in the matrix, potentially aiding in the stabilization of the gel structure. Panthenol might interacted with the aqueous environment and the polymer matrix via hydrogen bonding and van der Waals interactions, contributing to the gel's moisturizing capacity and elasticity. Collectively, these components may interact synergistically to form a hydrogel network, capable of both retaining moisture and delivering bioactive compounds effectively across the skin barrier.

Whereas, oil-in-water (O/W) emulsions were formed through the action of glyceryl stearate and ceteareth-20, which served as non-ionic emulsifiers. These agents stabilized dispersed oil droplets by reducing interfacial tension and forming a coherent interfacial film at the

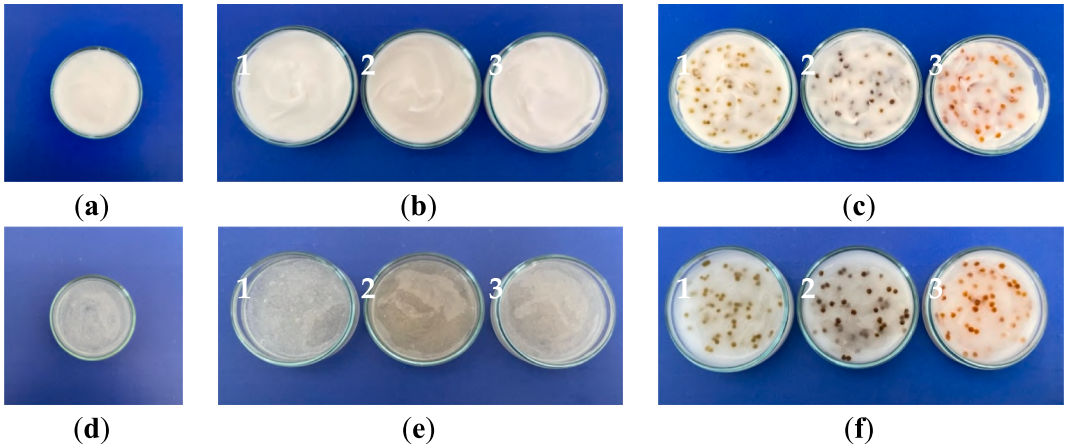


Fig. 7. The pictures of prepared base samples: (**a**) cream and (**d**) hydrogel, as well as creams with the addition of (**b**) extracts and (**c**) microparticles, and hydrogels with the addition of (**e**) extracts and (**f**) microparticles containing: (**1**) Sn; (**2**) Vt + Vo; (**3**) Gh + Pl + Am + Tc + Pe.

oil–water interface. Cetearyl alcohol functioned as both an emulsion stabilizer and a rheology modifier, contributing to the overall viscosity, consistency, and sensory profile of creams. Caprylic/capric triglycerides and isopropyl palmitate further acted as rheology modifiers, improving the spreadability, texture, and functional properties of the emulsion. Octyldodecanol contributed to emulsion stability and enhanced application properties by promoting uniform distribution on the skin. The aqueous phase, containing humectants such as glycerin, propylene glycol, panthenol, and allantoin, ensured moisture retention. Emulsion stabilization might be achieved through a combination of steric stabilization by non-ionic surfactants, hydrogen bonding within the aqueous matrix, and viscous structuring provided by fatty alcohols.

Phytochemicals present in plant extracts – such as flavonoids, phenolic acids, tannins, alkaloids, and polysaccharides – could significantly influence the physicochemical properties, structural integrity, and functional performance of polymer-based systems. These bioactive compounds may possess multiple functional groups (e.g., –OH, –COOH, $–NH_2$), which allows them to participate in non-covalent interactions with the polymer matrix, including hydrogen bonding with polymer chains (e.g., chitosan or xanthan gum), or electrostatic interactions with charged groups, such as the protonated amino groups ($–NH_3^+$) of chitosan. Therefore, the system's stability and functionality may also be influenced by the incorporation of plant-derived compounds.

Other researchers also investigated the possibility of using chitosan microparticles containing plant extract added to emulsions for cosmetic purposes. Mazutti et al. prepared formulations for topical treatment of cutaneous infections [84]. 5 % of chitosan microparticles loaded with *Eugenia dysenterica* leaves extract (EDA) were added to a stable emulsion containing Polawax® Wax, mineral oil, butylhydroxyethyloluene, EDTA, methylparaben, propylparaben, and glycerin. Their study confirmed the possibility of emulsion containing prepared extract encapsulated in chitosan microparticles as a suitable vehicle for topical administration of EDA for treating infections caused by *S. aureus*. Furthermore, Acosta et al. used chitosan microspheres to enclose olive leaf extract [85]. They prepared three o/w and w/o emulsions containing eucalyptus water, roses water, olive oil, calendula oil, jojoba oil, avocado oil, olivem® 1000, sodium stearoyl lactylate, beeswax, glycerol stearate, xanthan gum, shea butter, candy dye, chlorophyll dye, and 0.2 % of chitosan microspheres. Based on the extract's suitable *in vitro* release profile, they stated that these microspheres may be applied in cosmetic moisturisers.

3.4. Biophysical skin parameters

The biophysical skin parameters – skin colour, skin surface hydration and skin barrier quality – were examined using Courage + Khazaka probes. The results of preliminary studies of these analyses after application to the skin of herbal-based preparations are shown in Figs. 8–10.

Colour space L*a*b* coordinates express the values of skin colour measurements. Skin brightness is expressed as L*, whereas the location of measured values on the red-green axis is expressed as a* and on the blue-yellow axis – b*. Skin pigmentation is described by b* coordinate, while skin redness, microcirculation and erythema – by a*. Therefore, only a* parameter was considered in this study because prepared herbal dermatological products were not designed to affect the skin's brightness and pigmentation.

Preliminary to the application of products to the skin, control measurements were conducted. Samples were then applied and left to absorb to the skin. The colourimetric indications after applying dermatological preparations to the skin were compared to the control field (skin areas

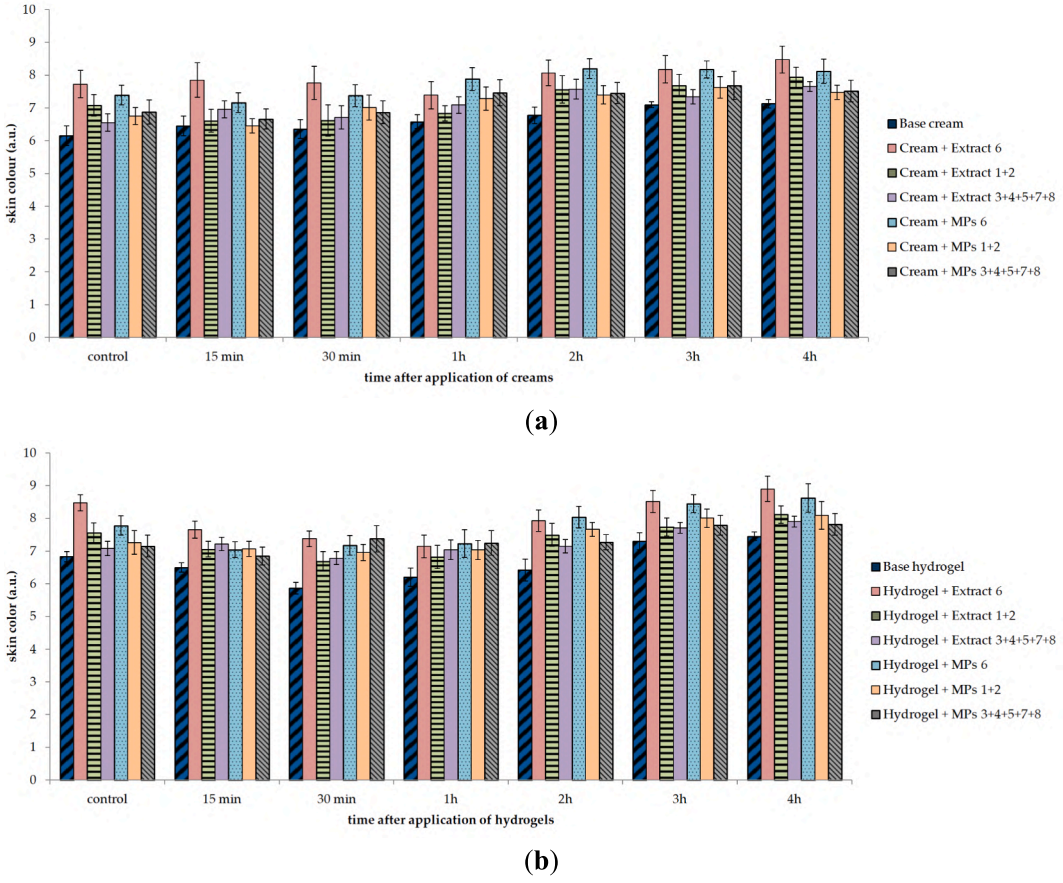


Fig. 8. Colourimetric measurements of skin before (control) and after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

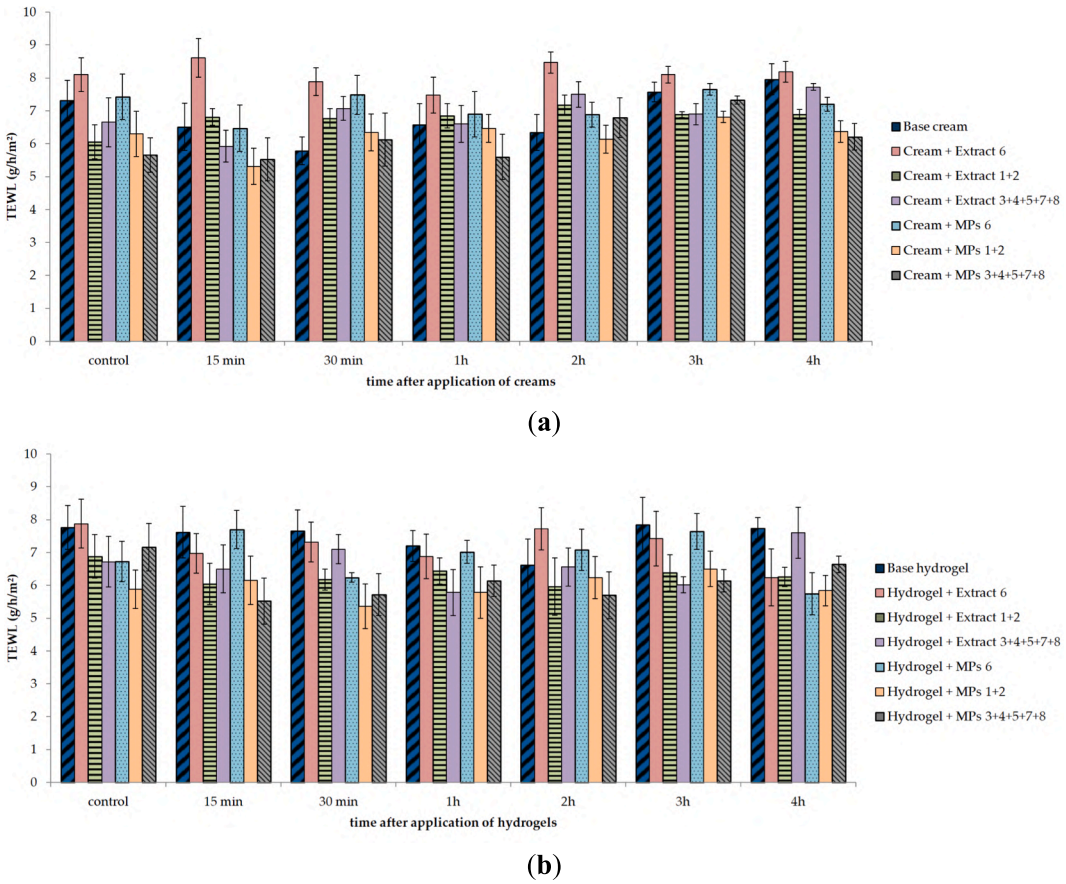


Fig. 9. Tewametric measurements of skin before (control) and after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

before the test) to assess significant changes in skin redness before and after the application of preparations to the skin. Due to its complex composition, plant extracts may cause adverse skin effects, such as allergic and irritant contact dermatitis [86]. As seen in Fig. 8, the application of obtained herbal preparations did not damage or irritate the skin or cause a statistically significant change in skin redness (erythema).

Water constantly evaporating from the human skin is an integral part of the body's metabolism. The level of transepidermal water loss (TEWL) indicates the permeability barrier function of skin since even slight damage to the barrier function causes a rise in the water loss. Therefore, this parameter is essential to evaluate the efficiency of topical applied products. Tewametric measurements after 15 min, 30 min, 1 h, 2 h, 3 h and 4 h of application of the herbal preparations were compared to the results taken for the skin area with applied base samples.

Based on the obtained outcome (Fig. 9), we can conclude that the application of prepared dermatological preparation did not cause deterioration of the epidermal permeability barrier function, and, hence, skin barrier integrity was maintained. Tewameter indicators have not significantly changed throughout this study, resulting in the TEWL values maintaining a stable level (approximately 6–7 g/h/m^2). Therefore, obtained phytoformulations showed slight occlusive effects, preventing the skin's moisture from evaporating in short-term experiments.

The *stratum corneum* hydration indirectly determines the skin moisture. The coreometer measurement depth is minimal, reaching solely to the *stratum corneum* to eliminate the impact of deeper skin layers. Moreover, due to electrical capacitance measurement, substances on the skin, such as salts or residues of topically applied products, have minimal influence on the corneometer indications. Skin surface hydration was measured before and then 15 min, 30 min, 1 h, 2 h, 3 h and 4 h after application of dermatological preparations. The results presented are the difference measurements between the test field and control field (non-treated by the products) at each time point (Fig. 10).

One can see that the application of all samples led to higher skin water content detected in the outermost skin layer. However, it did not occur in the same way for creams and hydrogel. After the application of creams, the corneometer indications were lower (~7 a.u.), and they increased over time (~12 a.u.). However, the sample containing microparticles with a loaded extract from *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* resulted in significantly higher skin hydration values (~14.5 a.u.). After 3 and 4 h, emulsions containing elderflower extract (~15 a.u.) and microparticles with *Viola tricolor* + *Veronica officinalis* extract (~14 a.u.) had significantly higher corneometric values compared to the control sample (base cream) application (~9 a.u.). In contrast, the skin hydration level was higher after applying hydrogels and decreased during the measurements. The introduction of extracts to the hydrogels increased its short-term skin hydration efficacy. Hydrogels containing all three extracts added directly to the hydrogel caused the highest rise in skin hydration values (~19 a.u.). However, over time, it decreased but remained higher (~13 a.u.) than for the base hydrogel application site (~7 a.u.). Therefore, phytoformulations prolonged skin hydration to at least 4 h in a single application.

Plant extracts are a source of numerous substances that may moisturise the skin. Prepared extracts were rich in polyphenols and flavonoids, containing several hydroxyl groups within their molecules that can form a hydrogen bond with water, thus binding water in the epidermis [87]. This contributed to herbal creams and hydrogels' higher skin hydration properties than base formulations.

Provided benefits on the skin conditioning result not only from

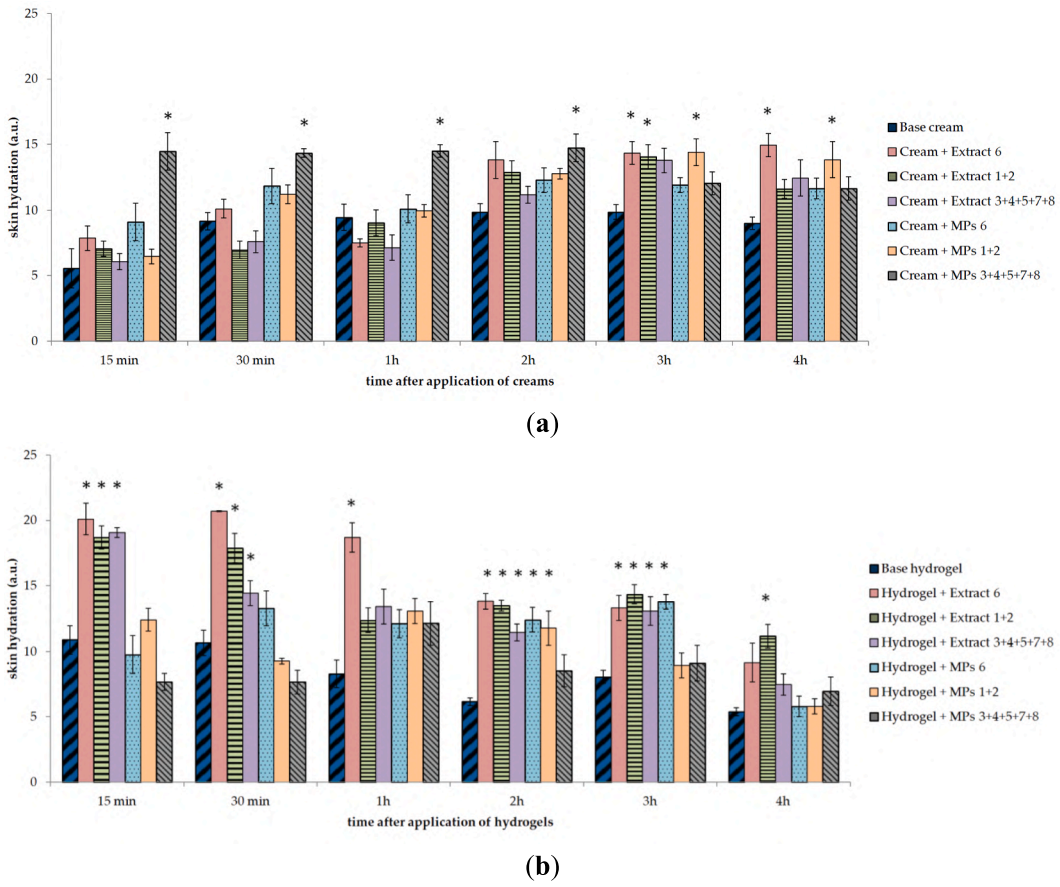


Fig. 10. Corneometric skin measurements after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. The results show differences in the corneometer indications between the test field and control field at the appropriate point. * indicates a difference at $p < 0.05$ between the results of tested samples compared to the base (**a**) cream and (**b**) hydrogel.

prepared extracts but also from the entire composition of formulations. The emulsion contains a mixture of emollients (cetearyl alcohol, caprylic/ capric triglicerydes, paraffinum liquidum, *Vitis vinifera* seed oil, *Prunus domestica* seed oil, octyldodecanol, isopropyl palmitate, glyceryl stearate) creating a temporary occlusive layer on the skin surface, which prevents excessive evaporation of water from the surface (providing an indirect moisturising effect), thereby conditioning the skin [88]. Tocopherol (vitamin E) has the ability to be incorporated into the lipid structures of cell membranes and the intercellular cement of the *stratum corneum*, thus strengthening the epidermal barrier [89]. Strengthening the epidermal barrier not only hinders the penetration of foreign substances and prevents irritation but also inhibits TEWL, thus improving skin hydration. These emulsions also contained humectants (glycerin, propylene glycol) that attract water, which is subsequently occluded by the film formed by emollients. Glycerin and propylene glycol, presented in all creams and hydrogels, are hydrophilic moisturising substances. Moreover, they have the ability to penetrate the *stratum corneum*, thanks to which they act as a penetration enhancer – thus facilitating the transport of other substances deep into the deeper skin layers [90]. Another hydrophilic moisturising substance – allantoin – provides soothing, irritation-relieving, anti-inflammatory effects and supports skin regeneration properties [91]. Panthenol also has an anti-inflammatory effect, accelerates the regeneration processes of the epidermis, soothes irritations and moisturises. Furthermore, it penetrates well into the epidermis, transforming into biologically active vitamin B5, which activates the division of epidermis and dermis cells and stimulates cell renewal [92].

Other studies applied similar methods to assess the effect of topical application of plant extract formulations to the skin using Courage + Khazaka skin probes. *Castanea sativa* leaf ethanol:water (7:3) extract was reported to positively impact the skin barrier integrity [93]. Application of creams containing different plant extracts, such as *Curcuma* and *Acerola* fruit extracts [94], olive leaf extract [95], *Hydrangea serrata* extract [96], *Centella asiatica, Momordica cochinchinensis* and *Phyllanthus emblica* extracts [97], led to increase in corneometric and decrease in tewametric indications and, hence, improving skin condition. However, serum with raspberry leaf cell culture extract, potentially hydrating and moisturising skin, did not cause a significant improvement in skin moisture or TEWL [98]. Hydrogel based on hydroxyethylcellulose with *Cannabis sativa* L. herb extract positively affected skin condition, indicating the protective effect against water loss from the epidermis during a short-time study performed on forearm skin [99]. De Melo et al. evaluated the impact on skin parameters of the immediate film-forming effect of hydrogel and cream containing *Kappaphycus alvarezii* and *Caesalpinia spinosa* extracts [100]. Their results showed that film formed on the skin surface reduced TEWL and improved *stratum corneum* on forearm skin after 1 h of application. Moreover, their extracts revealed more pronounced effects when added to the emulsion formulation instead of a gel.

Emulsion containing chitosan microparticles loaded with *Eugenia dysenterica* leaves extract was also obtained by Mazutti et al. [84]. They additionally performed *in vitro* skin penetration using Franz diffusion cells on the skin from porcine ears. It was established that active substance was delivered to the *stratum corneum* and the deeper skin layers. Chitosan has been reported to provide an enhancer effect by secondary structure changes of keratin and water content in *stratum corneum* [101]. However, the emulsion formulation loaded with chitosan microparticles significantly increased the amount of active substance that penetrated

the deeper skin layers, which could be ascribed to the effect of the emulsifier presented in the emulsion on the *stratum corneum* lipid arrangements [102,103].

4. Conclusions

In this research, we obtained aqueous and ethanol extracts from eight medicinal plants common in Poland (herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers of *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome of *Potentilla erecta*), as well as their blends. We noticed that aqueous extract from *Sambucus nigra* flowers (elderflower) and aqueous and ethanol extracts from *Veronica officinalis* herbs showed the highest level of TPC, TFC and antioxidant properties. Moreover, *Potentilla erecta* ethanol extract had the highest polyphenols content and antioxidant properties but the lowest content of flavonoids regardless of the extraction solvent type. Selected extracts with established phytochemical profiles (TPC, TFC, antioxidant activity: CUPRAC, FRAP, DPPH RSA) were successfully enclosed in chitosan microparticles with loading capacity ranging from 13.7 to 71.4 %. The *in vitro* release profile of extract from microparticles revealed initial burst release lasting 5 h, followed by a sustained release rate of up to 3 days. Afterwards, dermatological preparations in the form of emulsion and hydrogel containing plant extracts and chitosan microparticles loaded with extracts were obtained. Prepared extracts were protected in chitosan microparticles and mechanically released during the spreading to the skin. Preliminary studies of biophysical skin parameters were instrumentally conducted after the application of phytoformulations. Applying herbal preparations did not cause skin irritation or disintegrity of skin barrier function, but it led to higher hydration of the outermost skin layer. Emulsion with microparticles containing *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* aqueous extract exhibited the best short-term properties of *stratum corneum* hydration. In terms of hydrogels – the sample containing free extracts from *Sambucus nigra* and *Viola tricolor* + *Veronica officinalis* showed better hydration of the *stratum corneum*. The application of hydrogel resulted in a higher outermost skin layer hydration level compared to emulsions during the first 30 min. However, after that time, creams had better *stratum corneum* hydration properties until the end of these short-term preliminary studies. Therefore, developed phytoformulations may find an application as skin conditioning products.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Nicolaus Copernicus University in Toruń (KB 67/2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

CRediT authorship contribution statement

Weronika Walendziak: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Natalia Rodríguez Villegas:** Investigation. **Timothy E.L. Douglas:** Writing – review & editing, Supervision. **Justyna Kozlowska:** Writing – review & editing, Supervision, Resources, Conceptualization.

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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Article

Design, Optimization, and Characterization of Freeze-Dried Emulsions Based on Sodium Alginate and Whey Protein Isolate Intended for Cosmetic and Dermatological Applications

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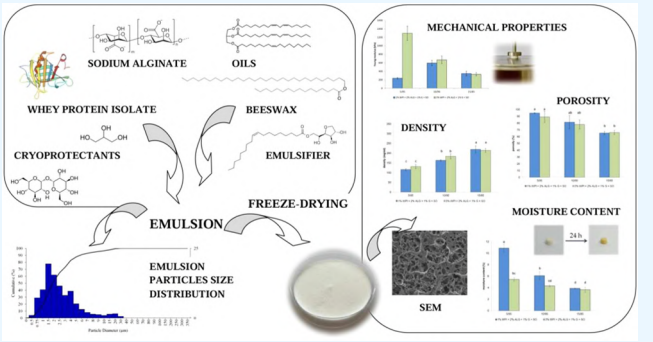
ABSTRACT: Traditional water-based emulsions dominate personal care products, offering minimal skincare benefits but consuming significant amounts of water resources. This sustainable prototype of biopolymer-based skincare products reduces water usage due to the potential to reuse water sublimed during freeze-drying and enhances biopolymer-based materials' performance. This study presents the development and characterization of freeze-dried emulsions formulated with biopolymers, specifically sodium alginate and whey protein isolate, aimed at cosmetic and dermatological applications. Emulsions were modified with cryoprotectants, including glycerin, propylene glycol, sorbitol, mannitol, and trehalose, as well as oils (sunflower oil or sea buckthorn oil), beeswax, and Span 80 as an emulsifier. The methodology involved varying the time and speed of emulsion homogenization to optimize the size distribution of the oily phase droplets. The prepared freeze-dried emulsions were characterized by scanning electron microscopy (SEM), mechanical properties, residual moisture content, porosity, and density measurements. The physicochemical properties of obtained matrices significantly depended on the concentration of WPI, aqueous-to-oily phase mixing ratios, and the addition of different types and concentrations of cryoprotectants, oils, and beeswax. The results revealed that the obtained materials exhibited promising porosity (59−95%) and density (varying from 116 to 308 mg/mL), low residual moisture content (from 2.3 to 10.9%), and favorable mechanical properties (ranging from 240 kPa to 1.7 MPa), positioning them as novel materials with potential for skin application. Optimization and a combination of existing technologies for a sustainable, functional skincare solution show a superior performance over conventional formulations in terms of shelf life, microbial stability, reduced preservatives, and more efficient transport and storage.



1. INTRODUCTION

Emulsions are dispersed systems composed of two mutually immiscible liquids: the internal (the dispersed phase) is finely dispersed in the external (the continuous phase) with the help of surface-active agents. There are several distinguished types of emulsions.[1,2] The oil phase dispersed in the aqueous phase constitutes an oil-in-water emulsion (O/W), whereas a water-in-oil (W/O) system is when the oil is in the continuous phase and the water is in the dispersed phase. Multiple emulsions are also possible, including water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O) emulsions, whose smaller droplets are dispersed in larger ones.[3] The basis of the oily phase may be various oils, such as sunflower oil and sea buckthorn oil, owing to their beneficial properties as skin conditioning agents. Sunflower oil, rich in vitamin E, oleic acid, and linoleic acid, provides skin hydration, barrier repair, and antioxidant protection, making it a widely used emollient in skincare formulations.[4] Sea buckthorn oil, known for its high content of carotenoids, flavonoids, and essential fatty acids, offers strong anti-inflammatory, regenerative, and antioxidant effects, promoting skin conditioning and protection against oxidative stress.[5] Beeswax contributes to the emulsion due to its stabilizing properties.[6] Essential components of emulsions are emulsifiers that lower the surface tension between two phases and stabilize the system by ensuring its thermodynamic stability. These compounds are made of the hydrophilic part ("head") and the hydrophobic part ("tail"), which are appropriately placed around the internal phase droplets. However, surfactant molecules tend to quickly adsorb and desorb from the droplets, affecting the emulsions' stability. Moreover, the phases of emulsions can be separated through

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Table 1. Composition of the Prepared Materials[a]

sample	oily/aqueous phase ratio	aqueous phase			oily phase		
		polymers		cryoprotectant	oil	emulsifier	wax
1% WPI + 2% ALG + 1% G + SO_5/95	5/95	1% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
1% WPI + 2% ALG + 1% G + SO_10/90	10/90	1% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
1% WPI + 2% ALG + 1% G + SO_15/85	15/85	1% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% G + SO_5/95	5/95	3% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% G + SO_10/90	10/90	3% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% G + SO_15/85	15/85	3% of WPI	2% of ALG	1% of G	SO	1% of Span 80	-
3% WPI + 2% ALG + SO_10/90	10/90	3% of WPI	2% of ALG	-	SO	1% of Span 80	-
3% WPI + 2% ALG + 3% G + SO_10/90	10/90	3% of WPI	2% of ALG	3% of G	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% PG + SO_10/90	10/90	3% of WPI	2% of ALG	1% of PG	SO	1% of Span 80	-
3% WPI + 2% ALG + 3% PG + SO_10/90	10/90	3% of WPI	2% of ALG	3% of PG	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% S + SO_10/90	10/90	3% of WPI	2% of ALG	1% of S	SO	1% of Span 80	-
3% WPI + 2% ALG + 3% S + SO_10/90	10/90	3% of WPI	2% of ALG	3% of S	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% M + SO_10/90	10/90	3% of WPI	2% of ALG	1% of M	SO	1% of Span 80	-
3% WPI + 2% ALG + 3% M + SO_10/90	10/90	3% of WPI	2% of ALG	3% of M	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% T + SO_10/90	10/90	3% of WPI	2% of ALG	1% of T	SO	1% of Span 80	-
3% WPI + 2% ALG + 3% T + SO_10/90	10/90	3% of WPI	2% of ALG	3% of T	SO	1% of Span 80	-
3% WPI + 2% ALG + 1% G + SBO_10/90	10/90	3% of WPI	2% of ALG	1% of G	SBO	1% of Span 80	-
3% WPI + 2% ALG + 1% G + SO + 1% B_10/90	10/90	3% of WPI	2% of ALG	1% of G	SO	1% of Span 80	1% of B
3% WPI + 2% ALG + 1% G + SBO+ 1% B_10/90	10/90	3% of WPI	2% of ALG	1% of G	SBO	1% of Span 80	1% of B
3% WPI + 2% ALG + 1% G + SO + 3% B_10/90	10/90	3% of WPI	2% of ALG	1% of G	SO	1% of Span 80	3% of B
3% WPI + 2% ALG + 1% G + SBO + 3% B_10/90	10/90	3% of WPI	2% of ALG	1% of G	SBO	1% of Span 80	3% of B

[a]WPI: Whey protein isolate; ALG: sodium alginate; G: glycerin; PG: propylene glycol; S: sorbitol; M: mannitol; T: trehalose; SO: sunflower oil; SBO: sea buckthorn oil; B: beeswax.

flocculation, coagulation, coalescence, creaming, sedimentation, and Oswald ripening. The average droplet size and distribution play an important role in these processes along with the emulsions' pH, viscosity, and added ingredients.[3,7] The thermodynamic instability may be overcome by freeze-drying of emulsions.[8] During freeze-drying under low pressure and reduced temperature, water is removed via sublimation from frozen samples.[9] Resulting porous materials combine the advantages of both forms: emulsions and freeze-dried matrices. However, some mechanical stress may occur during the freeze-drying process, potentially destabilizing the emulsified system. Therefore, several parameters must be carefully and thoroughly chosen, such as the proper selection of emulsifiers and cryoprotectants. Cryoprotectants prevent damage from irreversible aggregation and fusion of internal phase droplets during freezing. Many polyols and sugars (including monosaccharides, disaccharides, and polysaccharides), such as glycerin, propylene glycol, sorbitol, mannitol, and trehalose, play the role of cryoprotectants. Emulsions have found applications in cosmetic,[10,11] pharmaceutical,[12,13] medical,[14,15] and food industries.[16,17] However, there are not many studies regarding freeze-dried emulsions, including their applications in medical (for vessel clips[18]) and pharmaceutical (as adsorbents for pharmaceutical pollutants,[19] for intravenous injection of bufadienolides,[20] and vaccine adjuvants[21]) industries.

Whey protein isolate (WPI) and sodium alginate (ALG) are widely used biopolymers in various applications due to their complementary functional properties and biocompatibility. Whey protein isolate, a highly purified form of whey protein with a protein content exceeding 90% and richness in essential amino acids, is derived from milk whey through filtration. It exhibits remarkable emulsifying, stabilizing, and film-forming properties, making it highly suitable for creating structured emulsions.[22] WPI contributes to forming stable interfacial layers around dispersed oil droplets, enhancing the physical stability of emulsions.[23] Its proteinaceous nature also aids in water binding and gelation, critical for maintaining the freeze-dried systems' structural integrity.[24] However, sodium alginate, a naturally occurring polysaccharide extracted from brown seaweed, is composed of repeating units of β-D-mannuronic acid and α-L-guluronic acid. It is a thickening, gelling, and stabilizing agent in various formulations.[25] In emulsion-based systems, sodium alginate contributes to the viscosity and stability of the aqueous phase, providing a matrix-enhancing encapsulation of oil droplets and reducing water mobility.[26,27] This characteristic is especially advantageous in freeze-dried formulations, where moisture control is essential for maintaining the product stability. Combining WPI and sodium alginate in emulsion-based systems offers synergistic advantages for developing freeze-dried products with tailored textural properties. The structural network formed by these biopolymers plays a crucial role in water immobilization, cryoprotection, and sublimation behavior during the freeze-drying process.

A robust ecological trend can now be seen in cosmetic chemistry (the so-called ecological cosmetics) in terms of both formulation[28,29] and packaging.[30,31] Research focuses on biodegradable packaging and natural ingredients; however, no one seems to notice the escalating global water crisis.[32] Water is the main constituent of numerous cosmetic and personal care products including emulsions. It can be found in skin, body, hair, oral, and sun care products. However, water has no cosmetic effect on the skin; it is only the base component and solvent of other ingredients in most cosmetic forms. Due to the rapidly shrinking resources of clean and accessible water, reducing water usage for formulating products

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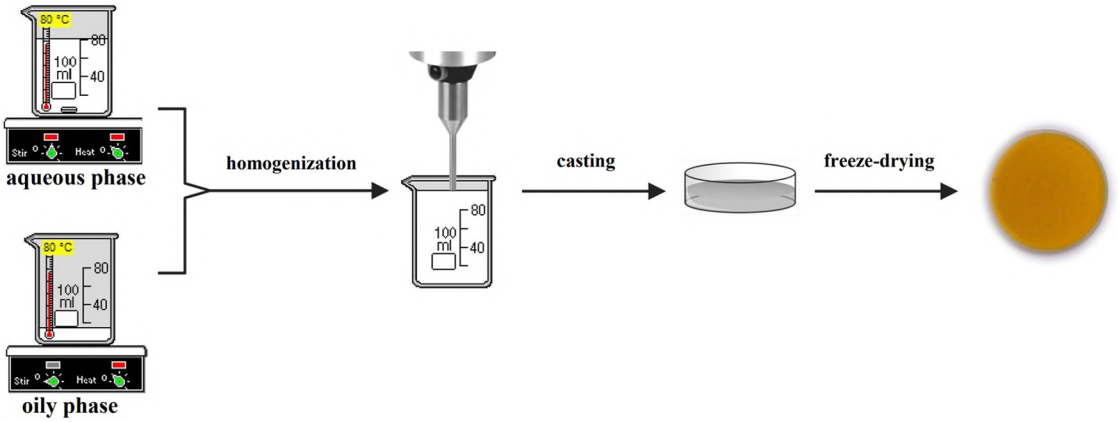

Figure 1. Preparation method for freeze-dried emulsions.

is a responsible attitude toward climate change and the global water crisis.

Developing a methodology for obtaining a prototype with reduced water consumption is one possible solution to reduce the overuse of water in cosmetic chemistry. These advanced materials also have many other advantages, including an increased shelf life and stability, reduced proneness to microbial growth (potentially reducing preservatives' content), and more accessible storage and transport (due to the reduced weight). Moreover, dry-form materials are practical and travel-friendly; they are lighter and may be packed into hand luggage when passing through airport security. This enhanced cosmetic form is eco-friendly not only because of sustainable water management but also because of the packaging; materials in the dry form allow the reduction of plastic packaging and, thus, plastic waste. Reducing the amount of plastic packaging will be possible because the prototype is stored in a dry form and has a smaller volume and size than traditional emulsions. Moreover, this optimized form presents opportunities in product development that require reduced water usage to prepare and apply the material.

Therefore, the main goal of this research was to develop a preparation methodology for freeze-dried emulsions based on biopolymers (sodium alginate and whey protein isolate), cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose), oils (sunflower oil and sea buckthorn oil), beeswax, and emulsifiers (Span 80). Different times and speeds of emulsion homogenization were investigated in order to determine the narrow size distribution of the smallest dispersed phase droplets in the emulsion continuous phase. Freeze-dried emulsions were characterized via scanning electron microscopy (SEM), mechanical properties, residual moisture content, porosity, and density measurements. Prepared freeze-dried emulsions are advanced, highly effective materials with potential skin applications intended for cosmetic and dermatological applications.

2. MATERIALS AND METHODS

2.1. Materials. Sodium alginate was acquired from BÜCHI Labortechnik AG (Flawil, Switzerland). Span 80, D-sorbitol, and beeswax were obtained from Sigma-Aldrich (Poznan, Poland). Glycerin and propylene glycol were supplied from Chempur (Piekary Slaskie, Poland). D-Mannitol and D-(+)-trehalose dihydrate were received from Pol-Aura (Poznan, Poland). Isopropanol was purchased from Stanlab (Lublin, Poland). All of the mentioned chemicals were of analytical grade. Sunflower oil and sea buckthorn oil were obtained from Nanga (Zlotow, Poland). Whey protein isolate (BiPRO) with 97.7% protein and 75% β-lactoglobulin in DM was obtained from Davisco Foods International Inc. (Eden Prairie, MN).

2.2. Preparation Method of Materials. Materials based on whey protein isolate, sodium alginate, cryoprotectants, and oily substances were prepared by freeze-drying of the O/W emulsions. Compositions of fabricated materials are presented in Table 1. Emulsions were obtained with three oily-to-aqueous mixing ratios (5/95, 10/90, and 15/85). Different concentrations of WPI (1 or 3%), different types and concentrations of cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose) (1 or 3%), different types of oils (sunflower or sea buckthorn), and different concentrations of beeswax (1 or 3%) were investigated. Concentrations were calculated based on the total mass of the emulsion. The preparation methodology is presented in Figure 1. Aqueous and oily phases were heated to 70−80 °C and mixed and homogenized using different times (1, 3, or 5 min) and rotation speeds (15,000 or 20,000 rpm) (T25 digital ULTRA-TURRAX disperser, IKA Werke, Staufen, Germany). After evaluating oily droplet sizes of prepared emulsions, 3 min and 20,000 rpm homogenization parameters were selected as optimal for preparing other samples. Afterward, they were frozen (−20 °C) on glass plates and freeze-dried (−55 °C, 5 Pa, 24 h) (α 1−2 LD plus lyophilizator, Martin Christ, Osterode am Harz, Germany).

2.3. Characterization of the Materials. *2.3.1. Emulsion Droplet Size Distribution.* The analysis of droplet size distributions of emulsions based on whey protein isolate, sodium alginate, cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose), and lipids (sunflower oil, sea buckthorn oil, and beeswax) were carried out using a laser diffraction particle size analyzer (SALD-2300 with the SALD-MS23 sampler, Shimadzu, Kyoto, Japan). A small amount of emulsions was added to the sampler's dispersion bath containing distilled water. During circulation, the oily emulsion droplets were dispersed between the flow cell and the dispersion bath and irradiated with a laser beam in the measurement unit. The light intensity distribution of scattered light was used to calculate the oil droplet size distribution using Wing SALD II software (ver. 3.1.0, Shimadzu, Kyoto, Japan). The width of the droplet size distribution (span) was calculated using eq 1

$$\text{span} = (X_{90} - X_{10})/X_{50} \quad (1)$$

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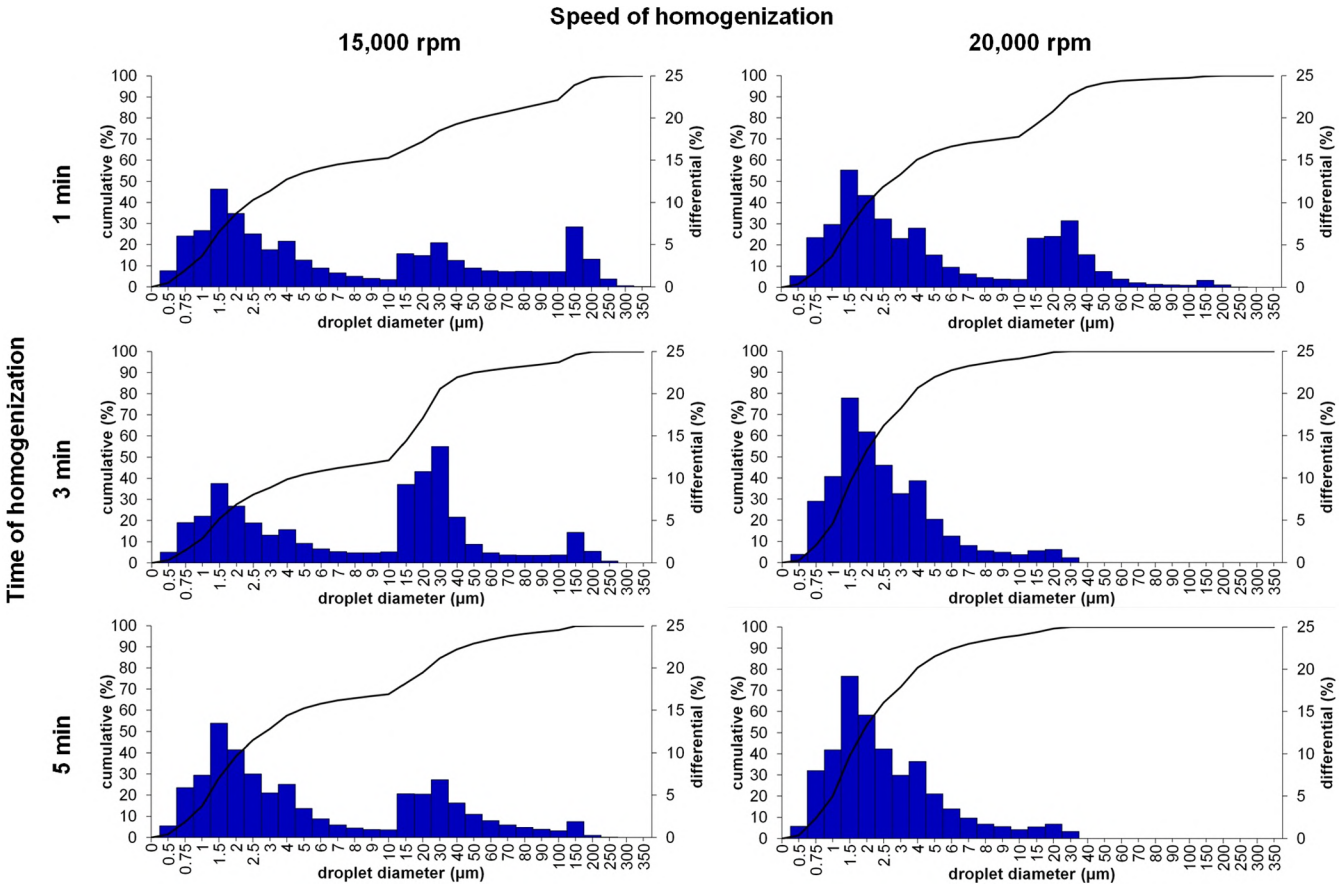

Figure 2. Droplet size distribution of the 3% WPI + 2% ALG + 1% G + SO_10/90 emulsion depending on the time (1, 3, and 5 min) and speed (15,000 and 20,000 rpm) of homogenization.

where X_{10}, X_{50}, and X_{90} represent the volume percentages of oil droplets (10, 50, and 90% undersize, respectively).

2.3.2. Imaging. Scanning electron microscopy (SEM) imaging (Quanta 3D FEG scanning electron microscope, Quorum Technologies, Lewes, U.K.) was applied in order to evaluate the structures and cross-sections of the obtained porous materials. A thin layer of gold and palladium (SC7620 mini sputter coater/glow discharge system, Quorum Technologies, Lewels, U.K.) was spread on the surfaces of the freeze-dried emulsions before the analysis.

2.3.3. Mechanical Properties. A mechanical testing machine (Shimadzu EZ-Test EZ-SX, Kyoto, Japan) fitted with a 50 N load cell was used to investigate the freeze-dried emulsions' mechanical properties. The compression results (5 mm/min compression speed) of seven cylindrical samples of each material type with a diameter of 10 mm were recorded using the Trapezium X Texture program (version 1.4.5.). The Young's modulus and compressive maximum force were calculated from the stress−strain curves.

2.3.4. Porosity and Density Measurements. The liquid displacement method was employed in order to perform porosity (ϵ) and density (d) measurements of porous matrices. Isopropanol was selected as a nonsolvent of used polymers.[33] The graduated cylinder was filled with isopropanol (V_1). Weighed materials (W) were put into the graduated cylinder and left for 5 min. Subsequently, the isopropanol volume was noted (V_2), and it was reread after careful removal of the materials (V_3). Measurements of the porosity (eq 2) and density (eq 3) of materials were carried out in triplicate and calculated as follows

$$\epsilon\ (\%) = (V_1 - V_3)/(V_2 - V_3)\cdot 100 \tag{2}$$

$$d = W/(V_2 - V_3) \tag{3}$$

2.3.5. Residual Moisture Content. The residual moisture contents of freeze-dried emulsions based on WPI, sodium alginate, different cryoprotectants, and oily substances were assessed as the percentage of the water removed from the samples dried to the constant weight (eq 4). Weighed samples (1 cm × 1 cm) (W_w) were dried at 105 °C for 24 h and then weighed again (W_d). The measurements were carried out in triplicate and calculated as follows

$$\mathrm{MC}\ (\%) = (W_w - W_d)/W_w\cdot 100 \tag{4}$$

2.3.6. Statistical Analysis. The Past 4.09 program (PAleontological Statistics Software, Oslo, Norway) was used to carry out one-way ANOVA with Tukey's pairwise analysis to compare results statistically. Data are shown as the mean ± SD for each experiment with p-values ≤0.05 considered significant.

3. RESULTS AND DISCUSSION

3.1. Emulsion Droplet Size Distribution. In order to optimize the preparation method of emulsions, different times (1, 3, and 5 min) and speeds (15,000 and 20,000 rpm) of emulsion homogenization were examined. 3% WPI + 2% ALG + 1% G + SO_10/90 was chosen as an exemplary emulsion. The emulsion droplet size distribution was expressed as the

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mean droplet size and span, which measures the breadth of the distribution.

The oily droplet size distribution charts and characteristics of these droplets are presented in Figure 2 and Table 2,

Table 2. Characteristics of 3% WPI + 2% ALG + 1% G + SO_10/90 Emulsion Droplets Depending on the Time (1, 3, and 5 min) and Speed (15,000 and 20,000 rpm) of Homogenization[a]

sample (time and speed of homogenization)	mean droplet size (μm ± SD)	droplet size (μm)			
		X_{10}	X_{50}	X_{90}	span
1 min, 15,000 rpm	6.64 ± 0.79	0.82	3.81	108.69	28.33
1 min, 20,000 rpm	3.96 ± 0.59	0.84	2.72	28.90	10.33
3 min, 15,000 rpm	7.53 ± 0.68	0.93	10.88	50.89	4.59
3 min, 20,000 rpm	2.02 ± 0.34	0.79	1.88	5.72	2.62
5 min, 15,000 rpm	4.58 ± 0.65	0.84	2.89	44.09	14.99
5 min, 20,000 rpm	2.03 ± 0.35	0.76	1.86	6.20	2.92

[a] X_{10}, X_{50}, and X_{90} represent the volume percentages of droplets (10, 50, and 90% undersize, respectively).

respectively. Based on the obtained results, one can conclude that the size distributions of oily droplets in the obtained emulsions depend on the rotation speed of the homogenization of water and oily phases as well as the time of homogenization. The span ranged from 2.62 for the sample homogenized for 3 min at 20,000 rpm to 28.33 for the sample fabricated at 1 min and 15,000 rpm. The mean droplet size ranged from 2.02 to 7.53 μm with smaller mean droplet sizes and spans for samples homogenized at higher speeds (20,000 rpm instead of 15,000 rpm) regardless of the homogenization time. However, homogenization during 1, 3, and 5 min at 15,000 rpm created emulsions with larger mean droplet sizes and polydispersity, which may indicate incomplete homogenization. One can also conclude that all prepared samples were macroemulsions based on the diameters of the oily droplet sizes.

Distribution of the sizes of oil droplets within an emulsion is a crucial parameter impacting the stability, texture, appearance, functional properties, and performance of the emulsion, as well as the bioavailability of active ingredients.[34,35] Uniformity, represented as a lower span and smaller size of the oily droplets, can improve the emulsion's stability by increasing the emulsion's surface area and thus reducing the coalescence or phase separation.[36,37] Therefore, the most desired results were obtained for samples developed using a homogenizer for 3 and 5 min at 20,000 rpm. As presented in Figure 2, these samples presented very similar droplet diameters with a more narrow distribution. However, in order to prepare other samples, the parameters of time and speed were selected as 3 min and 20,000 rpm, respectively.

After establishing the time and speed of homogenization, different oily-to-aqueous phase ratios (5/95, 10/90, and 15/85) of samples containing 1 or 3% WPI, 2% sodium alginate,

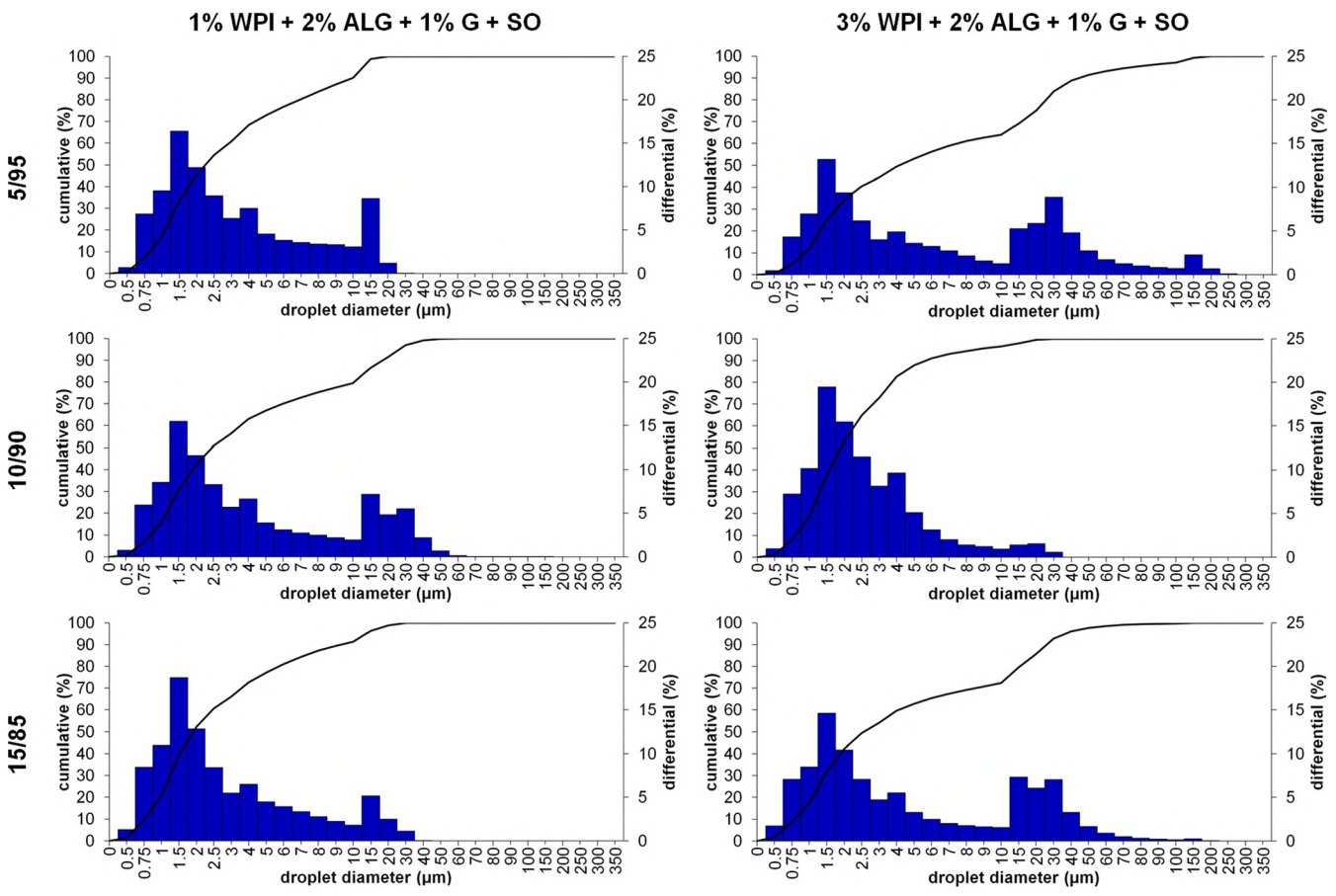

Figure 3. Droplet size distribution of samples containing 1 and 3% WPI, 2% sodium alginate, 1% glycerin, and sunflower oil with Span 80 at different oily-to-aqueous phase ratios: 5/95, 10/90, and 15/85.

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Table 3. Characteristics of Emulsion Droplets in Samples Based on Biopolymers (Sodium Alginate and Whey Protein Isolate), Cryoprotectants (Glycerin, Propylene Glycol, Sorbitol, Mannitol, and Trehalose), Oils (Sunflower Oil and Sea Buckthorn Oil), Beeswax, and Emulsifiers (Span 80), As Well As Different Oily-to-Aqueous Phase Ratios (5/95, 10/90, and 15/85)[a]

		droplet size (μm)			
sample	mean droplet size (μm ± SD)	X_{10}	X_{50}	X_{90}	span
1% WPI + 2% ALG + 1% G + SO_5/95	2.55 ± 0.40	0.81	2.23	9.95	4.10
1% WPI + 2% ALG + 1% G + SO_10/90	3.21 ± 0.50	0.84	2.46	18.40	7.15
1% WPI + 2% ALG + 1% G + SO_15/85	2.28 ± 0.41	0.75	1.90	9.36	4.53
3% WPI + 2% ALG + 1% G + SO_5/95	5.48 ± 0.65	0.94	4.17	43.94	10.30
3% WPI + 2% ALG + 1% G + SO_10/90	2.02 ± 0.34	0.79	1.88	5.72	2.62
3% WPI + 2% ALG + 1% G + SO_15/85	3.65 ± 0.58	0.78	2.56	25.32	9.58
3% WPI + 2% ALG + SO_10/90	2.39 ± 0.35	0.94	2.25	8.44	3.33
3% WPI + 2% ALG + 3% G + SO_10/90	3.30 ± 0.56	1.08	2.54	23.73	8.91
3% WPI + 2% ALG + 1% PG + SO_10/90	2.23 ± 0.39	0.78	1.94	8.61	4.04
3% WPI + 2% ALG + 3% PG + SO_10/90	3.66 ± 0.56	0.86	2.55	25.69	9.75
3% WPI + 2% ALG + 1% S + SO_10/90	4.01 ± 0.59	0.92	2.74	32.12	11.38
3% WPI + 2% ALG + 3% S + SO_10/90	6.00 ± 0.65	0.99	4.24	44.46	10.24
3% WPI + 2% ALG + 1% M + SO_10/90	3.95 ± 0.61	0.82	2.67	32.52	11.89
3% WPI + 2% ALG + 3% M + SO_10/90	3.29 ± 0.52	0.86	2.46	21.69	8.48
3% WPI + 2% ALG + 1% T + SO_10/90	3.68 ± 0.54	0.88	2.69	24.56	8.82
3% WPI + 2% ALG + 3% T + SO_10/90	5.05 ± 0.66	0.90	3.30	47.56	14.16
3% WPI + 2% ALG + 1% G + SBO_10/90	4.01 ± 0.60	0.85	2.70	31.16	11.23
3% WPI + 2% ALG + 1% G + SO + 1% B_10/90	21.56 ± 0.63	1.82	32.37	101.88	3.09
3% WPI + 2% ALG + 1% G + SBO + 1% B_10/90	20.02 ± 0.65	1.63	31.16	101.30	3.20
3% WPI + 2% ALG + 1% G + SO + 3% B_10/90	11.60 ± 0.69	1.14	17.99	75.89	4.15
3% WPI + 2% ALG + 1% G + SBO + 3% B_10/90	11.40 ± 0.67	1.18	16.97	75.49	4.38

[a]X_{10}, X_{50}, and X_{90} represent the volume percentages of droplets (10, 50, and 90% undersize, respectively).

1% glycerin in the aqueous phase, and sunflower oil with Span 80 in the oily phase were investigated. As shown in Figure 3, the oily droplet size distribution differed depending on the amount of the oily phase in the system and the amount of whey protein isolate in the aqueous phase. For materials developed with 1% WPI, the amount of oily phase droplets with larger diameters was lower than that of samples containing 3% WPI at 5/95 and 15/85 oily-to-aqueous phase ratios. This observation is also noted in Table 3, which contains the characteristics of these droplets. Higher spans and mean droplet sizes were noted for samples containing 3% WPI in 5/95 and 15/85 oily-to-aqueous phase ratios. For samples containing 1% WPI, the span was from 4.10 to 7.15, and the mean droplet size was from 2.28 to 3.21 μm, whereas for materials with 3% WPI at 5/95 and 15/85 mixing ratios, the span was from 9.58 to 10.30 and the mean droplet size was from 3.65 to 5.48 μm (an exception was the 3% WPI + 2% ALG + 1% G + SO_10/90 sample with the lowest droplet diameter size and span). Based on the results, the 10/90 oily-to-aqueous phase ratio was chosen as the optimal amount of the oily phase in the emulsion system. Larger oily droplet diameters and a wider size distribution were noted for samples containing higher amounts of WPI, which could be associated with the hydrophobic areas in WPI.[38] Due to the amino acid sequences and three-dimensional structures of β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins, WPI consists of hydrophobic and hydrophilic regions. Hydrophobic areas are correlated with nonpolar side chains of amino acids such as leucine, valine, and phenylalanine. In contrast, hydrophilic regions are linked with polar or charged side chains of serine, glutamine, and lysine.[39] The balance between these two regions can lead to the additional stabilization of emulsions since the hydrophobic parts adsorb into the oil droplets, preventing their coalescence and aggregation.[40,41] Furthermore, the larger oil content was associated with the increase in polydispersity of the emulsion and the formation of larger emulsion droplet sizes.[42,43]

Samples containing 3% WPI, 2% sodium alginate in an aqueous phase, and sunflower oil with the emulsifier in the oily phase were modified with different types and concentrations of cryoprotectants: glycerin, propylene glycol, sorbitol, mannitol, and trehalose. Figure 4 presents their oily droplet size distribution. The size distribution of the dispersed phase droplets in the emulsion continuous phase slightly depended on the presence of cryoprotectants in the sample composition. Samples containing 1% of the addition of the cryoprotectant had a mean droplet diameter of 2.02−4.01 μm and a span value from 2.62 to 11.89, whereas 3% of cryoprotectant addition led to a mean droplet size from 3.29 to 6.00 μm and a span value from 8.48 to 14.16 (Table 3). The sample without cryoprotectants in the material composition had a 2.39 μm mean droplet size and a 3.33 span value. Higher characteristics of the oily droplets in samples containing higher amounts of cryoprotectants might be connected with their effects on the emulsions' viscosity and interfacial properties.[44] Increasing the viscosity of the continuous phase may result in a restricted movement of the oil droplets and emulsifier effectiveness.[45] The bimodal distribution of emulsion droplets may also reflect multiple droplet populations.

Figure 5 presents the oily droplet size distribution of samples containing the same aqueous phase: 3% WPI, 2% sodium alginate, and 1% glycerin, with an alteration regarding the oily phase. Materials were fabricated using sunflower oil or sea buckthorn oil with the addition of 1 or 3% beeswax. One can see a shift in the droplet size distribution. After the addition of beeswax into the emulsion composition, the number of droplets with higher diameters was larger than that of the smaller droplets. Therefore, the addition of beeswax led to a

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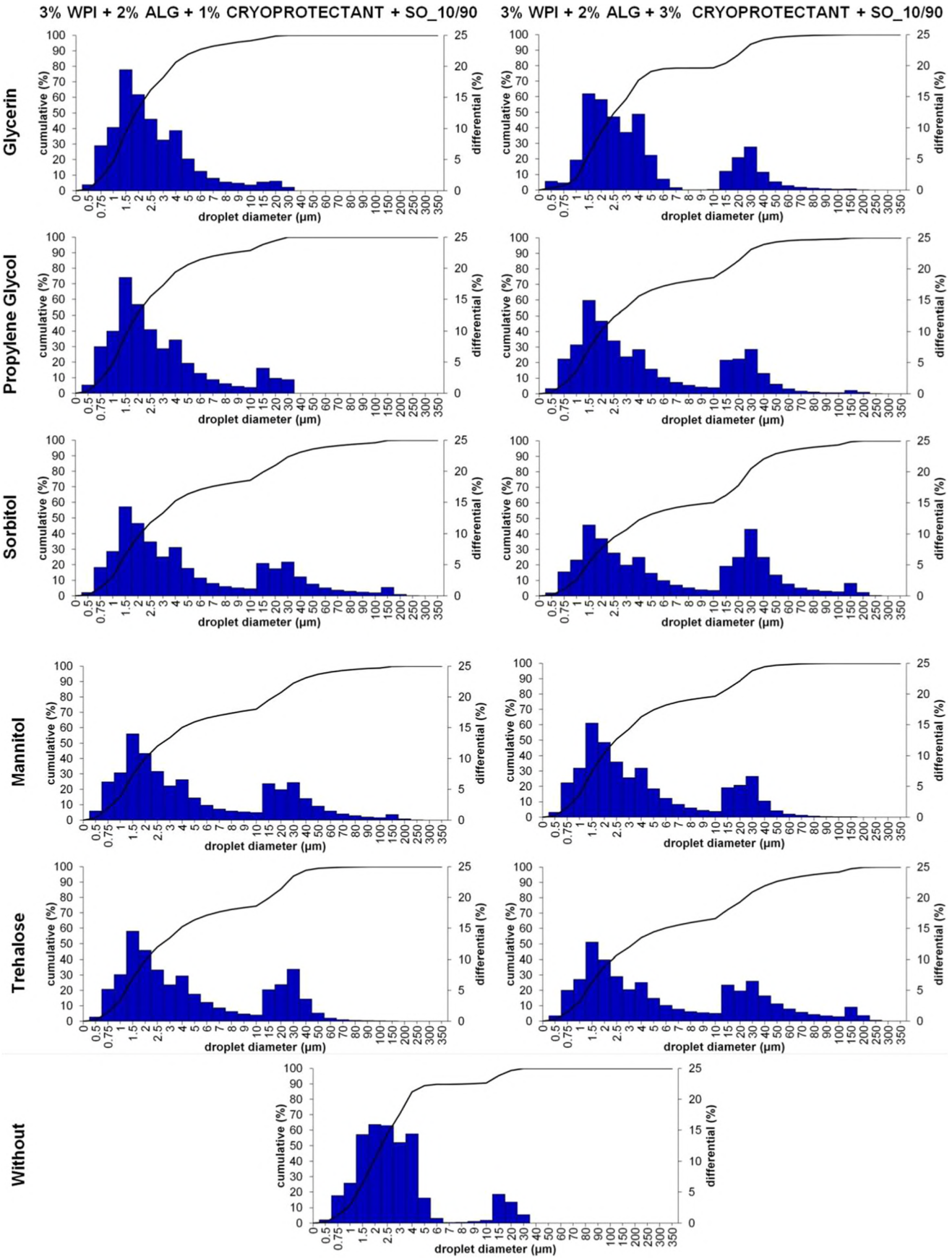

Figure 4. Droplet size distribution of samples containing 3% WPI, 2% sodium alginate, sunflower oil, and Span 80 with and without the addition of 1 or 3% of cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose) with a 10/90 oily-to-aqueous phase ratio.

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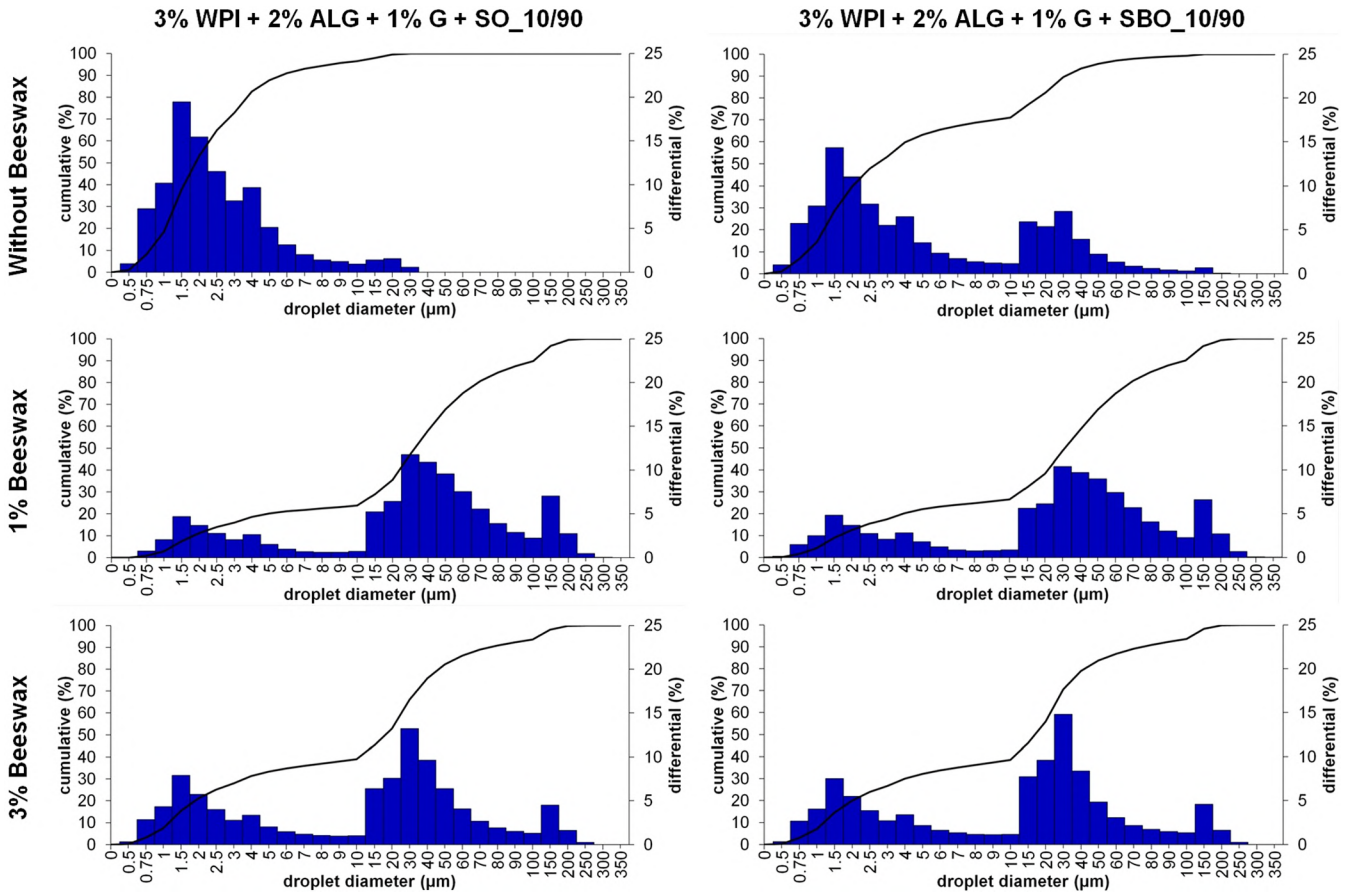

Figure 5. Droplet size distribution of samples containing 3% WPI, 2% sodium alginate, 1% glycerin in the aqueous phase, and sunflower oil or sea buckthorn oil with 1 of 3% addition of beeswax and emulsifiers (Span 80) in the oily phase with a 10/90 oily-to-aqueous phase ratio.

significant rise in the mean droplet size from 2.02−4.01 to 11.40−21.56 μm depending on the amount of beeswax; a lower amount of beeswax caused a higher rise in oily droplet sizes (Table 3). The span value of droplets was lower for samples containing beeswax and was from 3.09 to 4.38, whereas the sample with sea buckthorn oil and emulsifiers in the oily phase had a span value of 11.23. Therefore, despite a narrow distribution of oily droplets containing beeswax, their diameter was higher than for samples without beeswax. Higher oily droplet diameters may be connected with a higher density and viscosity of the oily phase after the addition of beeswax, which also led to a more homogeneous internal network.[46,47] Samples with broader or bimodal droplet size distributions may demonstrate reduced homogeneity. However, the freeze-drying process can induce or contribute to structural rearrangements within the emulsion matrix, potentially leading to partial homogenization and modification of the physico-chemical properties.

3.2. Appearance and Structure of Materials. Freeze-drying of prepared emulsions based on biopolymers (sodium alginate and whey protein isolate), cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose), oils (sunflower oil and sea buckthorn oil), beeswax, and emulsifiers (Span 80) resulted in the fabrication of three-dimensional matrices. Pictures and SEM images showing the structure of obtained materials are presented in Figure 6. FD emulsions were soft and spongy; however, some compositions tend to be slightly more hard, brittle, and rigid. SEM images revealed that all matrices exhibited a complex internal structure featuring irregular interconnected macropores. However, several samples such as 3% WPI + 2% ALG + SO_10/90, 3% WPI + 2% ALG + 3% S + SO_10/90, 3% WPI + 2% ALG + 1% T + SO_10/90, 3% WPI + 2% ALG + 1% G + SBO_10/90, and 3% WPI + 2% ALG + 1% G + SO + 3% B_10/90 had a more linear and irregularly arranged structure with a longitudinal alignment of pores throughout the length of the sponges. These samples also appear to contain more lamellar or channel-like structures formed by the alignment of pores in a single direction. Samples containing sunflower oil as the basis of the oily phase had a whitish color, whereas samples with sea buckthorn oil had a vibrant orange color due to the intense color of this oil.

Porous structure formation is influenced by the nucleation of ice grains within the polymer network, which are replaced by macropores during sublimation. However, during the freezing of samples, some mechanical stress may occur, including aggregation of the oily phase, which could potentially destabilize the emulsified system. To avoid such damage, cryoprotectants such as glycerin, propylene glycol, sorbitol, mannitol, or trehalose are employed due to their help in the prevention of ice crystallization. Do Vale Morais et al. developed a freeze-dried microemulsion for drug delivery purposes, evaluating different types and concentrations of cryoprotectants (mannitol, glucose, lactose, sorbitol, and maltose).[48] Their results indicated maltose as the most effective cryoprotectant. Iyer et al. examined the effect of the addition of sucrose, trehalose, and mannitol to freeze-dried emulsions as vaccine drug products.[21] They concluded that sucrose appeared to be the most effective in the preservation of

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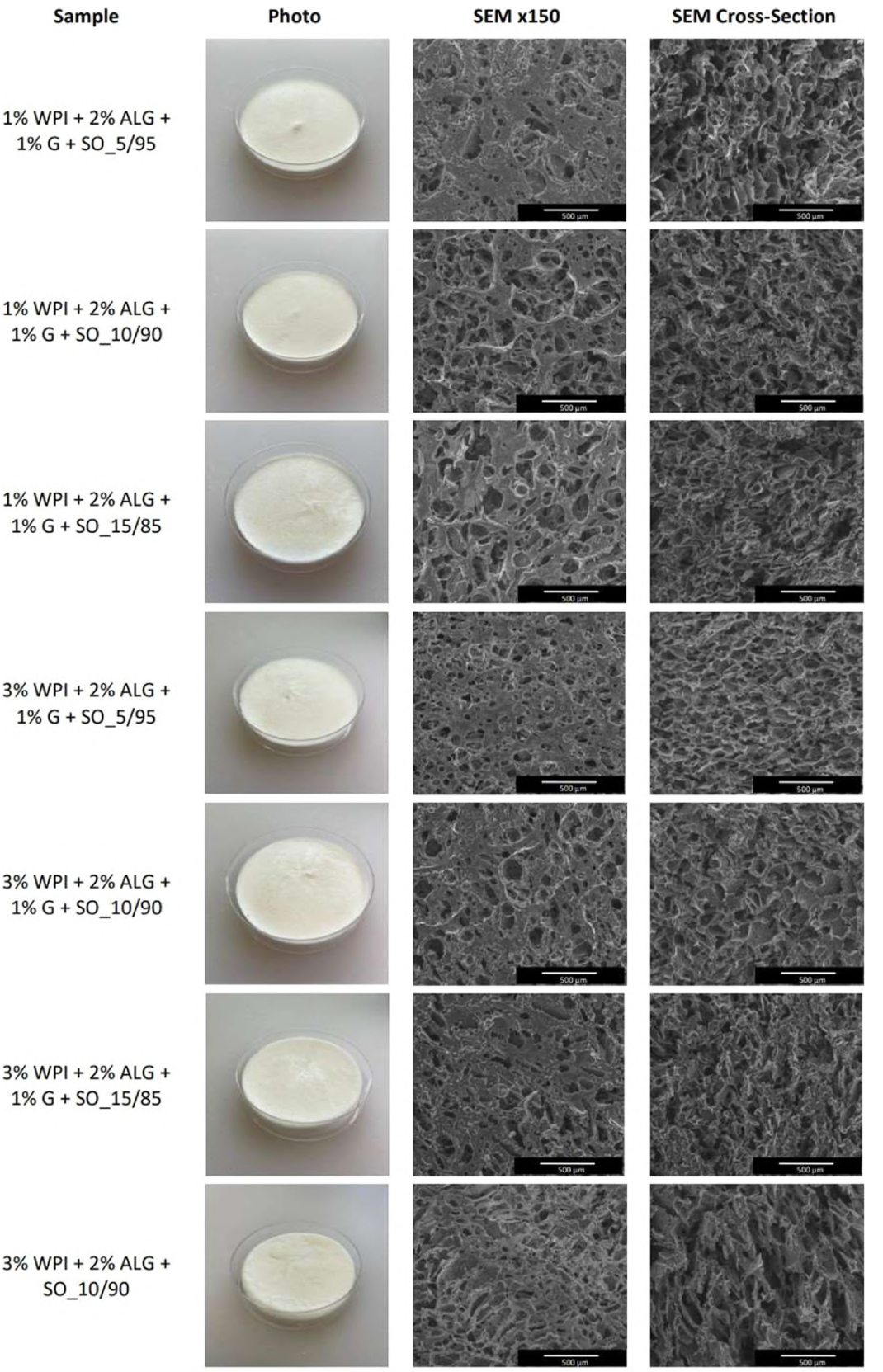

Figure 6. continued

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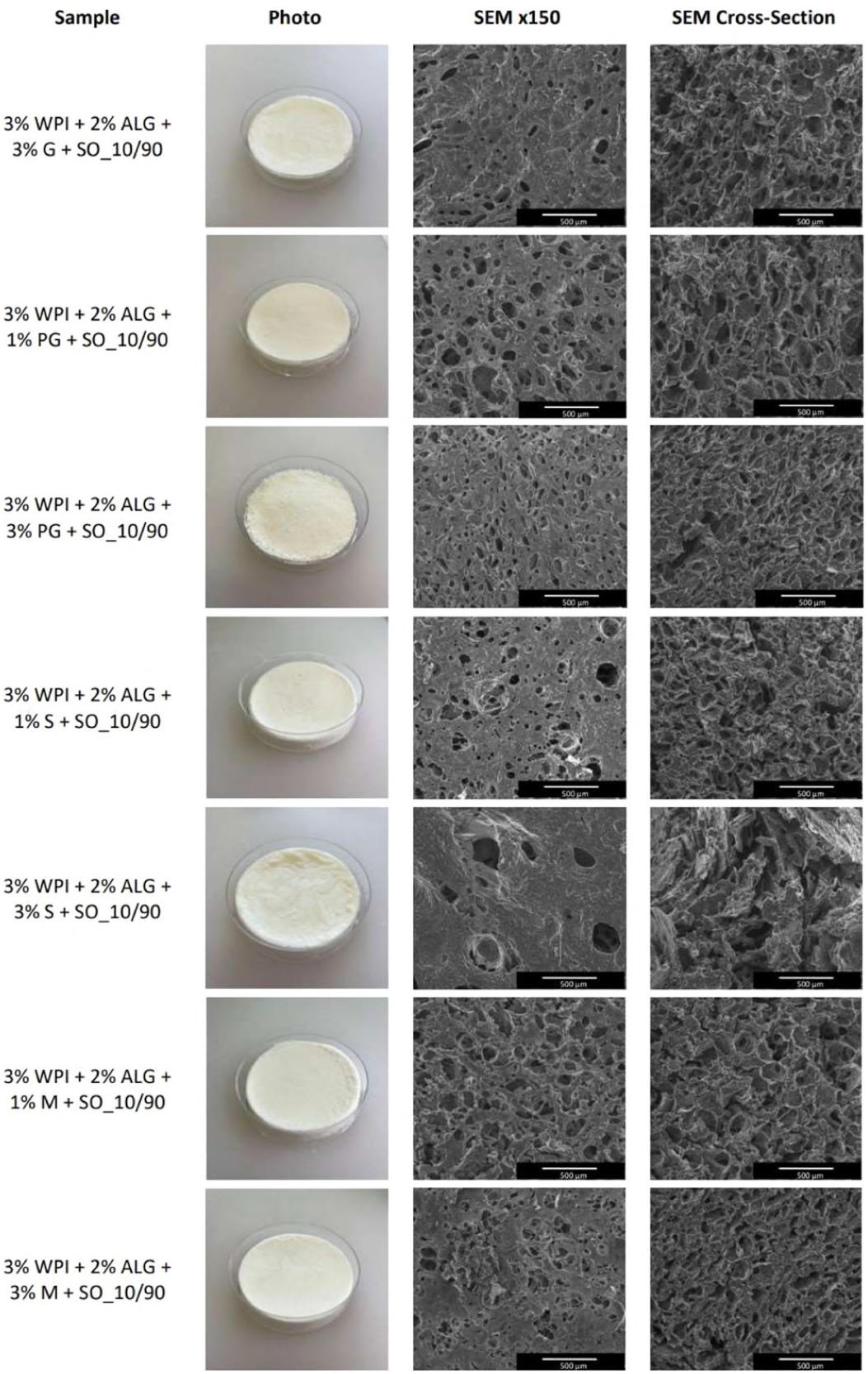

Figure 6. Pictures of obtained materials (the diameter of the container is 60 mm) and SEM images of their structures at a magnification of ×150 (scale bar = 500 μm) and cross-sections at a magnification of ×150 (scale bar = 500 μm).

the droplet size. Nevertheless, in another study, mannitol was superior to trehalose, lactose, and glycine as a protective agent during evening primrose oil microemulsion freeze-drying.[49]

3.3. Mechanical Properties. Mechanical properties during compression of prepared freeze-dried emulsions were evaluated using the Young's modulus and compressive

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Table 4. Mechanical Properties of Prepared Freeze-Dried Emulsion Matrices Based on Biopolymers (Sodium Alginate and Whey Protein Isolate), Cryoprotectants (Glycerin, Propylene Glycol, Sorbitol, Mannitol, and Trehalose), Oils (Sunflower Oil and Sea Buckthorn Oil), Beeswax, and Emulsifiers (Span 80), As Well As Different Oily-to-Aqueous Phase Ratios (5/95, 10/90, and 15/85) during Compression

sample	Young's modulus (kPa)	compressive maximum force (N)
1% WPI + 2% ALG + 1% G + SO_5/95	239.5 ± 18.3	4.02 ± 0.24
1% WPI + 2% ALG + 1% G + SO_10/90	598.1 ± 62.9	9.04 ± 0.25
1% WPI + 2% ALG + 1% G + SO_15/85	350.1 ± 57.6	6.69 ± 1.19
3% WPI + 2% ALG + 1% G + SO_5/95	1296.3 ± 168.3	12.85 ± 2.29
3% WPI + 2% ALG + 1% G + SO_10/90	671.2 ± 87.5	11.90 ± 0.88
3% WPI + 2% ALG + 1% G + SO_15/85	331.2 ± 37.3	8.76 ± 0.93
3% WPI + 2% ALG + SO_10/90	509.5 ± 28.4	10.91 ± 0.73
3% WPI + 2% ALG + 3% G + SO_10/90	326.0 ± 38.8	8.22 ± 0.72
3% WPI + 2% ALG + 1% PG + SO_10/90	1699.4 ± 197.4	19.46 ± 0.32
3% WPI + 2% ALG + 3% PG + SO_10/90	589.5 ± 80.1	18.70 ± 0.74
3% WPI + 2% ALG + 1% S + SO_10/90	1122.7 ± 122.4	21.67 ± 1.37
3% WPI + 2% ALG + 3% S + SO_10/90	349.2 ± 17.0	17.18 ± 3.51
3% WPI + 2% ALG + 1% M + SO_10/90	1446.3 ± 137.2	19.74 ± 2.41
3% WPI + 2% ALG + 3% M + SO_10/90	1154.5 ± 143.9	19.95 ± 2.88
3% WPI + 2% ALG + 1% T + SO_10/90	1545.4 ± 95.5	14.96 ± 0.66
3% WPI + 2% ALG + 3% T + SO_10/90	1359.6 ± 207.2	28.20 ± 1.09
3% WPI + 2% ALG + 1% G + SBO_10/90	705.9 ± 84.1	11.86 ± 0.54
3% WPI + 2% ALG + 1% G + SO + 1% B_10/90	906.4 ± 63.5	10.67 ± 0.70
3% WPI + 2% ALG + 1% G + SBO + 1% B_10/90	844.6 ± 76.2	9.44 ± 0.48
3% WPI + 2% ALG + 1% G + SO + 3% B_10/90	1098.6 ± 102.8	13.76 ± 0.55
3% WPI + 2% ALG + 1% G + SBO + 3% B_10/90	960.8 ± 145.1	11.69 ± 0.68

maximum force (Table 4). Mechanical properties influence the product handling, storage durability, and user experience. Freeze-dried emulsions with higher mechanical strength resist crumbling and maintain structural integrity during transport while still being soft enough to be rehydrated and applied without difficulty. The Young's modulus and compressive maximum force significantly depended on the composition of materials: the oily-to-aqueous phase ratio, concentration of WPI, presence and concentration of cryoprotectants, alteration of the oily phase-oil type, and the addition of beeswax. The values of the compressive maximum force differed from ∼4 N (for sample 1% WPI + 2% ALG + 1% G + SO_5/95) to ∼28 N (for sample 3% WPI + 2% ALG + 3% T + SO_10/90) with similar dependencies as the Young's modulus. The Young's modulus of samples varied from ∼240 kPa to ∼1.7 MPa. The amount of WPI in the sample with a 5/95 oily-to-aqueous phase ratio played a crucial role, namely, for the 1% WPI + 2% ALG + 1% G + SO_5/95 sample, the Young's modulus was the lowest (240 kPa) and for the 3% WPI + 2% ALG + 1% G + SO_5/95 sample, it was significantly higher (1.3 MPa). In other mixing ratios, the content of WPI did not notably alter the Young's modulus; however, its value was higher for samples with a 10/90 oily-to-aqueous ratio (∼600−670 kPa) compared to a 15/85 ratio (∼330−350 kPa). It has been found that increasing the WPI concentration increases the mechanical strength of materials up to a point, after which it decreases.[50] A higher WPI concentration in freeze-dried materials tends to form a denser and more interconnected network through enhanced hydrogen bonding and van der Waals forces, contributing to their mechanical stiffness.[51] The oil-to-water ratio also influences the material's ability to resist deformation due to the plasticizing effect of more oil in the matrix composition.[52,53] The oil might also act as a lubricant between the protein molecules, decreasing the material's resistance to applied stress and potentially disrupting the formation of a strong biopolymeric network, resulting in a more flexible structure.

Materials without cryoprotectants in their composition exhibited a Young's modulus of ∼510 kPa. Furthermore, the presence of different cryoprotectants played a crucial role in the materials' mechanical resistance to compression. This parameter was also higher for samples containing 1% cryoprotectant addition. The highest Young's modulus was noted for 1% addition of propylene glycol (∼1.7 MPa), 1 and 3% addition of mannitol (∼1.4 and ∼1.2 MPa, respectively) and trehalose (∼1.5 and ∼1.4 MPa, respectively), and 1% addition of sorbitol (∼1.1 MPa). Lower values of the Young's modulus exhibited materials containing a 3% addition of glycerin (326 kPa), propylene glycol (∼590 kPa), and sorbitol (∼350 kPa). Significant differences in the Young's modulus between the concentrations of cryoprotectants were recorded for glycerin, propylene glycol, and sorbitol. However, solely a 3% addition of glycerin and sorbitol decreased the Young's modulus below the value noted for the sample without cryoprotectants. The higher Young's modulus of samples is related to materials more resistant to deformation under stress. More stiff and rigid matrices are more likely to retain their shape under compression and will not deform easily under applied force, maintaining their structure. Cryoprotectants also work as plasticizers, which are added to increase flexibility and reduce the brittleness of freeze-dried matrices.[54] Their influence on mechanical properties depends on their concentration, molecular structure, and, hence, formed interactions with other components of matrices.[55,56] They reduce intermolecular interactions between the protein and polysaccharide molecules within the matrix. Differences in the mechanical properties of samples containing different protective agents might be attributed to their structure. Glycerin and propylene glycol act similarly due to the similar structure of small hydrophilic molecules with the ability to

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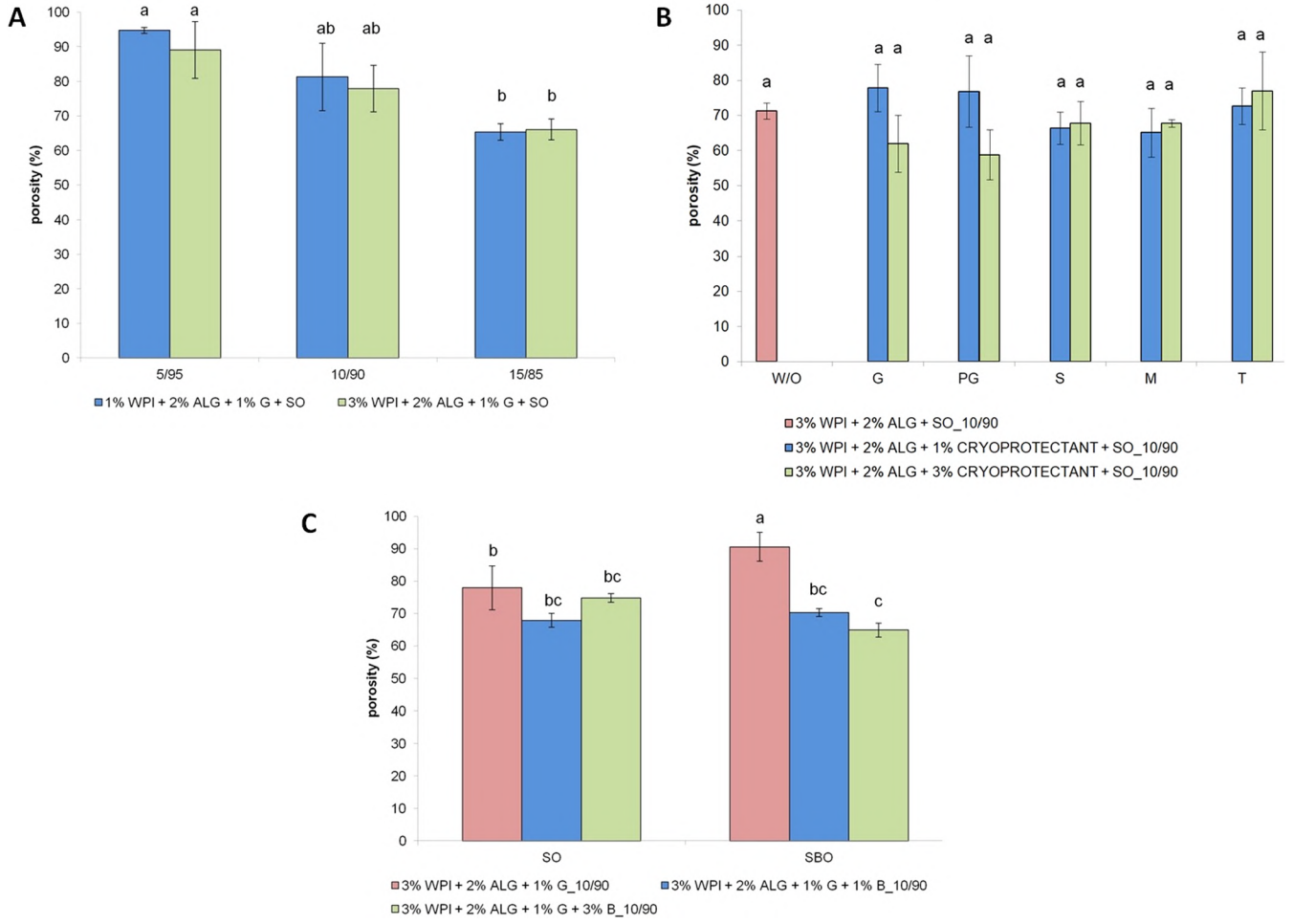

Figure 7. Porosity of freeze-dried emulsions with altering: (A) concentration of whey protein isolate at different oily-to-aqueous phase mixing ratios; (B) type and concentration of cryoprotectants (as well as a sample without the addition of cryoprotectants); and (C) type of oil and concentration of beeswax. Bars not sharing the same letter are significantly different ($p \leq 0.05$).

form hydrophilic bonds. Due to their low molecular weight, they can easily diffuse into polymer chains, disrupting polymer interactions by increasing chain mobility and thus decreasing stiffness. Glycerin has three hydroxyl groups, while propylene glycol has two, which can affect their plasticizing ability. Sorbitol and mannitol as sugar alcohols have six hydroxyl groups, presenting a larger, more complex molecule. Their multiple hydroxyl groups allow them to interact with emulsion constituents. However, mannitol tends to have a worse plasticizing ability than sorbitol due to its more crystalline structure. Trehalose is a disaccharide with nonreducing linkage by an α,α-1,1-glycosidic bond, which makes it more stable than other sugars. Owing to its structure, it provides a more cryoprotectant than plasticizing effect. Compared to glycerin and sorbitol, mannitol and trehalose tend to have a weaker plasticizing effect, especially in higher concentrations, contributing to reinforced polymer alignment and intermolecular spacing during freezing, resulting in a higher Young's modulus.

The addition and further increase in the concentration of beeswax resulted in higher Young's modulus values for both sunflower oil (~0.9 and 1.1 MPa, respectively) and sea buckthorn oil (~0.8 and 1 MPa, respectively). The rise in the Young's modulus observed after incorporating beeswax into the matrix was likely due to its ability to blend with and bind within the biopolymer network.[57] Beeswax may fill interstitial voids and reinforce the structural integrity of the matrix, leading to a denser and more cohesive material with enhanced mechanical resistance. However, the oil type did not lead to notably different values of the Young's modulus; the sample with sunflower oil had 671.2 ± 87.5 kPa, while the material containing sea buckthorn oil had 705.9 ± 84.1 kPa.

3.4. Porosity and Density Measurements. The liquid displacement method was employed in order to examine the porosity (Figure 7) and density (Figure 8) of freeze-dried emulsions. Porosity influences the material's ability to absorb and retain water upon rehydration rapidly, enabling efficient transformation back into the emulsion during application. Highly porous structures facilitate a quicker dissolution and better spreadability on the skin. Density, however, affects the product weight and packaging; lower-density formulations offer benefits in terms of lightweight packaging and reduced transport costs, aligning with sustainability goals.

The porosity varied from 59 to 95%. The porosity of prepared matrices significantly depends on the oily-to-aqueous phase mixing ratios; the higher the contribution of the oily phase in the material composition, the lower is their porosity (Figure 7A). The highest porosity (95%) was observed for the 1% WPI + 2% ALG + 1% G + SO_5/95 sample. Materials obtained with a 15/85 oily-to-aqueous phase ratio, regardless of the WPI concentration, had a porosity of ~65%. Creating a

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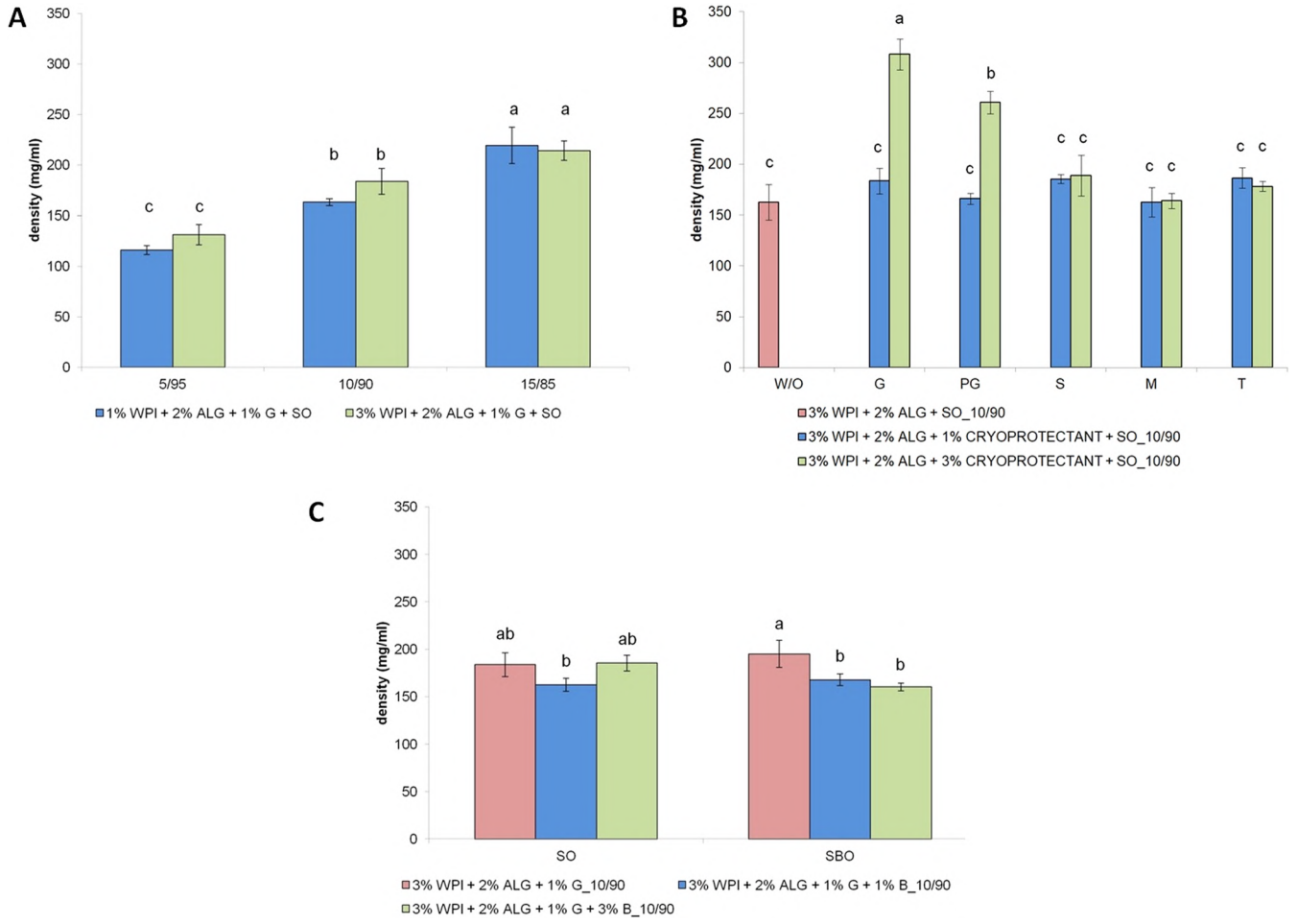

Figure 8. Density of freeze-dried emulsions with altering: (A) concentration of whey protein isolate at different oily-to-aqueous phase mixing ratios; (B) type and concentration of cryoprotectants (as well as a sample without the addition of cryoprotectants); and (C) type of oil and concentration of beeswax. Bars not sharing the same letter are significantly different ($p \leq 0.05$).

porous structure depends on the formation of ice crystals that sublime during freeze-drying. Increasing the oil phase contribution reduces the pore amount, thus reducing the samples' porosity. Samples with a higher aqueous phase content exhibited greater porosity due to more water available to form ice crystals, which later sublimated to leave behind larger or more numerous pores. Conversely, increasing the oil phase reduced porosity, as lipids do not sublime, thereby displacing water and decreasing the extent of pore formation. However, we did not observe significant differences in samples containing different whey protein concentrations.

The porosity of samples containing different types and concentrations of cryoprotectants and samples without the addition of cryoprotectants did not show statistically significant differences (Figure 7B). For these samples, the porosity ranged from 59 to 78%. This indicates that cryoprotectants were evenly distributed throughout the aqueous phase. This uniform distribution led to consistent water removal patterns and similar porosity of obtained samples despite differences in the cryoprotectant type and concentration.

Freeze-dried emulsions based on biopolymers and glycerin in the aqueous phase and sea buckthorn oil with an emulsifier in the oily phase showed 91% porosity (Figure 7C). The higher porosity observed for the sample containing sea buckthorn oil might be attributed to its higher polyunsaturated fatty acid content having more polar double bonds increasing their interaction with the aqueous phase. Modifying this sample with the addition of beeswax led to decreased material porosity (70% porosity for the sample containing 1% beeswax and 65% for the sample with 3% beeswax). The addition of beeswax into prepared samples created more dense and compact matrices due to its hydrophobicity, reducing the porosity. However, matrices containing sunflower oil as the basis of the oily phase did not exhibit significant differences in porosity values (68−78%).

The porosity of emulsion-based materials had been determined as 79−85% with decreased porosity for samples with a higher polymer concentration,[58] 85−90% with decreasing values for samples with an increased contribution of the hydrophobic phase,[59] and 88−98% depending on the polymer content and volume of the internal phase.[60] Furthermore, sodium alginate-based matrices with polymer concentrations ranging from 4 to 16% had interconnected porosities of 83 to 58%, respectively, and total porosities ranging from 85 to 80%, respectively, with lower porosity for materials containing a higher polymer concentration.[61] In comparison, Autissier et al. found that a decrease in freeze-drying pressure significantly increased the sample porosity from 33 to 68%.[62]

The density of prepared materials ranged from ∼116 to ∼308 mg/mL. The density of fabricated matrices did not significantly depend on the concentration of whey protein

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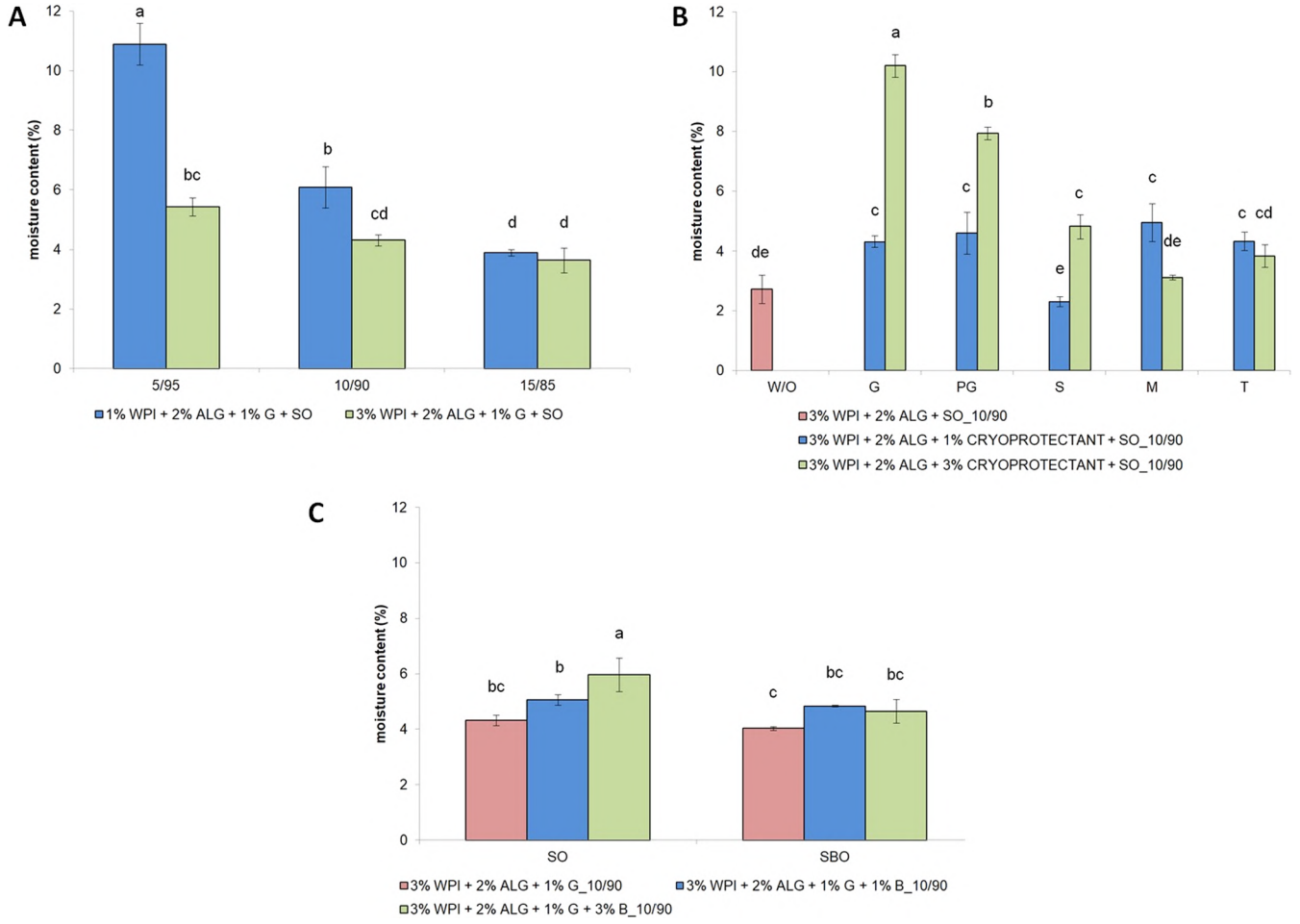

Figure 9. Residual moisture content of freeze-dried emulsions with altering: (A) concentration of whey protein isolate at different oily-to-aqueous phase mixing ratios; (B) type and concentration of cryoprotectants (as well as a sample without the addition of cryoprotectants); and (C) type of oil and concentration of beeswax. Bars not sharing the same letter are significantly different ($p \leq 0.05$).

isolate (Figure 8A). Nonetheless, the higher the oily-to-aqueous phase mixing ratio, the higher the density of samples, from 115 to 131 and 214−219 mg/mL. Density results are consistent with those obtained for the porosity of matrices. As the oily phase did not sublime during freeze-drying, its higher contribution resulted in an increased density by reducing the porosity of the matrix. The proportion of the material that remained postdrying also increased, contributing to a higher solid mass per unit volume, thereby increasing the density.

The sample presenting the highest density contained a 3% addition of glycerin (∼308 mg/mL) and a 3% addition of propylene glycol (∼261 mg/mL) (Figure 8B). Glycerin and propylene glycol are more hygroscopic and have a stronger affinity for water molecules, allowing them to retain water in the matrix more effectively. This can contribute to these samples' higher density and a higher residual moisture content. They could also penetrate and occupy intrapolymer spaces, especially at higher concentrations, reducing the extent of porous voids by limiting the expansion of ice crystals during freezing, hence increasing the density. Moreover, they are smaller and more flexible molecules than sorbitol, mannitol, and trehalose, providing easier integration into the matrix and increasing the samples' density. Materials not modified with the addition of cryoprotectants and samples containing 1% glycerin and propylene glycol, 1 and 3% sorbitol, mannitol, and trehalose did not show statistically significant differences in density (∼163−189 mg/mL).

Materials containing sunflower oil did not exhibit differences in density after the addition of beeswax (∼162−185 mg/mL; Figure 8C), whereas freeze-dried emulsions containing sea buckthorn oil had a higher density (∼195 mg/mL) than samples modified with the addition of beeswax (∼160−168 mg/mL).

Our results are in line with those obtained by other research groups. It was established that materials based on high internal phase emulsions using freeze-drying, vacuum-drying, and heat-drying presented densities from 19 to 350 mg/mL.[63] A higher internal phase volume led to higher-density materials due to the decreased pore volume. Moreover, Manzocco et al. determined that the freeze-dried whey protein isolate aerogel had 220 mg/mL density, while the sample prepared using supercritical drying had 290 mg/mL density.[64] The porosity of emulsion-templated materials containing poly-(hydroxybutyrate-co-valerate) had a density ranging from 196 to 310 mg/mL.[58]

3.5. Residual Moisture Content. The residual moisture content of samples was evaluated as the percentage of water loss during the drying of samples. This parameter is essential to shelf stability. A lower moisture content reduces microbial growth risk, potentially minimizing or eliminating the need for

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preservatives, which is particularly advantageous for sensitive-skin products.

The resulting moisture content of the freeze-dried emulsion ranged from ∼2.32 to ∼10.89% (Figure 9). Its values significantly depended on the aqueous/oily phase ratio, namely, the more the oily phase amount, the lower is the moisture content of materials. The highest moisture content was observed for the sample containing 1% WPI and 1% glycerin at a 5/95 mixing ratio (Figure 9A). Matrices prepared with a 15/85 mixing ratio had a ∼3.64−3.90% moisture content with no significant differences. The moisture content of freeze-dried materials depends on biopolymer concentrations and, hence, the matrice network. WPI forms a heat-induced gel network influenced by the degree of protein hydration and unfolding.[65] Therefore, the higher the WPI concentration, the lower is the moisture content.[66] Meanwhile, sodium alginate's ability to retain water is attributed to its carboxyl groups.[67] Materials based on WPI and alginate were reported to have a moisture content of 6.50%.[68]

Furthermore, the residual moisture content was also significantly influenced by the concentration and type of cryoprotectants; the highest values were noted for samples with 3% glycerin (∼10.20%) and propylene glycol (∼7.94%) (Figure 9B) due to their strong hydrogen bonding capacity, which hindered complete moisture removal during sublimation, while the lowest residual moisture content of all samples was observed for materials with 1% sorbitol. A low moisture content was also detected for materials not modified with the addition of cryoprotectants (∼2.73%). Different cryoprotectants vary in hygroscopicity, influencing the amount of the residual moisture content retained after freeze-drying.[69,70] The lower moisture content of samples containing sorbitol, mannitol, and trehalose may be attributed to their lower hygroscopicity, resembling to absorb moisture from the environment compared to glycerin and propylene glycol. Furthermore, glycerin is highly hygroscopic, which can result in the resorption of moisture. However, trehalose is considered a preferable cryoprotectant for biomolecules due to its lack of internal hydrogen bonds, allowing for more flexible hydrogen bond formation during freeze-drying.[71]

Altering the oil type and addition of beeswax contributed to the moisture content varying from ∼4 to ∼6% (Figure 9C). The composition of the oil phase, namely, the different types of oil, influences water distribution within the freeze-dried emulsion. Sea buckthorn oil is rich in polyunsaturated fatty acids, increasing oil polarity, which may facilitate better water dispersion and removal during freeze-drying.[72] The presence of beeswax in samples may contribute to a higher residual moisture content due to its hydrophobic nature.[73,74] Therefore, beeswax may entrap water within the freeze-dried matrix by limiting the diffusion and sublimation of water molecules. The precise selection of cryoprotectants and the optimization of biopolymer ratios, in conjunction with appropriate emulsifier and oil phase compositions, govern the extent of water immobilization and sublimation dynamics during the drying process, thereby critically impacting the resulting moisture content.

3.6. General Discussion. Traditional water-based emulsions dominate personal care products, although water as their base component offers minimal skincare benefits while consuming significant resources. The development of sustainable, biopolymer-based skincare products presents an innovative approach to reducing water usage and enhancing the performance of cosmetic materials. This study demonstrates the feasibility of freeze-dried emulsions formulated with biopolymers (sodium alginate and whey protein isolate), cryoprotectants (glycerin, propylene glycol, sorbitol, mannitol, and trehalose), oils (sunflower oil and sea buckthorn oil), beeswax, and emulsifiers (Span 80). Since all ingredients are either food-grade biopolymers, plant-derived oils, or cosmetic-grade waxes and polyols, commonly used in topical formulations, their established regulatory status and history of safe cosmetic use support their expected safety for cosmetic and dermatological applications.

The physicochemical properties of the freeze-dried emulsions were significantly influenced by factors such as the WPI concentration, aqueous-to-oily phase ratios, and the type and concentration of cryoprotectants, oils, and beeswax. The materials exhibited promising porosity (59−95%) and density variations (116−308 mg/mL), contributing to their lightweight nature and efficient reconstitution. Furthermore, the low residual moisture content (2.3 to 10.9%) enhances these emulsions' stability and shelf life. The mechanical properties of the obtained materials ranged from 240 kPa to 1.7 MPa, demonstrating their robustness for application in skincare. These properties suggest that the FD emulsions maintain integrity during handling and storage, making them suitable for practical use in cosmetic formulations. One of the primary advantages of freeze-dried emulsions is their potential to reduce microbial growth. Traditional water-based emulsions require preservatives to prevent contamination; however, the removal of water from the formulation minimizes microbial growth. This makes the freeze-dried emulsions particularly beneficial for individuals with allergies or sensitivities to preservatives.

Although freeze-drying is known to be energy-intensive and may pose scalability challenges in industrial cosmetic production, several strategies can be employed to mitigate these limitations. For instance, integrating energy recovery systems in freeze-drying equipment or combining freeze-drying with predrying methods (such as microwave-assisted drying) can significantly reduce the total energy consumption. Process optimization through batch scheduling and load maximization can also improve the energy efficiency. Despite the initial energy costs, freeze-dried emulsions offer distinct advantages that may offset these inputs in a full lifecycle analysis. Key environmental benefits include reduced water consumption (water sublimed during freeze-drying can be reused in the next step of production, decreasing overall water usage), lower packaging waste (the dry form of these emulsions reduces the need for plastic packaging, as they are lighter and more compact than traditional emulsions), and efficient transport and storage (the reduced mass and volume of freeze-dried emulsions facilitate more efficient logistics, leading to a lower carbon footprint in distribution). The development of freeze-dried emulsions represents a significant advancement in cosmetic chemistry and materials science. Upon contact with a minimal amount of water just before application to the skin, the freeze-dried materials rapidly reconstitute into soft, gel-like emulsions, consistent with their original composition of biopolymers, oils, and humectants. Such textures are particularly desirable in cosmetic and dermatological applications, offering favorable spreadability, skin adherence, and a pleasant sensory profile during topical use. This novel formulation method offers several advantages over traditional emulsions, including an extended shelf life due to reduced

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microbial growth, increased stability under varying storage conditions, and the potential for customized skincare solutions by varying the compositions of biopolymers, cryoprotectants, oils, and active ingredients. When considering the broader environmental and economic context, these benefits position freeze-dried emulsions as a promising, sustainable alternative in the cosmetic industry.

The findings of this study underscore the potential of freeze-dried emulsions as a sustainable and functional alternative to conventional skincare products by combining existing technologies with optimized formulations. Their successful implementation could revolutionize the personal care industry, aligning with the growing consumer demand for eco-friendly and high-performance skincare solutions. However, formulation optimization, such as exploring additional biopolymers and active ingredients, rehydration behavior, long-term stability studies, and evaluating biophysical skin parameters using probands, remains a key area required for future investigation to enhance the performance of FD emulsions.

4. CONCLUSIONS

In conclusion, freeze-dried emulsions using sodium alginate and whey protein isolate offer a promising approach for sustainable cosmetic and dermatological products. This study explored cryoprotectants, oils, and beeswax to refine the preparation method and enhance the product quality through physicochemical characterization. Optimizing the homogenization time and speed was key to achieving a desirable droplet size distribution. Prepared freeze-dried emulsions had a complex porous structure, and their physicochemical properties significantly depended on the oily-to-aqueous phase mixing ratio, concentration of WPI, type and concentration of the cryoprotectant, type of oil, and the addition and concentration of beeswax. Different concentrations of WPI did not affect the samples' porosity and density. At the same time, materials with lower amounts of WPI had a lower oily droplet size and span, compressive strength, and higher moisture content. The higher content of the oily phase in the emulsion composition led to a decrease in porosity and residual moisture content as well as an increase in density. Although the type and concentration of the added cryoprotectant had a slight difference for the porosity, span, and oily droplet size, a 3% addition of glycerin and propylene glycol led to higher values of density and residual moisture content, whereas a higher Young's modulus was observed for samples with mannitol, trehalose, and 1% addition of propylene glycol and sorbitol. The addition of beeswax resulted in larger oily droplets with a narrow distribution, a higher Young's modulus, and lower porosity and density in samples containing sea buckthorn oil and higher moisture content in materials with sunflower oil. The characterization results indicate that the physicochemical properties of these biopolymer-based freeze-dried emulsions contribute to their potential for an extended shelf life and reduced microbial growth. Additionally, these formulations offer environmental benefits by reducing water usage and plastic packaging waste, supporting the shift toward more sustainable cosmetic technologies. Overall, this research highlights the potential of freeze-dried biopolymer emulsions as tailorable, eco-friendly alternatives in the cosmetic and dermatological industries.

■ ASSOCIATED CONTENT

Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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W.W. and J.K.: Conceptualization, data curation, and formal analysis. W.W.: Methodology, software, investigation, visualization, and writing—original draft preparation. J.K.: Resources. W.W., J.K., and T.D.: Writing—review and editing. J.K. and T.D.: Supervision. All authors have read and agreed to the published version of the manuscript.

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Notes

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Article

Physicochemical Properties of Freeze−Dried Bigel-Based Materials Composed of Sodium Alginate/Whey Protein Isolate Hydrogel and Ethylcellulose/Sunflower Oil Oleogel

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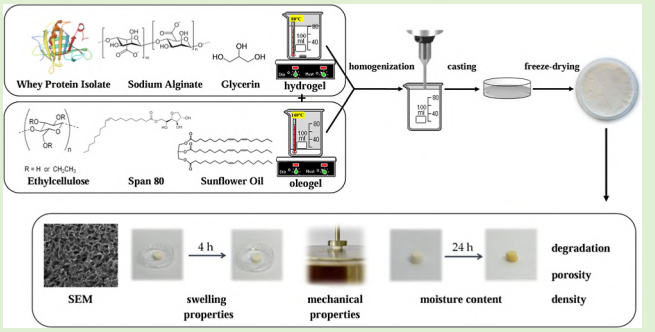
ABSTRACT: Freeze−drying bigels is a novel technique for developing functional materials for dermatological and cosmetic use, leveraging the benefits of two structured phases. This study optimized freeze−dried bigels composed of whey protein isolate (WPI)/sodium alginate/glycerin hydrogel and ethylcellulose (EC)/Span 80/sunflower oil oleogel at varying hydrogel/oleogel ratios. The materials showed swelling ratios from 50% to 255%, with higher values for a lower oleogel content and higher polymer concentration. The higher oleogel content extended the degradation from a few hours to 7 days. The polymer concentrations and hydrogel/oleogel ratios influenced Young's modulus (1.25−3.7 MPa). Porosity varied from 35% to 58%, and density varied from 100 to 200 mg/mL. The residual moisture content (5% to 20%) increased with EC content and decreased with WPI and oleogel content. These findings underscore the role of polymer concentrations and phase ratios in tuning the physicochemical properties of freeze−dried gels, positioning them as promising biomaterials for skincare and cosmetic applications.



1. INTRODUCTION

Hydrogel is a three-dimensional network of hydrophilic polymer chains that can hold large amounts of water. Hydrogels are materials widely investigated for wound healing, cosmetic, biomedical, pharmaceutical, drug delivery, and tissue engineering applications.[1,2] Whey protein isolate (WPI) and sodium alginate can be classified as relatively cheap and versatile polymers, creating hydrogel networks. WPI is a byproduct of the dairy industry, obtained during the industrial production of cheese from bovine milk. Caseins and whey protein constitute the main proteins in ruminant milk. Lipids, carbohydrates, and lactose are removed during the purification process, resulting in a product containing at least 90% proteins. The main components of WPI are β-lactoglobulin and α-lactalbumin; however, glycomacropeptide, immunoglobulins, bovine serum albumin, lactoferrin, lysozyme, prosthetic peptones, and others are also present.[3] Sodium alginate is a linear block copolymer composed of α-L-guluronic and β-D-mannuronic acid residues connected by a glycosidic bond. Homogeneous blocks composed only of the residue of one or the other acids are separated by blocks of random or alternating units of α-L-guluronic and β-D-mannuronic acids.[4] This naturally occurring anionic polysaccharide is obtained from the cell walls of marine algae, mainly brown algae (*Phaeophyceae*).[5]

Oleogel is usually prepared by gelation of polymeric organogelators or through self-assembly and noncovalent bonds with low-molecular-weight organogelators, such as fatty acids, fatty alcohols, waxes, lecithin, cyclodextrins, and others.[6] Ethylcellulose (EC) may be employed as a polymeric organogelator for different oils due to its ability to structure liquid oil directly.[7] It is a cellulose derivative, a polysaccharide linear polymer that goes through a glass transition at around 140 °C. The structure of EC-based oleogel significantly depends on the fatty acid profile of oil.[8] Saturated oil tends to create a softer gel, whereas with the increase of unsaturation, the hardness of the gel also increases. This is due to the more efficient packing of unsaturated lipids into the EC network, resulting in the increase in the oil density and hence promoting the gel strength.[9,10] Several oils have already been reported to be used in the preparation of oleogels, with sunflower oil among them.[11] Sunflower oil primarily comprises linoleic acid, a polyunsaturated fat, and oleic acid, a monounsaturated fat.[12] The oil also contains a large amount of tocopherols. Therefore,

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Table 1. Compositions of Prepared Freeze−Dried Bigels[a]

sample	ratio hydrogel/oleogel	composition of materials (%, w/w)				
		hydrogel			oleogel	
		WPI	ALG	G	EC	Span 80
10% EC + 1% WPI + 2% ALG	5/95	1	2	1	10	1
	10/90	1	2	1	10	1
	15/85	1	2	1	10	1
10% EC + 3% WPI + 2% ALG	5/95	3	2	1	10	1
	10/90	3	2	1	10	1
	15/85	3	2	1	10	1
15% EC + 1% WPI + 2% ALG	5/95	1	2	1	15	1
	10/90	1	2	1	15	1
	15/85	1	2	1	15	1
15% EC + 3% WPI + 2% ALG	5/95	3	2	1	15	1
	10/90	3	2	1	15	1
	15/85	3	2	1	15	1

[a]WPI—whey protein isolate; ALG—sodium alginate; G—glycerin; and EC—ethylcellulose.

it is a suitable oil to select in order to prepare materials intended for the skin.

Bigels are semisolid systems formed by combining a hydrogel, based on hydrophilic polymers, and an oleogel—oil gelled with an organogelator. These two immiscible gels are mixed at a high shear rate and specific temperature. There are several parameters crucial to the physicochemical properties of bigel-based materials, such as the composition of aqueous and oily phases gelled with suitable polymers (hydrophilic for hydrogels and lipophilic or amphiphilic for oleogels), the concentration of gelling agents, as well as mixing proportion of oleogel and hydrogel.[13,14] The main mechanism behind the bigels formation is the physical interactions between the two structured phases, including hydrogen bonding, which has been found to play an important role in the structure of bigels.[15,16]

Bigels' many advantages over other semisolid formulations resulting from combining two structured phases have drawn recent scientific attention, mainly concerning food applications[17−20] and topical administration of drugs and active substances.[21−23] These systems present better physicochemical properties and stability than single gel.[24,25] Moreover, they enable the delivery of both hydrophilic and lipophilic ingredients individually and simultaneously, as well as the control of their release due to blending both structured phases.[26] Nonoily nature, easy spreadability to the skin, and enhancement of the *stratum corneum* hydration are their further assets.[27] The tailorable properties of bigels, owing to the modification of each gel composition and their combinations, make them suitable materials for various applications.

Modification of bigels by freeze−drying may lead to further enhancement of their characteristics for cosmetic and dermatological applications. Freeze−drying is a dehydration process that involves removing the solvent via sublimation of frozen samples at a reduced temperature and under reduced pressure. This leads to obtaining almost anhydrous, light, and porous materials with a three-dimensional structure. Research on sodium alginate and WPI-based hydrogels has shown that freeze−drying leads to optimal porosity and water absorption for effective wound healing and drug delivery.[28,29] Hydrogels based on sodium alginate have been shown to enhance moisture retention and biodegradability, while WPI improves mechanical strength and bioactive compound encapsulation.[30,31] Similarly, studies on oleogel-hydrogel systems suggest that freeze−drying improves the oil binding capacity and mechanical strength of materials.[32,33] Hydrophilic−lipophilic balance of bigels is crucial for maintaining their stability,[34,35] and freeze−drying may disrupt this balance, leading to phase separation. To address this, incorporating stabilizers or cryoprotectants could help maintain the structural integrity of freeze−dried bigels.[36] Cryopreservation is expected to enhance porosity and water absorption due to the prevention of ice crystal formation while also improving elasticity by preventing excessive protein aggregation and maintaining a more flexible, stable structure.[37] To the best of our knowledge, there are few reports of freeze−dried bigel-based materials, mainly for medical uses, revealing significant changes in their physicochemical properties.[38,39] However, the effect of freeze−drying on bigels remains underexplored, highlighting the need for further investigation. Moreover, the idea of using such materials for cosmetic purposes is an innovation in cosmetic chemistry.

This study explores the impact of freeze−drying on bigels, addressing a gap in current research where most studies focus on bigels, oleogels, or freeze−dried hydrogels. By modifying the structure through freeze−drying, we aim to enhance porosity, swelling behavior, mechanical strength, and degradation control, making these materials more suitable for dermatological and transdermal drug delivery applications. These findings will support the development of next-generation bigel-based formulations with optimized functional properties, which will contribute to pharmaceutical, biomedical, and cosmetic applications, offering stable, effective, and scalable solutions for advanced skincare and medical treatments.

This research aimed to optimize the methodology for obtaining materials based on freeze−dried gels and characterize these materials. The hydrogel comprised sodium alginate, WPI, and glycerin, whereas the oleogel was composed of sunflower oil, EC, and Span 80. They were blended at different hydrogel/oleogel ratios using a homogenizer, frozen, and freeze−dried. Subsequently, they were characterized by scanning electron microscopy (SEM), degradation properties, mechanical properties, moisture content, swelling properties, porosity, and density. Freeze−drying of bigels exhibits an

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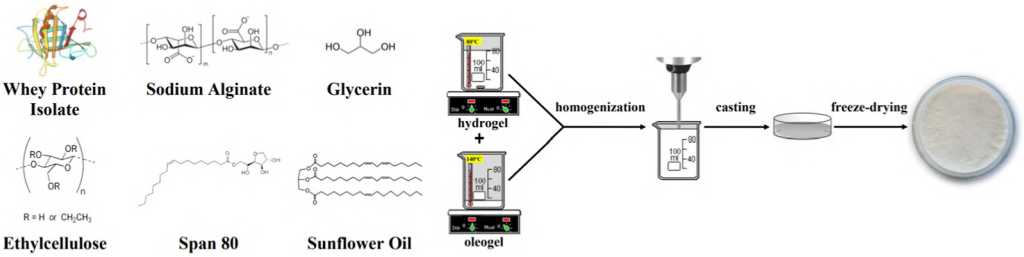

Figure 1. Freeze−dried bigel-based materials preparation method.

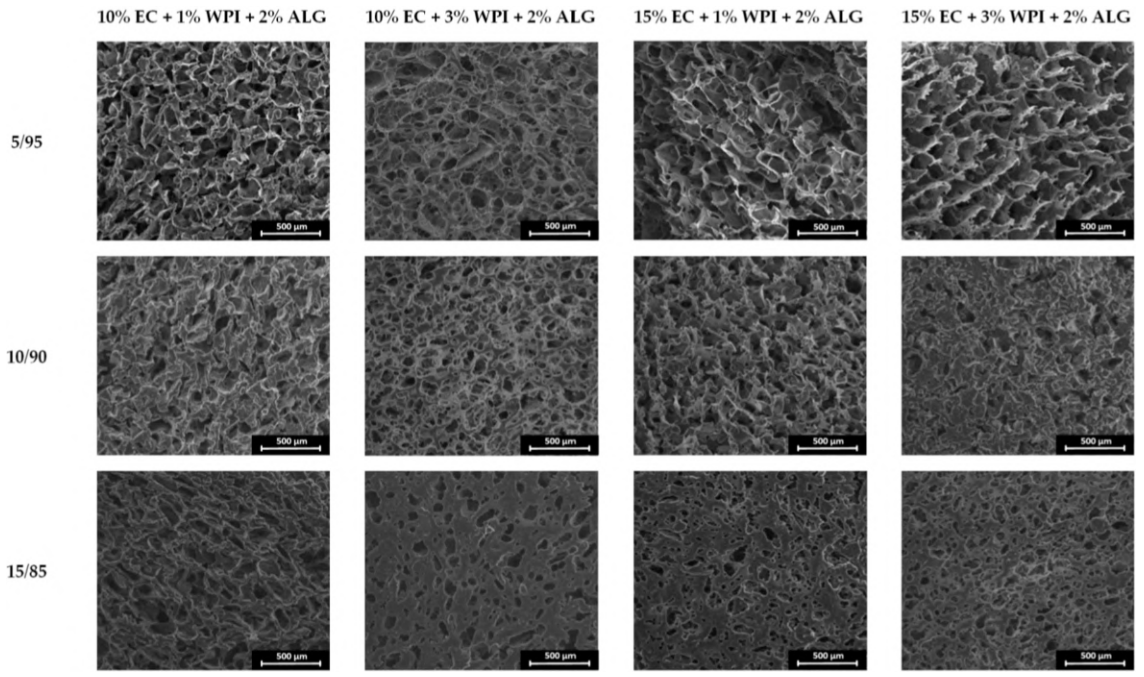

Figure 2. Structure of the obtained freeze−dried bigels in magnification ×150 (scale bar = 500 μm).

unprecedented approach to formulating modern functional materials that may be implemented in dermatological and cosmetic applications.

2. MATERIALS AND METHODS

2.1. Materials. WPI (BiPRO, Davisco Foods International Inc., Eden Prairie, MN) with 97.7% protein and 75% β-lactoglobulin in DM (according to the manufacturer's specification) was used. Sodium alginate (ALG) was obtained from BÜCHI Labortechnik AG (Flawil, Switzerland) with the viscosity average molecular weight equal to 55,800 for K = 0.0178 cm^3/g and a = 1.[40] EC and Span 80 were acquired from Sigma-Aldrich (Poznan, Poland). Glycerin, sodium phosphate, and disodium phosphate were purchased from Chempur (Piekary Slaskie, Poland). Isopropanol was supplied from Stanlab (Lublin, Poland). Sunflower oil was obtained from Nanga (Zlotow, Poland). All chemicals used were of analytical grade.

2.2. Materials Preparation. Bigels were obtained by mixing hydrogel (containing 1% or 3% of WPI, 2% of ALG, and 1% of glycerin dissolved in water) and oleogel (composed of 10% or 15% of EC and 1% of Span 80 dissolved in sunflower oil) in three oleogel/hydrogel mixing ratios, 5/95, 10/90, and 15/85 (Table 1). Both phases were heated on the magnetic stirrer at a speed of 400 rpm until suitable temperatures of both gels were obtained: hydrogel to 70−80 °C, whereas oleogel to 140 °C which is a temperature of EC dissolution (Figure 1). Afterward, they were mixed and homogenized (20,000 rpm, 3 min) (T25 digital ULTRA-TURRAX disperser, IKA Werke, Staufen, Germany). The solutions were cast on glass plates that were subsequently frozen (−20 °C) and freeze−dried (−55 °C, 5 Pa, 24 h) (ALPHA 1−2 LD plus lyophilizator, Martin Christ, Osterode am Harz, Germany).

2.3. Materials Characterization. *2.3.1. Imaging.* Structures and cross sections of obtained porous materials were evaluated by SEM imaging (Quanta 3D FEG scanning electron microscope, Quorum Technologies, Lewes, UK). Before the analysis, the surfaces of the materials were covered with thin layers of gold and palladium (SC7620 mini Sputter Coater/Glow Discharge System, Quorum Technologies, Lewels, UK).

2.3.2. Swelling Properties. In order to establish the swelling properties of 3D samples, weighed dry samples (W_d) were immersed in buffer saline (PBS, pH 5.7) for 4 h. After that time, samples were removed from PBS solution and weighted (W_w). Measurements were carried out in triplicate. The swelling ratio (1) was expressed as the percentage ratio of increased weight to the initial weight, as follows

$$\text{Swelling ratio } (\%) = (W_w - W_d)/W_w \times 100 \quad (1)$$

2.3.3. Degradation Properties. Degradation of bigel-based materials were conducted by determining percentage weight loss of samples incubated in PBS (pH = 5.7) at room temperature. Weighed samples (W_b) were put in 24-well polystyrene plates and immersed in PSB for 6, 12, 18 h, 1, 2, 3, 5, and 7 days. After each incubation time, samples were removed from the PBS and rinsed with deionized water three times. Frozen samples (−20°) were lyophilized (−55 °C, 5 Pa, 24 h) (ALPHA 1−2 LD plus freeze-dryer, Martin Christ, Osterode am Harz, Germany) and weighed again (W_a). The percentage weight loss was carried out in triplicate and calculated according to eq 2

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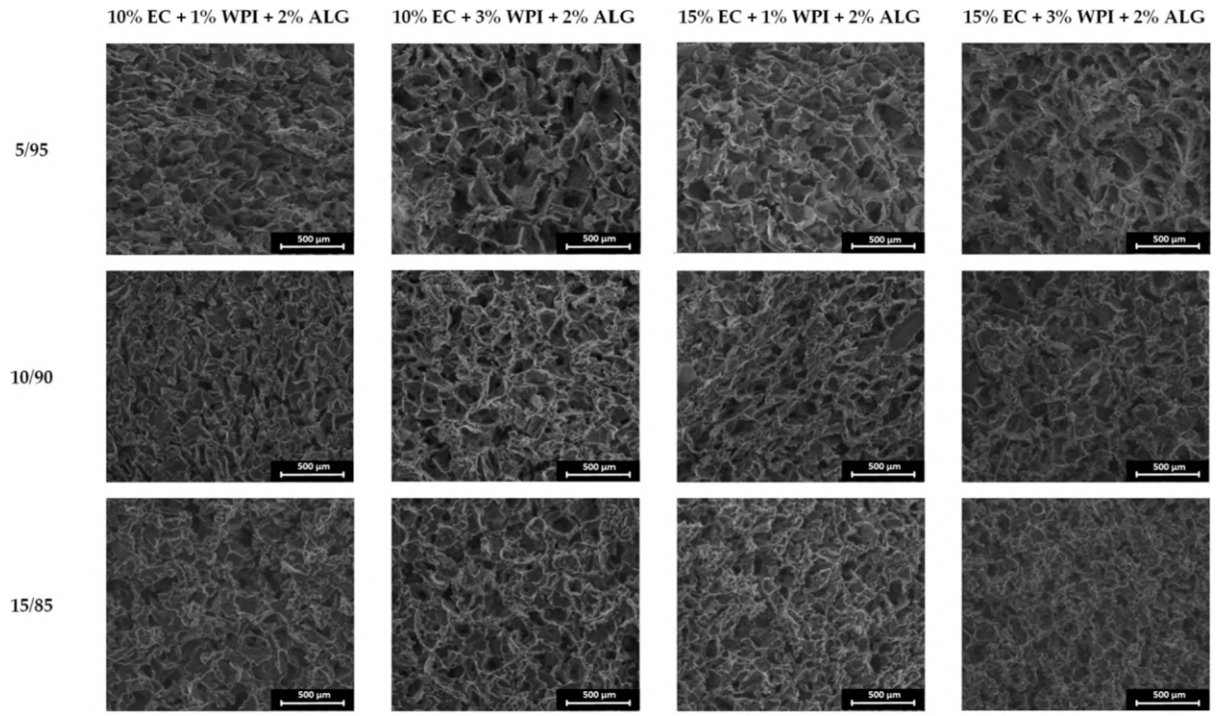

Figure 3. Cross-section of the obtained freeze−dried bigels in magnification ×150 (scale bar = 500 μm).

$$\text{Weight loss}\ (\%) = (W_b - W_a)/W_b \times 100 \quad (2)$$

2.3.4. Mechanical Properties. Mechanical properties of freeze−dried bigels were established using a mechanical testing machine (Shimadzu EZ-Test EZ-SX, Kyoto, Japan) fitted with a 50 N load cell. Cylindrical samples with a 10 mm diameter were compressed at a 5 mm/min compression speed. Stress−strain curves were recorded using the Trapezium X Texture program (version 1.4.5.), from which Young's modulus, compressive strength, and yield strength were calculated as the average values of seven measurements.

2.3.5. Porosity and Density Measurements. The porosity (E) and the density (d) of the obtained 3D materials were evaluated by a liquid displacement method using isopropanol as nonsolvent of used polymers: WPI, sodium alginate, and EC.[41] Moreover, isopropyl alcohol is able to easily permeate through the matrices and not cause swelling or shrinkage. Weighed samples (W) were placed in the graduated cylinder previously filled with isopropanol (V_1). Samples were left for 5 min, and after that the total volume of isopropanol and isopropanol-impregnated sample level (V_2) was read. Subsequently, materials were carefully removed from the cylinder. The residual isopropyl alcohol volume (V_3) was then recorded. Measurements of the matrices were performed in triplicate. Equations 3 and 4 were used to calculate the porosity and the density of samples, respectively

$$\text{E}\ (\%) = (V_1 - V_3)/(V_2 - V_3) \times 100 \quad (3)$$

$$d = W/(V_2 - V_3) \quad (4)$$

2.3.6. Residual Moisture Content. The residual moisture contents of weighed matrices (1 × 1 cm) (W_b) were evaluated as the weight loss of samples dried at 105 °C for 24 h to a constant weight. Subsequently, dried samples were weighed again (W_a). The measurements were carried out in triplicate. The residual moisture contents, defined as the percentage of the water removed from the samples, were calculated as follows (eq 5)

$$\text{Moisture content}\ (\%) = (W_b - W_a)/W_b \times 100 \quad (5)$$

2.4. Statistical Analysis. In order to statistically compare results, one-way ANOVA with Tukey's pairwise was performed using the Past 4.09 program (PAleontological Statistics Software, Oslo, Norway). Data are shown as the mean ± standard deviation for each experiment. p-Values ≤ 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1. Structure of Materials. Freeze−drying of the prepared bigels resulted in obtaining three-dimensional matrices that were soft and spongy. Their structure and cross sections are presented in Figures 2 and 3, respectively. These materials had complex, porous structures with irregular, interconnected macropores. Freeze−dried emulsions maintained a robust porous matrix with an intact structural integrity. Moreover, these materials did not exhibit phase-separated regions. However, an evident difference between samples containing more oleogels was observed. In samples with less oleogel content, the pore walls displayed a rough and wrinkled texture, whereas in materials prepared with 15/85, the pore inner walls were more smooth. The porous network was interspersed with ice crystal imprints, forming a more honeycomb-like structure in samples prepared by using a 5/95 oleogel/hydrogel mixing ratio. Meanwhile, numerous droplet imprints were visible in freeze−dried emulsions containing a 15/85 mixing ratio, suggesting retention of the original emulsion structure. It has been found that the nucleation of ice strongly affects the pore formation.[42] Since each pore results from the growth of one to a few ice grains within the polymer network, the ice grains are replaced by macropores during the sublimation. Therefore, the resulting structure of the materials is highly porous. The freeze−dried emulsion with higher WPI content exhibited a more compact, uniform, and homogeneous porous structure. Meanwhile, an increased EC concentration resulted in stable, well-formed pores. In lower oleogel/hydrogel mixing ratios, pore walls were wrinkled and folded, giving the structure a rough and irregular texture.

The synthesis process of freeze−dried bigels has a relatively low cost due to the use of widely available and biodegradable raw materials, such as sodium alginate, WPI, glycerin,

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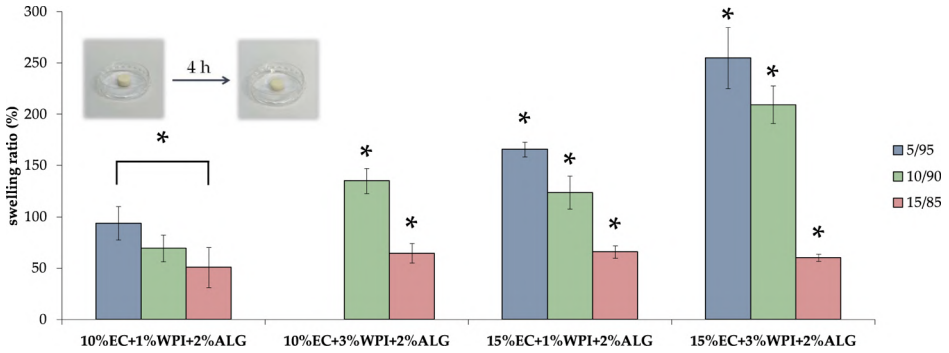

Figure 4. Swelling ratio of freeze−dried bigels. The pictures present an exemplary sample (15% EC + 1% WPI + 2% ALG) before and after 4 h of incubation in PBS buffer. ANOVA-one way with Tukey's pairwise (Cl = 95%) was performed to compare the results statistically. Significant differences among one group of materials with different oleogel/hydrogel mixing ratios were marked on the graph with (*).

sunflower oil, and EC. The primary contributors to energy use are heating and freeze−drying. While the environmental impact is low, optimizing freeze−drying efficiency and sourcing renewable energy could further enhance sustainability.

The long-term stability of freeze−dried bigel-based hybrid materials is high due to the strong interactions between the hydrogel and oleogel phases, forming a robust and cohesive network. WPI, sodium alginate, and EC provide structural reinforcement, while freeze−drying significantly enhances durability by reducing water content and preventing microbial growth.

Reports regarding freeze−dried bigels are limited. Martín-Illana et al. focused on formulating these materials with vaginally controlled release of tenofovir. Freeze−dried bigels containing pectin, chitosan, or hypromellose[38] and guar gum hydrogel and sesame oil containing Span 60 or Span 60 and Tween 60 as surfactants[39] had porous structures. They also noticed that the microstructure of the materials depended on the type of polymer used and their concentrations. Smaller pores were observed in samples with a larger amount of polymers. This can be ascribed to the greater viscosity and denser polymeric framework produced, resulting in smaller water droplets being trapped inside the network that were sublimated during freeze−drying, thus creating smaller pores.

3.2. Swelling Properties. Swelling properties were determined by immersing samples in PBS at pH 5.7 for 4 h (Figure 4). The swelling ratio of all porous materials increased due to the solvent uptake from the surrounding medium. The resulting overall swelling ratio differed from 50 to 255%. One sample (10% EC + 3% WPI + 2% ALG_5/95) dissolved. Samples containing 10% EC and 1% WPI + 2% ALG presented the lowest swelling properties in the 50 − 100% range. Increasing the WPI content to 3% resulted in higher swelling ratio (65 − 135%). Furthermore, samples containing a higher amount of EC in oleogel, i.e., 15% and 1% of WPI with 2% of sodium alginate developed swelling measurement values (65 − 165%) similar to 10% EC + 3% WPI + 2% ALG, but higher than 10% EC + 1% WPI + 2% ALG. The highest swelling properties had materials containing the highest amount of EC and WPI, ranging from 60 to 255%. Therefore, increasing the concentrations of WPI and EC in samples with 5/95 and 10/90 oleogel-to-hydrogel mixing ratios resulted in an increase in swelling ratios. Bigels with 15/85 oleogel/hydrogel proportions did not show significant differences in terms of polymer concentrations. Meanwhile, increasing oleogel content in the material composition led to a decrease in samples' swelling properties. The lowest swelling properties of samples containing the highest content of oleogel may be attributed to their lower disintegration due to the higher amount of oil in the material composition. Based on the obtained results, one can conclude that the swelling ratio significantly depended on the polymers content and the oleogel/hydrogel mixing ratio. It was higher when EC and WPI concentrations were higher and the oleogel/hydrogel mixing ratio was lower. Materials with a higher concentration of polymers and a lower proportion of oleogel were able to uptake more swelling medium, resulting in higher swelling properties. A higher amount of polymers with hydrophilic groups increased sponges' hydrophilicity and enhanced the absorption of water molecules, increasing their swelling properties.[43]

Other studies supported our findings. Alginate bigel-based beads also exhibited a lower swelling rate with the increase in the oleogel content.[44] Freeze−dried bigels prepared by Martín-Illana et al. based on guar gum hydrogel and sesame oil, adding Span60 or Span60 and Tween60 as surfactants, had a maximum swelling degree from 60 to 260%.[39] They also noticed that a smaller amount of polymers and a higher oil content led to a lower swelling ratio. This may be ascribed to the more consistent microstructure of the materials, allowing them to maintain their structure for extended periods. Manzocco et al. obtained WPI materials using freeze−drying and supercritical drying and characterized them by water uptake capacity.[45] Despite the difference in these samples' morphology, porosity, and kinetics of water absorption in time, their swelling ratio was approximately 45 − 50%. Nonetheless, the water uptake led to the destruction of materials. Sodium alginate is a component of numerous freeze−dried materials. Gelatin/alginate sponges have been reported to have a swelling ratio in the range between 100 to 900%, depending on the time of immersion and the proportions of polymers.[46] Higher swelling properties and faster degradation of samples comprising a greater amount of alginate may be linked to larger pores in sponge-like materials, which boosted the swelling rate. However, the swelling capacity of chitosan/alginate/hyaluronic acid materials was revealed to have values from 75 to 175%, with higher water uptake ability of samples containing more alginate.[47] Therefore, several parameters impact the swelling properties of porous materials, such as porosity, the hydrophilicity of polymers, the structure of polymer networks, and the interactions between polymer chains.[48]

3.3. Degradation Properties. Freeze−dried polymeric bigels were degraded in PBS (pH 5.7) for 7 days. Ours freeze−dried bigel-based materials were fabricated from degradable

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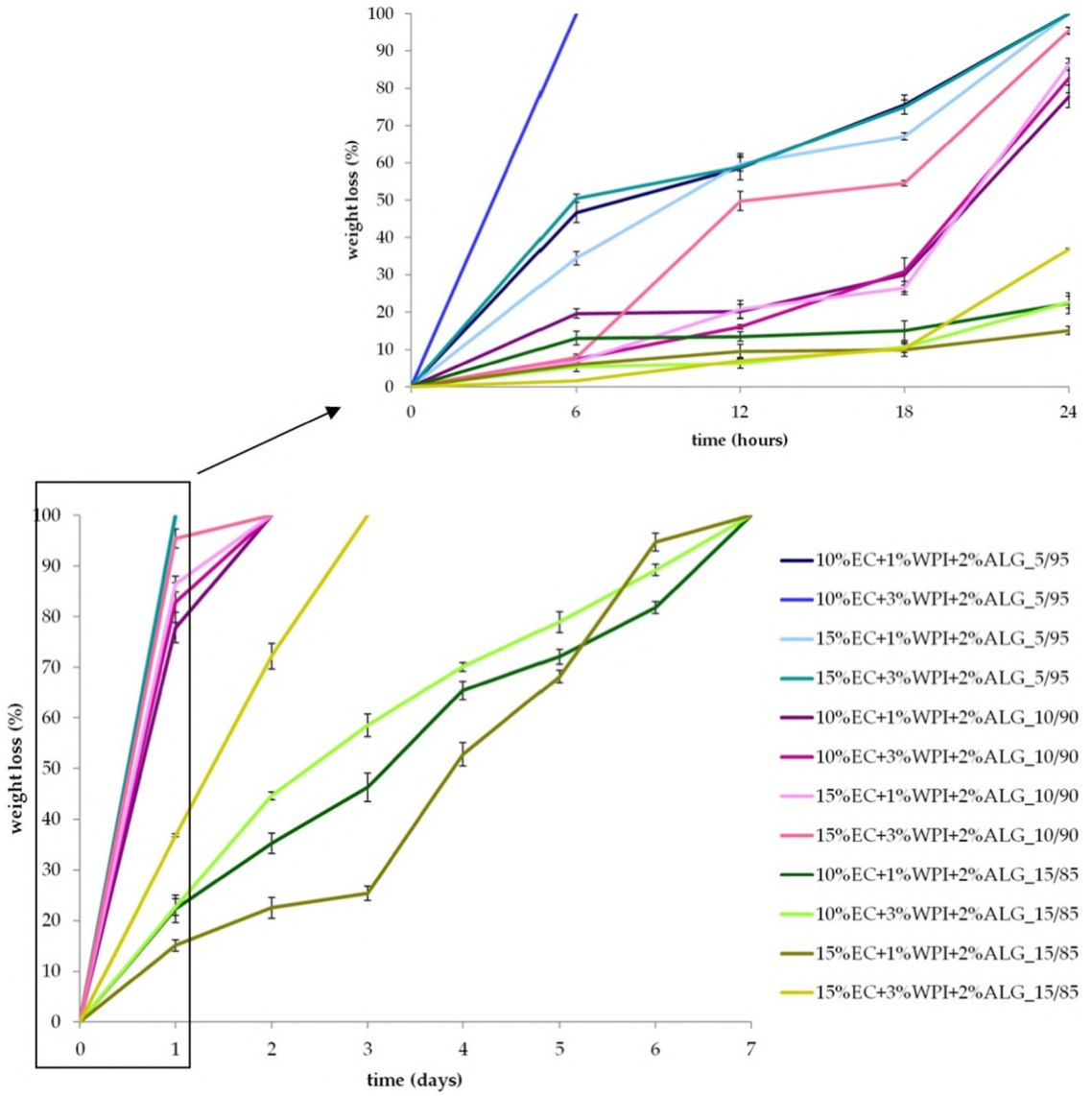

Figure 5. Values of weight loss during degradation measurements of freeze−dried bigels.

polymers: WPI, sodium alginate, and EC. Samples were not cross-linked since we designed these materials to be easily dissolved back to bigels immediately before their topical use on the skin as a skin-conditioning product. Therefore, degradation measurements were carried out in pH corresponding to the skin's natural pH.

According to the results (Figure 5), the percentage weight loss strongly depended on the hydrogel/oleogel mixing ratio. Therefore, samples containing less oleogel phase (5/95) degraded within the first 24 h. Freeze−dried bigels obtained with a 10/90 oleogel/hydrogel mixing ratio fully degraded after 2 days. Degradation of materials containing the highest amount of oleogel in their composition occurred within 7 days of analysis. Therefore, an important observation was that introducing a higher contribution of hydrophobic oleogel delayed the degradation of bigels, providing protection from degradation processes. The higher contribution of oleogel in the samples' composition resulted in a lower degradation rate due to the hydrophobic nature of the oleogel, which reduces water uptake and minimizes susceptibility to hydrolytic degradation. EC in the oleogel forms a stable nonpolar matrix that shields the material from environmental moisture, enhancing its structural integrity over time. This protective effect contrasts with hydrogel-rich compositions, which are more prone to water absorption and subsequent degradation due to their hydrophilic characteristics. However, samples containing higher concentrations of polymers, 15% of EC in oleogel, and 3% of WPI in hydrogel with 10/90 and 15/85 mixing ratios tend to have higher percentage weight loss than other samples. The higher degradation in samples with increased polymer content (15% EC and 3% WPI) is due to the hydrophilic nature of WPI, which enhances swelling and allows for greater medium penetration into the matrix. This increased medium uptake accelerates hydrolytic degradation, particularly in hydrophilic components such as WPI and sodium alginate, weakening the structural stability. Sodium alginate plays a critical role in the structural stability and swelling behavior of bigel-based materials. Its hydrophilic nature promotes water absorption and swelling, enhancing medium penetration into the matrix and potentially accelerating degradation. The degradation of samples is in agreement with the results obtained for the swelling properties. Increased

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Table 2. Mechanical Properties of the Obtained Freeze−Dried Bigels Based on WPI, Sodium Alginate (ALG), and Ethylcellulose (EC) during Compression

sample	Young's modulus (MPa)	compressive maximum force (N)	yield strength (N/mm^2)
10% EC + 1% WPI + 2% ALG_5/95	2.46 ± 0.45	15.5 ± 0.49	8.03 ± 0.27
10% EC + 1% WPI + 2% ALG_10/90	2.21 ± 0.62	12.7 ± 0.42	5.69 ± 0.65
10% EC + 1% WPI + 2% ALG_15/85	1.91 ± 0.64	9.70 ± 0.37	3.59 ± 0.27
10% EC + 3% WPI + 2% ALG _5/95	1.25 ± 0.22	4.36 ± 0.40	1.83 ± 0.48
10% EC + 3% WPI + 2% ALG _10/90	1.31 ± 0.44	5.12 ± 0.34	2.13 ± 0.41
10% EC + 3% WPI + 2% ALG _15/85	2.70 ± 0.46	5.54 ± 0.24	2.22 ± 0.52
15% EC + 1% WPI + 2% ALG_5/95	3.73 ± 0.77	12.7 ± 0.37	7.26 ± 0.37
15% EC + 1% WPI + 2% ALG_10/90	2.32 ± 0.90	12.0 ± 0.20	6.46 ± 0.40
15% EC + 1% WPI + 2% ALG_15/85	1.78 ± 0.22	9.82 ± 0.47	5.52 ± 0.14
15% EC + 3% WPI + 2% ALG_5/95	1.29 ± 0.47	5.71 ± 0.08	3.07 ± 0.19
15% EC + 3% WPI + 2% ALG_10/90	1.43 ± 0.45	5.78 ± 0.08	2.65 ± 0.32
15% EC + 3% WPI + 2% ALG_15/85	1.94 ± 0.20	5.84 ± 0.17	3.24 ± 0.29

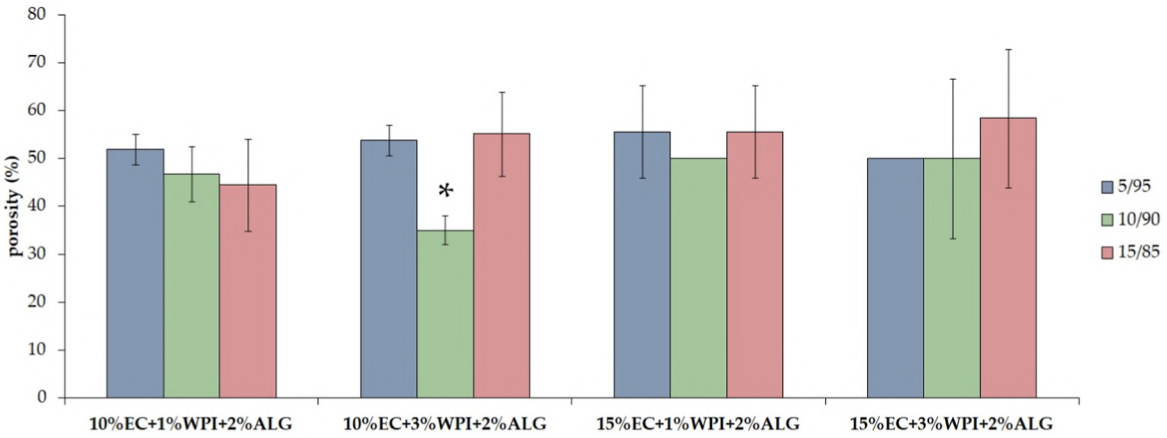

Figure 6. Porosity of freeze−dried bigels based on WPI, sodium alginate, and ethylcellulose (EC). ANOVA-one way with Tukey's pairwise (Cl = 95%) was performed to statistically compare the results. Significant differences among one group of materials with different oleogel/hydrogel mixing ratios were marked on the graph with (*).

swelling properties can enhance medium penetration into the polymer matrix, accelerating their degradation process.[49] Hence, the higher the medium uptake abilities, the higher the degradation of the obtained bigel-based materials. This observation is strictly related to their composition (content of polymers) and oleogel/hydrogel mixing ratio.

The degradation rate of freeze−dried polymeric materials depends on several factors, such as their structure and composition, moisture content, and porosity.[50] It was noted that hydrolysis and proteolysis are essential mechanisms for the degradation of materials based on polysaccharides and proteins. This leads to the depolymerization of polysaccharides into monomers influenced by water molecules, leading to the cleavage of chemical bonds[51] or the breakdown of proteins into smaller peptides or amino acids[52] and, eventually, material breakdown. Nonetheless, incorporating lipids into freeze−dried polymeric materials may lead to complex changes in their degradation behavior, influenced by factors such as moisture absorption, chemical interactions, and mechanical properties.[53,54] The hydrophobic nature of oleogel can reduce the polymer matrix's overall water absorption, slowing down hydrolytic degradation. Quickly degradable materials are increasingly sought by scientist for various applications due to their environmental benefits and potential for sustainability.[55] Some critical applications may include packaging, pharmaceuticals, agriculture, and personal care products.

3.4. Mechanical Properties. The values of Young's modulus and compressive and yield strength significantly depended on the content of polymers: WPI in hydrogel and EC in oleogel, as well as their mixing ratio (Table 2). Samples containing 3% of WPI exhibited a rise in the values of Young's modulus and compressive and yield strengths with an increasing oleogel/hydrogel mixing ratio. However, the mechanical parameters decreased with the increasing oleogel/hydrogel mixing ratio in samples containing 1% of WPI. Young's modulus decreased with a higher oleogel-to-hydrogel mixing ratio since the oleogel phase was softer and less rigid than the hydrogel phase. As the oleogel content increased, it disrupted the denser and more interconnected network provided by the hydrogel, reducing the material's overall stiffness. This shift in composition led to a more compliant structure, resulting in lower resistance to deformation under stress and, consequently, a lower Young's modulus. The results indicated that samples containing 1% WPI, 2% sodium alginate, and 15% EC in a 5/95 oleogel/hydrogel mixing ratio (~3.7 MPa) were the stiffest materials showing the most resistance to compression.

Bigel-based beads containing alginate hydrogel and glycerol monostearate oleogel exhibited a decrease in Young's modulus and hardness with the increase in oleogel contribution.[44] However, increasing the oleogel content led to an increase in hardness (from ~ 0.1 N to ~ 2 N) of gelatin-glycerol monostearate bigels with the best mechanical properties for

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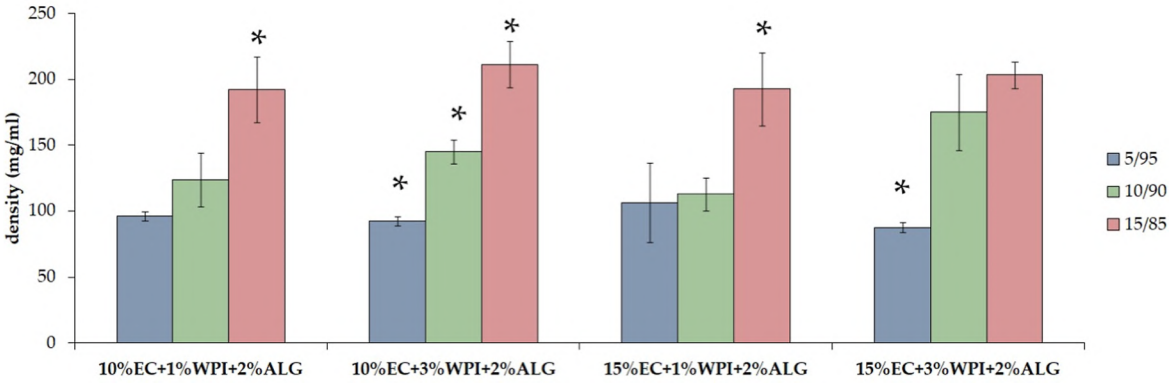

Figure 7. Density of freeze−dried bigels based on WPI, sodium alginate, and EC. ANOVA-one way with Tukey's pairwise (CI = 95%) was performed to statistically compare the results. Significant differences among one group of materials with different oleogel/hydrogel mixing ratios were marked on the graph with (*).

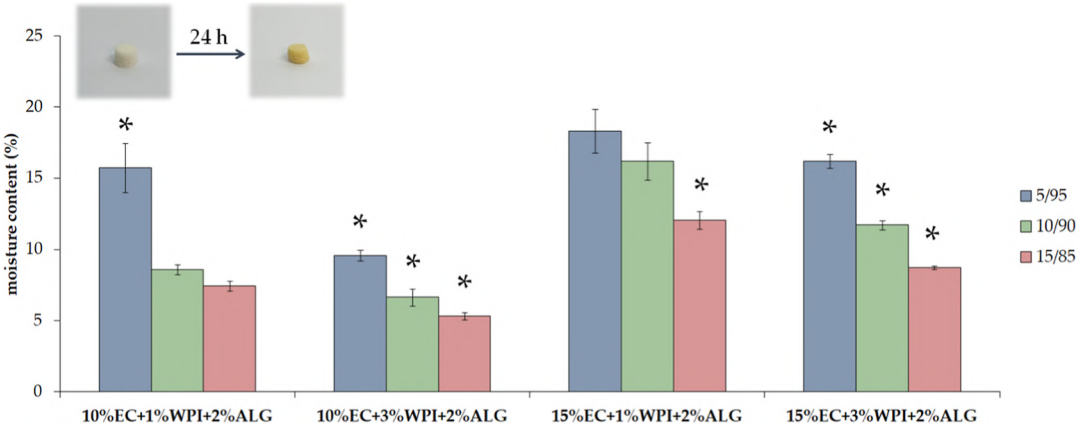

Figure 8. Residual moisture content of freeze−dried bigels. The pictures present an exemplary sample (15% EC + 1% WPI + 2% ALG) before and after 24 h of drying at 105 °C. ANOVA-one way with Tukey's pairwise (Cl = 95%) was performed to statistically compare the results. Significant differences among one group of materials with different oleogel/hydrogel mixing ratios were marked on the graph with (*).

samples homogenized for 3 min.[56] Freeze−dried bigels based on organogel (sesame oil and Span 60) and hydrogel (pectin, chitosan or HPMC and Tween60) were increasingly deformable, and they presented hardness from 1.21 to 6.48 N.[38] Aerogel prepared from an oil-in-water emulsion containing WPI and soybean oil modified by the addition of guanidinium hydrochloride presented high yield stress (1.4 MPa) and Young's modulus (16.9 MPa).[57] The authors linked it to the cellular structure and the synergistic effect of enhanced intermolecular disulfide bonds and oil droplets working as cross-linkers. Chen et al. obtained foam-like materials based on WPI and blends of WPI with alginate in order to improve their mechanical properties during compression.[58] It revealed that pure WPI aerogels were very brittle (0.18−1.6 MPa) depending on the concentration of WPI. However, the greater addition of alginate resulted in further modulus improvement from 0.48 to 12.9 MPa. Alginate/gelatin materials had a maximum elastic stress of around 0.4 and 0.2 MPa and Young's modulus of approximately 3.5−4 MPa,[59] 6.7 MPa, and after additional modification with usnic acid 2.3 and 21.1 MPa.[60] However, porous materials based on alginate blended with different polymers have not always been shown to exhibit enhanced mechanical properties. Adding alginate to bacterial cellulose sponges decreased Young's modulus from 3 MPa to less than 1 MPa.[61] In comparison, alginate/chitosan sponges were noticed to have a less defined microstructure than the single component sponges, resulting from a polymeric network being more randomly ordered during the freezing of samples.[62] The compression force of chitosan samples was ∼5.5 N and that of alginate materials was less than 0.5 N, while their mixtures had compression force in between those results.

3.5. Porosity and Density Measurements. Porosity and density were evaluated via liquid displacement using isopropyl alcohol (Figures 6 and 7). Porosity showed no significant differences in terms of different polymer contents and the oleogel/hydrogel mixing ratio. The porosity of freeze−dried bigels based on EC/sunflower oil oleogel and WPI/alginate hydrogel ranged from 45 to 58%. The sample 10% EC + 3% WPI + 2% ALG_10/90 was an exception, which showed a significantly lower porosity (35%).

The formation of pores is strictly related to the nucleation of ice during the freezing of materials and the sublimation of ice crystals during the freeze−drying process.[42] Porosity and the shape and size of pores are linked to the morphology of ice crystals. Hence, these parameters can be adjusted depending on the purpose of the application of the materials. Freeze−dried materials based on alginate alone or in combination with different polymers, such as chitosan and gelatin, have been reported to have a wide range of porosity: 38−57%,[63] 30−90%,[64] ∼ 90%,[65] and 92%.[66]

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Based on the measurements (Figure 7), the density of freeze−dried bigels showed a difference from around 100 mg/mL (for samples with a 5/95 oleogel/hydrogel mixing ratio) to 200 mg/mL (for materials containing oleogel/hydrogel mixing ratio: 15/85). This indicates that this parameter increased with the increase of the oleogel ratio in materials. However, we did not observe differences in the density of samples containing different amounts of polymers: WPI in hydrogel or EC in oleogel.

Although xanthan and guar gum-based bigels displayed significantly higher densities (790−840 mg/mL[67]), the results of the materials after freeze−drying aligned with ours. Manzocco et al. fabricated WPI aerogels with low density ranging from 0.22 to 0.29 g/cm^3, which depended on the preparation method: freeze−drying of samples resulted in lower-density materials than supercritical−CO_2−drying.[45] However, Chen et al. observed differences in the density of WPI/alginate materials depending on the polymers contents. WPI-based porous materials exhibited density ranging from 0.112 to 0.245 g/cm^3, increasing with the higher content of WPI, whereas foam-like material based on alginate had a density of 0.047 g/cm^3.[58] They also prepared samples blending WPI and alginate with a density differing from 0.0592 to 0.129 g/cm^3, indicating that the addition of alginate decreased the density of the materials. Materials based on alginate blended with different components showed similar densities. Foams based on alginate, potato starch, and the clay mineral sepiolite prepared by Darder et al. presented density from 0.123 to 0.242 g/cm^3 with higher density for samples cross-linked with calcium ions.[68] The density of alginate/halloysite nanotube composite scaffolds ranged from 0.035 to 0.139 g/cm^3, with the lowest density for pure alginate samples.[69] The researchers explained the increased density of composite materials to the greater constituent content in the material volume since the water volume was adjusted during the preparation of samples.

3.6. Residual Moisture Content. The residual moisture content analysis was performed by drying the samples at 105 °C for 24 h (Figure 8). It is a crucial parameter determining the stability of obtained freeze−dried materials. The moisture content of freeze−dried bigels significantly depended on their composition due to their components' hydrophilic and hydrophobic characteristics, as well as their interactions during the freeze−drying process. The moisture content increased with higher EC content since it created a denser oleogel network that might entrap small amounts of residual water within its matrix. Conversely, increasing the WPI content reduced the moisture content as WPI promoted stronger protein−protein interactions during gel formation, which enhanced water removal during freeze−drying. Similarly, a higher oleogel-to-hydrogel mixing ratio decreases moisture content as the hydrophobic oleogel phase limits water retention. Therefore, the moisture content in these freeze−dried bigels was from 5 to almost 20%.

The results of residual moisture content measurements were in agreement with those of other studies. Freeze−dried hydrogels based on calcium alginate with loaded ciprofloxacin presented moisture content from 13.28% to 17.30%.[70] Meanwhile, sponges prepared from the alginate and acacia gum mixture cross-linked with $CaCl_2$ had moisture content in the 7−27% range with potential as wound dressing.[71] It was established that the residual moisture content is highly dependent on secondary drying temperatures.[72] Freeze−drying is a multistep process consisting of freezing the sample and primary drying, which is the sublimation of frozen water under vacuum. The last step is secondary drying, when unfrozen water from samples is removed by desorption at elevated temperatures. An optimal residual moisture content has been demonstrated not to be the lowest possible since very low moisture content accelerates oxidation and degradation of proteins.[73,74]

3.7. General Discussion. The exact mechanisms affecting the stability of freeze−dried bigels remain unclear, but they may be dictated by a combination of hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic associations between the hydrogel (WPI/sodium alginate/glycerin) and oleogel (EC/sunflower oil/Span 80) phases. Within the hydrogel, WPI—rich in β-lactoglobulin and α-lactalbumin—may have stabilized the network through protein−protein interactions, including disulfide bridge formation and hydrogen bonding,[75] as well as protein−polysaccharide interactions, namely electrostatic interactions with sodium alginate.[76] Meanwhile, sodium alginate not only enhanced structural integrity via electrostatic interactions but also contributed to water retention and swelling due to its highly hydrophilic carboxyl and hydroxyl groups. Furthermore, glycerin, acting as a plasticizer, disrupted excessive protein aggregation and enhanced the flexibility of the materials. The oleogel phase was structured by EC, which may have formed a gel network through hydrogen bonds during thermally induced gelation, with sunflower oil trapped within the polymeric matrix.[77,78] In addition, Span 80 may have acted as a nonionic surfactant to improve interfacial adhesion between the two immiscible gel phases, preventing phase separation during freeze−drying.

The porosity of these bigels was primarily influenced by the freeze−drying process, where ice crystal formation dictated the pore structure. Higher oleogel content led to a denser, more compact matrix (Figure 7), while a lower oleogel content resulted in an enhanced residual moisture content (Figure 8) and swelling properties (Figure 4). Swelling behavior will have been driven by the hydrophilicity of the polymer network; sodium alginate is highly hydrophilic and molecules in WPI contain hydrophilic regions. The presence of these molecules will have enabled water uptake, increasing swelling capacity. In contrast, the hydrophobic oleogel phase limited water penetration, thereby reducing swelling. The mechanical properties of the bigels, as shown in Table 2, depended on the balance between hydrogel elasticity and oleogel rigidity, with higher EC content increasing stiffness and Young's modulus. Degradation of the freeze−dried gels might have occurred through hydrogel dissolution and hydrolysis. Although higher WPI content may have increased stability, as discussed above, it may have accelerated breakdown due to increased water absorption and solvent accessibility, while higher EC content and oleogel-rich formulations may have provided a hydrophobic barrier that slowed degradation, extending it to 7 days (Figure 5). It should be noted that this discussion remains speculative; the elucidation of the exact mechanisms remains a topic for further study.

These molecular interactions allow for precise tuning of the bigels' physicochemical properties, enabling controlled swelling, degradation, and mechanical performance, making them promising candidates for dermatological applications, transdermal drug delivery, and advanced biomaterial formulations with tailored release and enhanced stability.

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4. CONCLUSIONS

In this study, functional freeze−dried bigels based on a WPI/sodium alginate/glycerin hydrogel and EC/Span 80/sunflower oil oleogel were successfully formulated. Physicochemical properties, such as swelling, degradation and mechanical properties, moisture content, and density significantly depended on the content of biopolymers in samples: WPI (1% or 3% in hydrogel) and EC (10% or 15% in oleogel), as well as the oleogel/hydrogel mixing ratio (5/95, 10/90, or 15/85). The lower the oleogel ratio and the higher the EC concentration in prepared samples, the higher their swelling ratio (from ∼50 to ∼ 255%) and moisture content (∼5 to ∼20%). The mechanical properties of freeze−dried bigels described as Young's modulus, compressive strength, and yield strength ranged from 1.25 to 3.7 MPa, with the highest result for the sample containing 15% of EC in oleogel and 1% of WPI in hydrogel with 5/95 oleogel/hydrogel mixing ratio. The porosity of formulated materials (from 45 to 58%) did not significantly differ in terms of studied variables except for the lowest value (35%) for the sample containing 10% EC in oleogel and 3% WPI in hydrogel with a 10/90 mixing rate. A rise in density (from ∼ 100 to 200 mg/mL) and prolonged degradation (from 6 h to 7 days) with the increase in the oleogel content in bigels was observed.

These findings highlight the potential of freeze−dried bigels as versatile and customizable biomaterials not only for dermatological and cosmetic applications. Considering both structured phases of bigels and resulting from these additional benefits, the possibility of simultaneous addition of hydrophilic and lipophilic active substances, stability, and good characteristics for skin application makes them promising candidates for advanced skincare formulations, wound healing applications, and transdermal drug delivery systems. Furthermore, their tunable swelling and degradation profiles offer opportunities for controlled release applications. Therefore, future work should focus on evaluating the biological compatibility and efficacy of these drugs in delivering active compounds for dermatological and cosmetic use. This involves conducting application tests after hydration, such as rheological evaluation, spreadability assessment, and in vitro analysis of skin biophysical parameters as well as optimizing their composition for enhanced bioactivity and incorporating therapeutic agents for specific applications to further expand their potential.

■ ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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Conceptualization, W.W. and J.K.; methodology, W.W.; software, W.W.; investigation, W.W.; resources, W.W. and J.K.; data curation, W.W.; formal analysis, W.W.; writing—original draft preparation, W.W.; writing—review and editing, W.W., J.K., and T.D.; visualization, W.W.; and supervision, J.K. and T.D. All authors have read and agreed to the published version of the manuscript.

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I hereby declare that in the publication:

W. Walendziak, N. R. Villegas, T. E. Douglas, J. Kozlowska, *Phytochemical studies of plant extracts enclosed in chitosan microparticles and the effect of phytoformulations on skin condition*, European Polymer Journal, 2025, 233, 113968

my contribution consisted of conceptualization, methodology, software, formal analysis, investigation, resources, data curation, visualization, writing – original draft preparation, writing – review and editing, project administration, funding acquisition.

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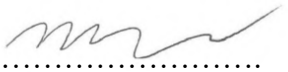

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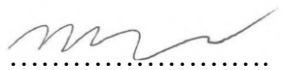

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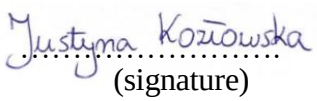

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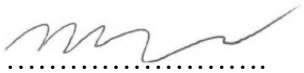

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