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Development and Optimization of an *In Vitro* Release Method for β-Arbutin from Chitosan–Hyaluronic Acid Films

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Abstract

The aim of this study was to develop and optimize an *in vitro* method for evaluating β-arbutin release from chitosan–hyaluronic acid films for cosmetic use and to investigate the effects of hydrodynamic conditions and formulation composition on release behavior. Films containing β-arbutin were prepared by solvent casting with chitosan and hyaluronic acid as film-forming polymers. Glycerol, sodium citrate, and tannic acid were added as additives. Release studies were conducted in phosphate-buffered saline (PBS, pH 5.5) at 32 ± 0.5 °C and 50 rpm, using different medium volumes (50–200 mL) and two agitation systems. β-Arbutin concentration was determined by a validated UV–Vis spectrophotometric method. The method showed excellent linearity (R^2 = 0.9994), with LOD and LOQ values of 0.0035 and 0.0105 mg/mL, respectively. Hydrodynamic conditions affected release profiles, analytical applicability, and discriminatory ability of the method. Among the tested conditions, experiments in 100 mL of medium using a shaking water bath provided the most suitable balance between reliable quantification and discriminatory ability for preliminary formulation screening. All films showed rapid β-arbutin release, while formulation additives affected the early-stage release behavior. The developed method is a suitable tool for preliminary evaluation and comparison of cosmetic polymeric films containing β-arbutin.

Key words: β-arbutin, chitosan–hyaluronic acid films, *in vitro* release, hydrodynamic conditions, cosmetic formulations

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Introduction

Hyperpigmentation disorders are among the most common aesthetic skin concerns and are often linked to excessive melanin production or uneven melanin distribution in the epidermis (1). Various depigmenting agents are therefore incorporated into cosmetic formulations designed for skin brightening and the treatment of hyperpigmentation. Among these, β-arbutin has attracted considerable attention due to its ability to inhibit tyrosinase activity and reduce melanin synthesis. Compared to hydroquinone, β-arbutin is considered a safer alternative with a more favorable safety profile while maintaining significant depigmenting activity (2, 3). Its natural origin is also important in the context of current cosmetic industry trends and consumer preferences for naturally derived ingredients and sustainable formulations (2, 4, 5).

In recent years, polymeric films have emerged as promising carriers for topical and cosmetic applications due to their ability to provide localized delivery of active substances, ease of application and removal, and favorable user acceptability (6). Films based on hydrophilic polymers are especially notable because they can rapidly hydrate and swell upon contact with aqueous media, which may facilitate the release of incorporated active compounds (7). Chitosan is widely studied in topical and cosmetic formulations because of its film-forming ability, biocompatibility, bioadhesive properties, and favorable safety profile. Chitosan-based films are increasingly investigated for cosmetic and dermal applications due to their ability to form flexible and bioactive matrices suitable for incorporating active compounds (8, 9). However, films made solely from chitosan may have limited stability in mildly acidic conditions corresponding to the physiological pH of the skin surface (10). To address these limitations, chitosan can be combined with oppositely charged polymers such as hyaluronic acid, leading to the formation of polyelectrolyte complexes (11). These systems often exhibit improved mechanical properties, enhanced stability, better hydration, and modified release characteristics compared to films based on individual polymers (12). Both chitosan and hyaluronic acid are naturally derived polymers with established safety for cosmetic and pharmaceutical applications (13). Hyaluronic acid is also a highly important and widely used ingredient in cosmetic and dermal formulations due to its excellent moisturizing properties, biocompatibility, and ability to improve skin hydration and elasticity. In addition, hyaluronic acid helps maintain skin barrier function and is frequently incorporated into topical formulations intended for skin care and regeneration (14).

Besides polymer composition, the incorporation of additives such as plasticizers and crosslinking agents can significantly influence film properties and the release behavior of active substances (15). Glycerol is commonly used as a plasticizer due to its compatibility with hydrophilic polymer matrices and its ability to increase polymer chain mobility and film flexibility (16). For polymeric films, ionic crosslinking agents may be particularly advantageous compared to covalent crosslinkers because they generally allow milder processing conditions, improved biocompatibility, and lower toxicity risk. Furthermore, ionic interactions can provide a suitable balance between film stability,

mechanical resistance, swelling behavior, and active substance release (17, 18). Among different ionic crosslinking agents, sodium citrate and tannic acid are especially notable due to their favorable safety profile and ability to establish intermolecular interactions within hydrophilic polymer networks (19–21). Such interactions may lead to the formation of denser polymer matrices and consequently modified diffusion and release characteristics of incorporated active compounds (22).

Although numerous studies have investigated polymeric films as drug carriers, considerably less attention has been given to the development and optimization of appropriate *in vitro* methods for evaluating the release of cosmetic actives from such systems (23, 24). Dissolution and release testing are considered critical quality and performance tools during the development of film dosage forms because they provide information about formulation performance, enable discrimination between formulations, and may help predict *in vivo* behavior (25). However, most available literature and standardized dissolution methodologies are primarily focused on pharmaceutical dosage forms intended for topical or transdermal (systemic) drug delivery (26–28). In contrast, cosmetic films are generally intended for localized and relatively short-term application on the skin surface, where the primary goal is local cosmetic activity rather than systemic absorption (23). Consequently, conventional dissolution methods used for pharmaceutical products may not be fully appropriate for cosmetic film formulations. For cosmetics, the experimental setup should provide sufficient sensitivity and discrimination while maintaining conditions relevant to topical application (29). Hydrodynamic conditions, including apparatus type, agitation pattern, and medium volume, can significantly affect diffusion layer thickness, mass transport, discrimination between formulations, and the analytical reliability of the method (30). Excessive medium volumes may cause pronounced dilution of the released active substance, resulting in concentrations that approach or fall below the limit of quantification (31). Furthermore, unlike conventional pharmaceutical dissolution testing, where sink conditions are strongly emphasized (32), complete sink conditions may not represent the most biorelevant approach for short-term cosmetic applications intended to achieve only local effects.

Previous studies involving polymeric films have often used paddle-over-disk systems (33), vertical diffusion cells (34, 35), or modified dissolution setups to evaluate release behavior (36–38). However, there is still no widely accepted or standardized approach for *in vitro* release testing of cosmetic films, especially those based on hydrophilic biopolymer matrices intended for short-term topical application.

The aim of this study was to develop and optimize an *in vitro* method for evaluating β-arbutin release from chitosan–hyaluronic acid films and to investigate the influence of hydrodynamic conditions and formulation composition on the release behavior of β-arbutin. Particular emphasis was placed on the effects of medium volume and apparatus type on method discrimination and analytical reliability to identify experimental conditions suitable for preliminary evaluation and comparison of cosmetic film formulations.

Experimental Section

Materials

β-Arbutin (Thermo Scientific, China, CAS 497-76-7) was used as the active substance. Chitosan (Sigma-Aldrich, China, degree of deacetylation 81.8%, molecular weight 50–190 kDa, CAS 9012-76-4) and hyaluronic acid (Alekpharm, Serbia, molecular weight 1000 kDa, CAS 9004-61-9) were used as film-forming polymers. Glycerol (Zorka Pharma, Serbia) was used as a plasticizer to improve film flexibility, while sodium citrate (Centrohem, Serbia) and tannic acid (Centrohem, Serbia) were used as mild ionic crosslinking agents to modify the polymer network structure. Glycerol, sodium citrate, and tannic acid were considered formulation additives.

Phosphate-buffered saline (PBS, pH 5.5) used as the release medium was prepared by dissolving 0.79 g $Na_2HPO_4 \cdot 2H_2O$, 7.41 g NaH_2PO_4 (anhydrous), and 8.00 g NaCl in distilled water to a final volume of 1000 mL.

Purified water was used for the preparation of all formulations and media. All substances were used without further purification.

Methods

Preparation of Films

Films were prepared using the solvent casting method. Chitosan stock solution (1% w/w) and hyaluronic acid stock solution (0.5% w/w) were prepared by dissolving the appropriate amount of polymer in acetate buffer at pH 5.0 under continuous stirring with a magnetic stirrer (C-MAG HS7, IKA, Germany) at 500 rpm for 24 h at room temperature (22–25 °C) to ensure complete polymer dissolution.

Polymer mixtures were then prepared by mixing equal volumes of the stock solutions. Hyaluronic acid solution was added gradually, dropwise, to the chitosan solution under continuous stirring at 800 rpm with a magnetic stirrer. In the final polymer mixture, the mass ratio of chitosan to hyaluronic acid was 2:1.

After preparing the polymer mixtures, one portion was reserved for the films without additives, while the remaining portions received one of the following additives: glycerol, sodium citrate, or tannic acid, each in an amount corresponding to 20% of the total polymer mass (chitosan + hyaluronic acid). The selected concentration was determined based on preliminary formulation experiments, which identified it as suitable for obtaining homogeneous films with appropriate integrity and handling properties for subsequent *in vitro* release studies. Thus, four formulations were prepared: one without additives and three with different additives. Sodium citrate and tannic acid were dissolved in a small amount of acetate buffer immediately before being added to the polymer mixtures.

Next, β-arbutin, previously dissolved in a small amount of acetate buffer, was added to all formulations at a concentration corresponding to 15% of the total polymer mass. It

should be noted that this value does not represent the final concentration of β-arbutin in the total film mass, as the films also contained plasticizer or crosslinking agents and residual moisture after drying. Therefore, the final β-arbutin concentration in the complete film formulations was lower and approximately matched concentrations commonly found in cosmetic products intended for topical application (39).

All mixing and homogenization steps were performed at room temperature (22–25 °C).

To ensure that all formulations were prepared with the same total amount of solvent and to maintain identical final polymer concentrations, the amount of acetate buffer used to dissolve sodium citrate, tannic acid, and β-arbutin was subtracted from the total buffer used for preparation of the polymer stock solutions.

The resulting mixtures were stirred for the additional 24 h at 800 rpm and then poured into silicone molds (5.5 × 12.5 cm). The films were dried under ambient conditions (22–25 °C, without controlled air humidity) for 4 days.

After drying, the films were removed from the molds and cut into 3 × 3 cm samples for further analysis. The films were evaluated as freshly prepared formulations. During the short storage period before analysis, they were protected from moisture, elevated temperature, and light to minimize possible changes in film properties and potential β-arbutin degradation.

Development of an *In Vitro* Method for Evaluating β-Arbutin Release from Films

In vitro release studies were conducted using film samples measuring 3 × 3 cm. The theoretical β-arbutin content in each film sample was 3 mg. Release experiments were performed in phosphate-buffered saline (PBS, pH 5.5) at 32 ± 0.5 °C with an agitation speed of 50 rpm. The selected medium and temperature were intended to simulate physiological skin conditions relevant for topical cosmetic application. The films were affixed to disks with a diameter of 41.2 mm using adhesive tape to ensure consistent contact between the film surface and the release medium throughout the experiment.

Different hydrodynamic conditions were systematically evaluated by varying both the apparatus type and the medium volume. For experiments using 50 and 100 mL of release medium, a shaking water bath (LSB18 Aqua Pro, Grant, United Kingdom) was used. Experiments with 150 and 200 mL of release medium were conducted using a rotating mini paddle apparatus (DT 126 light, Erweka, Germany). The shaker system provided oscillatory movement of the medium, while the rotating mini paddle apparatus created rotational hydrodynamic conditions. These differences were expected to affect diffusion layer thickness, mass transport, and consequently the release behavior of β-arbutin from the films. Smaller medium volumes (50 and 100 mL) could not be used with the rotating mini paddle apparatus due to technical limitations related to proper immersion and operation, while larger medium volumes (150 and 200 mL) were not feasible in the shaker system. These experimental limitations were considered during the interpretation of the results and method optimization.

Sampling was performed at 5, 10, 15, 20, 25, 30, 45, and 60 min. When 50 mL of medium was used, 1 mL aliquots were withdrawn; for all other medium volumes, 2 mL aliquots were sampled. All samples were filtered through MF-Millipore® membrane filters with a pore size of 0.45 μm. Immediately after sampling, the withdrawn medium volume was replaced with fresh PBS to maintain a constant release medium volume during the experiment.

β-Arbutin concentration was determined using a UV–Vis spectrophotometer (Evolution 300, Thermo Scientific, USA) at the absorption maximum of β-arbutin (λ = 280 nm), using a validated analytical method. Three consecutive absorbance measurements were performed for each collected sample, and the mean value was used to calculate the β-arbutin concentration. Placebo films corresponding to each investigated formulation were prepared under identical conditions, but without β-arbutin. They were subjected to the same release procedure under the corresponding experimental conditions and used as matrix blanks to correct for any potential UV absorbance originating from the film-forming polymers and additives. The percentage of released β-arbutin was calculated based on the experimentally determined β-arbutin concentration and the total medium volume, with the theoretical β-arbutin content in a 3×3 cm film sample (3 mg) considered as 100% release, according to Equation (1):

$$Q(\%) = (m_t / m_0) \times 100 \quad (1)$$

where m_t represents the amount of β-arbutin (mg) released at time t, and m_0 represents the theoretical β-arbutin content in the film sample (3 mg).

The results were expressed as the mean percentage of released β-arbutin as a function of time ± standard deviation (SD). All experiments were performed in triplicate.

Method development included systematic evaluation of the influence of apparatus type and medium volume on β-arbutin release profiles to identify hydrodynamic conditions that provide reproducible testing and adequate discriminatory ability for preliminary formulation screening.

In addition to hydrodynamic parameters, the influence of formulation composition, including the presence or absence of plasticizer (glycerol) and ionic crosslinking agents (sodium citrate and tannic acid), on β-arbutin release behavior was also investigated.

Validation of UV–Vis Spectrophotometric Method for β-Arbutin Determination

The calibration curve was constructed in PBS pH 5.5 over the concentration range of 0.02–0.15 mg/mL. This range was selected to encompass the expected β-arbutin concentrations in samples obtained during *in vitro* release studies while ensuring measurements remained within the reliable absorbance range of the spectrophotometer.

Absorbance values of the prepared standard solutions were measured at 280 nm, corresponding to the absorption maximum of β-arbutin. Linear regression analysis was performed to determine the calibration curve equation according to Equation (2):

$$y = kx + n \quad (2)$$

where y is absorbance, x is β-arbutin concentration (mg/mL), k is the slope, n is the intercept on the y-axis.

The coefficient of determination (R^2) was also calculated as a measure of method linearity. Method linearity was considered satisfactory when $R^2 \geq 0.99$.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH Q2(R1) guideline (40) using Equations (3) and (4):

$$LOD = 3.3\sigma / k \quad (3)$$

$$LOQ = 10\sigma / k \quad (4)$$

where σ is the standard error of the intercept obtained by linear regression analysis (defined by ICH Q2(R1) as the standard deviation of the response), k is the slope of the calibration curve.

The working range of the method was defined based on both calibration curve linearity and the reliable operating absorbance range of the spectrophotometer (0.10–1.00 absorbance units). Only results obtained within the validated analytical range were considered suitable for quantitative analysis.

Linear regression analysis, calculation of slope, intercept, standard error of the intercept, and statistical evaluation of intercept significance using Student's t-test (hypothesis n = 0) were performed using OriginPro 8.5 software (OriginLab Corporation, USA).

Results and Discussion

Validation of UV–Vis Spectrophotometric Method for β-Arbutin Determination

The calibration curve for β-arbutin determination in PBS at pH 5.5 at 280 nm is shown in Figure 1, and the corresponding validation parameters are summarized in Table I.

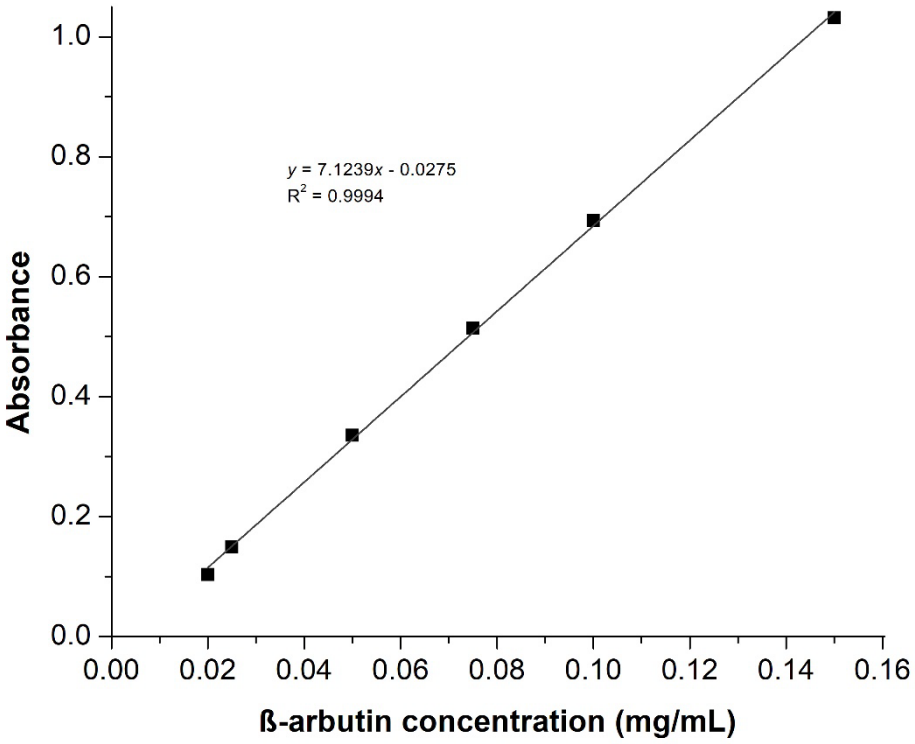

Figure 1. Calibration curve of β-arbutin in PBS pH 5.5 determined at 280 nm
Slika 1. Kalibraciona kriva β-arbutina u PBS puferu pH 5,5 određena na 280 nm

Table I Validation parameters of the developed UV–Vis spectrophotometric method for determination of β-arbutin in PBS pH 5.5
Tabela I Validacioni parametri razvijene UV–Vis spektrofotometrijske metode za određivanje β-arbutina u PBS puferu pH 5,5

Parameter	Value
Wavelength (nm)	280
Concentration range (mg/mL)	0.02–0.15
Calibration equation	$y = kx + n$
Slope (k)	7.1239
Intercept (n)	–0.0275
Standard error of intercept	0.0075
R^2	0.9994
LOD (mg/mL)	0.0035
LOQ (mg/mL)	0.0105
Reliable absorbance range	0.10–1.00

The investigated concentration range (0.02–0.15 mg/mL) was chosen to encompass the expected β-arbutin concentrations during *in vitro* release studies while ensuring measurements remained within the reliable operating absorbance range of the spectrophotometer. Since one aim of the study was to develop an *in vitro* release method suitable for preliminary evaluation of cosmetic films under different hydrodynamic conditions, particular attention was given to analytical sensitivity and reliability at lower analyte concentrations.

A high degree of linearity between β-arbutin concentration and absorbance was observed within the investigated range (R^2 = 0.9994), confirming the suitability of the developed UV–Vis spectrophotometric method for quantitative analysis of β-arbutin under the applied experimental conditions. The calibration curve demonstrated a linear relationship between concentration and absorbance throughout the range, and all experimentally obtained absorbance values remained within the reliable operating range of the spectrophotometer (0.10–1.00 absorbance units). Within this range, the spectrophotometer provides optimal linearity, signal-to-noise ratio, and analytical reliability.

The calculated LOD (0.0035 mg/mL) and LOQ (0.0105 mg/mL) values further confirmed adequate method sensitivity for monitoring β-arbutin release from the investigated polymeric films. The obtained LOQ value was particularly important when interpreting release experiments performed with larger medium volumes. Under these conditions, pronounced dilution of β-arbutin occasionally resulted in concentrations approaching or falling below the validated quantification limit, especially at early sampling points. As a result, the developed analytical method and its validated operating range directly influenced the interpretation of the release profiles and the discriminatory ability of the proposed *in vitro* release method.

The intercept of the calibration curve was statistically significantly different from zero according to Student's t-test (p = 0.0216). However, its absolute value remained low and did not significantly affect quantitative analysis within the validated concentration range. Similar findings have been reported in studies on calibration curve assessment and regression analysis in analytical chemistry, where minor non-zero intercepts may occur despite satisfactory method linearity and analytical performance (41, 42). The point (0, 0) was not included in the regression analysis because it did not represent an experimentally measured calibration standard, but rather a theoretical value. Including such a point may artificially improve the regression model and underestimate systematic deviation associated with experimental measurements (42). Therefore, the regression model was not forced through the origin, and standard linear regression based exclusively on experimentally obtained calibration points was applied. This approach is consistent with recommendations for calibration curve construction and validation in analytical chemistry (42). Minor deviations of this type are relatively common in UV–Vis spectrophotometric analysis and may arise from instrumental noise, baseline fluctuations, optical imperfections, or minor systematic deviations associated with spectrophotometric measurements (41, 42).

The validation procedure was performed in accordance with the ICH Q2(R1) (40) guideline for analytical method validation, which defines parameters required for the evaluation of analytical performance, including linearity, sensitivity, and quantification capability. Overall, the validation results confirmed that the developed UV–Vis spectrophotometric method was sufficiently reliable, sensitive, and suitable for further evaluation of β-arbutin release profiles from chitosan–hyaluronic acid films.

Influence of Hydrodynamic Conditions on β-Arbutin Release Profiles

Release profiles of β-arbutin from chitosan–hyaluronic acid films under different hydrodynamic conditions are shown in Figures 2–5. The tested conditions varied in both medium volume (50, 100, 150, and 200 mL) and the type of apparatus used for agitation. The experiments performed in 50 mL and 100 mL of medium were conducted using a shaking water bath, while those in 150 mL and 200 mL were performed with a rotating mini-paddle apparatus. The selected combinations of apparatus type and medium volume were based on technical feasibility and limitations of the available experimental systems. All experiments were conducted in PBS at pH 5.5, 32 ± 0.5 °C, and 50 rpm.

The results showed rapid release of β-arbutin from all films under all experimental conditions (Figures 2–5). The majority of incorporated β-arbutin was released within the first 10–15 min, after which the release profiles gradually reached a plateau. This behavior was expected given the high aqueous solubility of β-arbutin (39, 43) and the hydrophilic nature of chitosan–hyaluronic acid films, which quickly hydrate and swell upon contact with aqueous media. Rapid hydration and swelling of hydrophilic polymer matrices facilitate penetration of the release medium into the film structure and enable relatively fast diffusion of the dissolved active substance into the surrounding medium (44, 45). Similar release behavior has been reported for hydrophilic polysaccharide-based films and other rapidly swelling polymeric systems intended for topical and mucosal application, where diffusion through the hydrated matrix is the dominant release mechanism (46, 47).

Although rapid β-arbutin release was observed under all investigated conditions, visual comparison of the release profiles suggested that hydrodynamic conditions influenced the release behavior, particularly during the initial stage of the experiment. The results further indicated that the effects of medium volume and apparatus type could not be interpreted independently, because both parameters simultaneously influenced the hydrodynamic conditions, diffusion layer thickness, mass transport, and achievable analyte concentrations during the experiment. As β-arbutin release was completed within approximately 10–15 min under all investigated conditions, the observed differences between the release profiles were confined predominantly to the first few sampling points and should therefore be interpreted as preliminary trends rather than statistically confirmed differences.

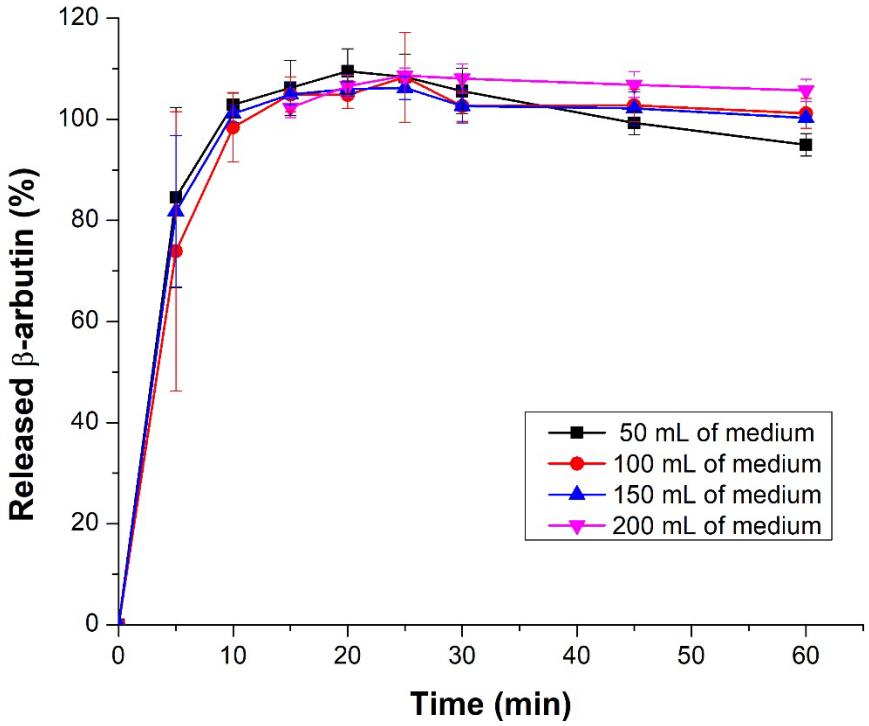

Figure 2. **Release profiles of β-arbutin from chitosan–hyaluronic acid films without additives under different hydrodynamic conditions; experiments were performed in PBS pH 5.5 at 32 ± 0.5 °C and 50 rpm using a shaking water bath (50 and 100 mL of release medium) and a rotating mini-paddle apparatus (150 and 200 mL of release medium). Data from early sampling points, at which β-arbutin could not be reliably quantified using the validated UV–Vis spectrophotometric method, were omitted from the graphical presentation**

Slika 2. **Profili oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline bez aditiva pod različitim hidrodinamičkim uslovima; eksperimenti su izvođeni u PBS puferu pH 5,5 pri temperaturi 32 ± 0,5 °C i brzini mešanja od 50 rpm korišćenjem vodenog kupatila sa „šejker“ dodatkom (50 i 100 mL medijuma) i aparature sa rotirajućom mini lopaticom (150 i 200 mL medijuma). Rezultati za početne vremenske tačke u kojima se koncentracija β-arbutina nije mogla pouzdano kvantifikovati primenom validirane UV–Vis spektrofotometrijske metode izostavljeni su iz grafičkog prikaza**

When experiments were conducted in 50 mL of medium using the shaker apparatus, relatively high β-arbutin concentrations were observed at the earliest sampling points due to the limited medium volume. These conditions allowed reliable analytical quantification throughout the experiment and produced release profiles with relatively low variability. However, the lower medium volume also reduced the concentration gradient between the film surface and the surrounding medium. According to diffusion theory, mass transport is driven by concentration differences between the donor and acceptor compartments (48), so these conditions may partially limit diffusion-driven transport of β-arbutin into the release medium. Under these conditions, release profiles were generally characterized by rapid initial release followed by an early establishment of a plateau phase.

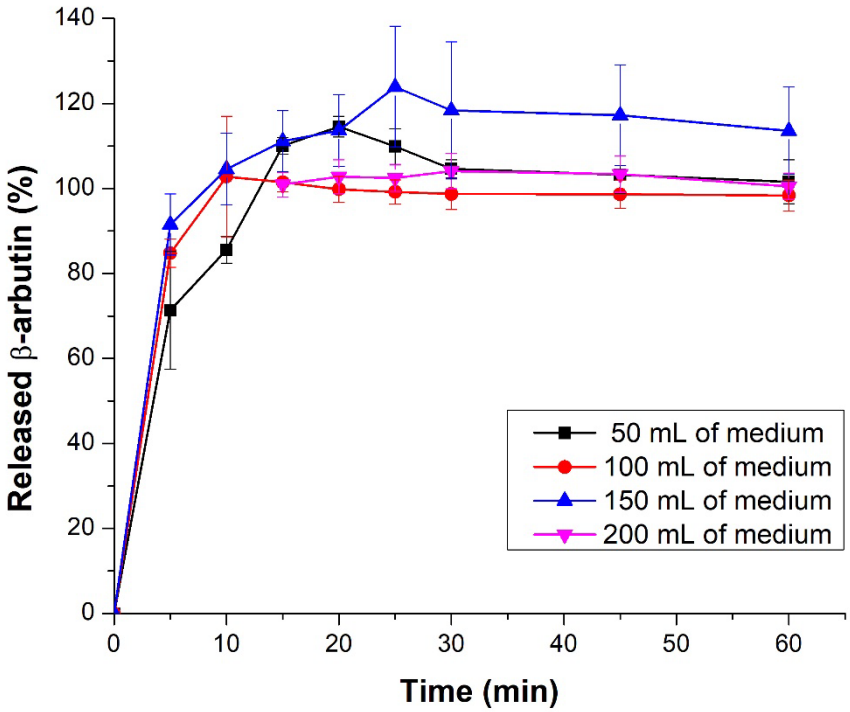

Figure 3. **Release profiles of β-arbutin from chitosan–hyaluronic acid films containing 20% glycerol under different hydrodynamic conditions; experiments were performed in PBS pH 5.5 at 32 ± 0.5 °C and 50 rpm using a shaking water bath (50 and 100 mL of release medium) and a rotating mini-paddle apparatus (150 and 200 mL of release medium). Data from early sampling points, at which β-arbutin could not be reliably quantified using the validated UV–Vis spectrophotometric method, were omitted from the graphical presentation**

Slika 3. **Profili oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline sa 20% glicerola pod različitim hidrodinamičkim uslovima; eksperimenti su izvođeni u PBS puferu pH 5,5 pri temperaturi 32 ± 0,5 °C i brzini mešanja od 50 rpm korišćenjem vodenog kupatila sa „šejker“ dodatkom (50 i 100 mL medijuma) i aparature sa rotirajućom mini lopaticom (150 i 200 mL medijuma). Rezultati za početne vremenske tačke u kojima se koncentracija β-arbutina nije mogla pouzdano kvantifikovati primenom validirane UV–Vis spektrofotometrijske metode izostavljeni su iz grafičkog prikaza**

Increasing the medium volume to 100 mL provided more favorable conditions for maintaining the concentration gradient while still ensuring sufficiently high β-arbutin levels for reliable UV–Vis quantification. Compared with experiments in 50 mL of medium, release profiles obtained in 100 mL generally showed slightly more pronounced release during the initial phase, which may be associated with improved diffusion conditions and enhanced mass transport. At the same time, β-arbutin concentrations remained within the validated working range of the analytical method throughout the experiment. As these differences were primarily observed during the first few sampling points, formulation-dependent differences appeared more pronounced than under the

other investigated hydrodynamic conditions, indicating improved discriminatory ability of the method. However, these observations are based solely on visual comparison of the release profiles and should therefore be interpreted as preliminary formulation-dependent trends rather than statistically confirmed differences.

Discriminatory ability is a key characteristic of dissolution and release testing methods, especially during preliminary formulation screening and optimization. A discriminatory method should detect formulation-related differences in release behavior while maintaining adequate analytical reliability and reproducibility (49). In the present study, based solely on visual comparison of the obtained release profiles, discriminatory ability appeared most pronounced under conditions using 100 mL of medium and the shaker apparatus, where formulation-dependent trends were more clearly observable than under the other investigated hydrodynamic conditions. These findings are particularly important because the primary objective of the proposed method was to identify experimental conditions that provide adequate discriminatory ability for preliminary formulation screening, rather than to establish statistically confirmed differences between formulations.

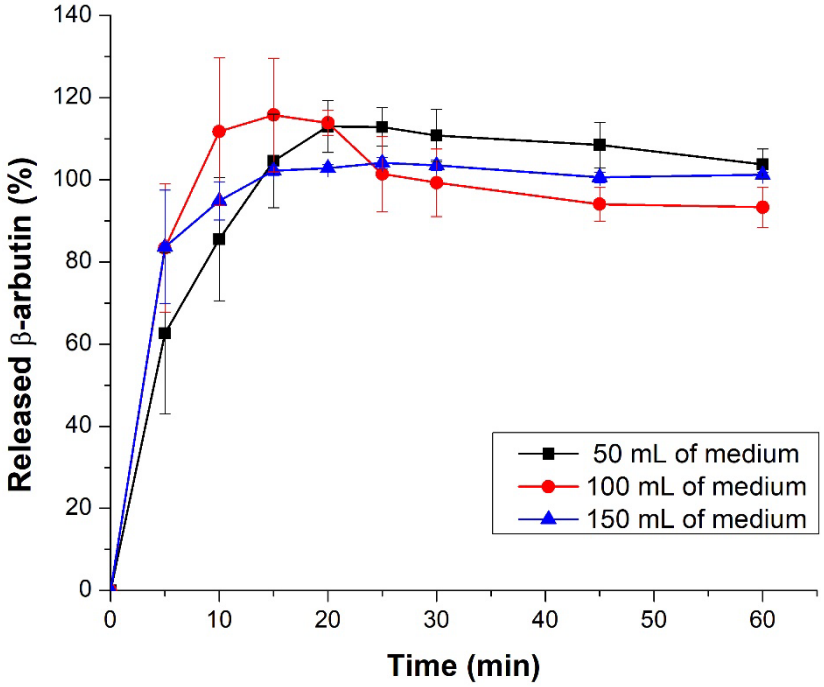

Figure 4. **Release profiles of β-arbutin from chitosan–hyaluronic acid films containing 20% sodium citrate under different hydrodynamic conditions; experiments were performed in PBS pH 5.5 at 32 ± 0.5 °C and 50 rpm using a shaking water bath (50 and 100 mL of release medium) and a rotating mini-paddle apparatus (150 mL of release medium)**

Slika 4. **Profili oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline sa 20% natrijum-citrata pod različitim hidrodinamičkim uslovima; eksperimenti su izvođeni u PBS puferu pH 5,5 pri temperaturi 32 ± 0,5 °C i brzini mešanja od 50 rpm korišćenjem vodenog kupatila sa „šejker“ dodatkom (50 i 100 mL medijuma) i aparature sa rotirajućom mini lopaticom (150 mL medijuma)**

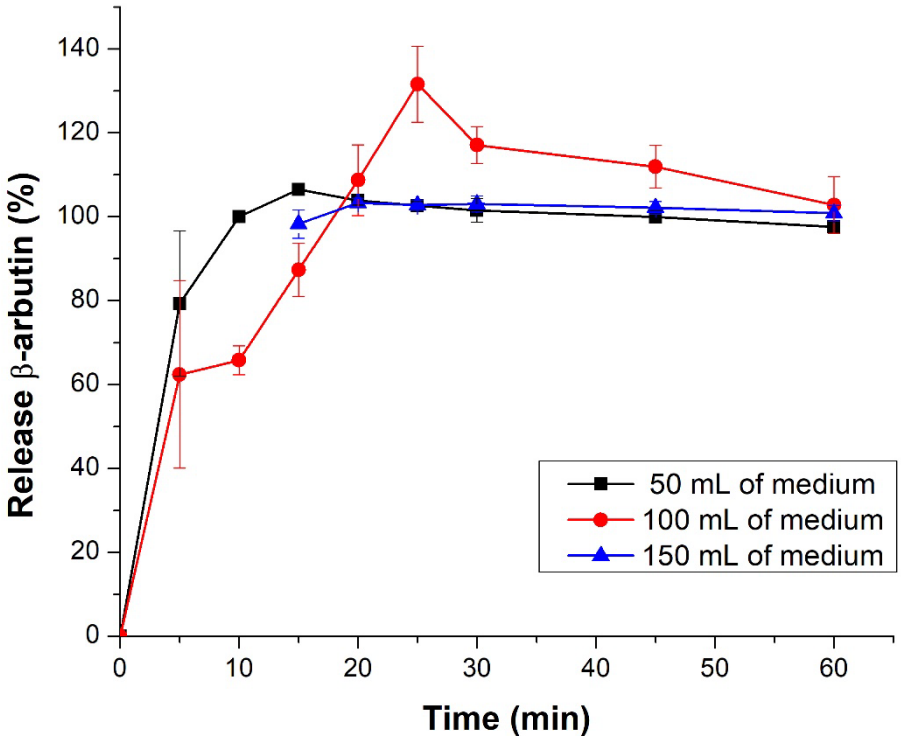

Figure 5. **Release profiles of β-arbutin from chitosan–hyaluronic acid films containing 20% tannic acid under different hydrodynamic conditions; experiments were performed in PBS pH 5.5 at 32 ± 0.5 °C and 50 rpm using a shaking water bath (50 and 100 mL of release medium) and a rotating mini-paddle apparatus (150 mL of release medium). Data from early sampling points, at which β-arbutin could not be reliably quantified using the validated UV–Vis spectrophotometric method, were omitted from the graphical presentation**

Slika 5. **Profili oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline sa 20% taninske kiseline pod različitim hidrodinamičkim uslovima; eksperimenti su izvođeni u PBS puferu pH 5,5 pri temperaturi 32 ± 0,5 °C i brzini mešanja od 50 rpm korišćenjem vodenog kupatila sa „šejker“ dodatkom (50 i 100 mL medijuma) i aparature sa rotirajućom mini lopaticom (150 mL medijuma), Rezultati za početne vremenske tačke u kojima se koncentracija β-arbutina nije mogla pouzdano kvantifikovati primenom validirane UV–Vis spektrofotometrijske metode izostavljeni su iz grafičkog prikaza**

At larger medium volumes (150 and 200 mL), experiments were conducted using the rotating mini-paddle apparatus. Compared with the shaker system, the rotating mini-paddle apparatus provides more uniform and controlled hydrodynamic conditions, characterized mainly by circular medium movement around the fixed film. In contrast, the shaker apparatus generates oscillatory movement of the medium, resulting in less uniform hydrodynamic conditions and periodically changing local flow characteristics near the film surface. These differences may significantly influence diffusion layer formation and local mass transport around the investigated films, thereby affecting release behavior (50, 51).

The results further suggested that not only medium volume, but also the mode of agitation may have influenced β-arbutin release behavior. Hydrodynamic conditions generated by the shaker and rotating mini-paddle apparatus differed considerably in medium movement and local flow characteristics around the film surface. Consequently, the obtained release profiles reflected not only intrinsic formulation properties, but also differences in local hydrodynamic conditions generated by the experimental setup. These findings emphasize the importance of careful selection and optimization of hydrodynamic conditions during the development of *in vitro* release methods for topical polymeric systems.

Despite potentially more controlled hydrodynamic conditions, experiments at larger medium volumes showed pronounced dilution of β-arbutin, particularly at early sampling points. In several cases, measured concentrations approached or fell below the validated limit of quantification of the developed UV–Vis spectrophotometric method and were therefore excluded from graphical presentation. Under these conditions, the influence of analytical sensitivity on interpretation of release profiles became particularly important. These findings suggest that larger medium volumes, although theoretically more favorable for maintaining sink conditions, may simultaneously compromise analytical sensitivity and reliable quantification when highly water-soluble active substances are investigated.

Sink conditions generally refer to experimental conditions in which the volume of release medium is sufficiently high to ensure that the concentration of the released active substance remains substantially below its saturation solubility throughout the experiment, thereby preventing concentration-dependent limitation of diffusion. In practice, sink conditions are generally considered to be achieved when the volume of release medium is sufficient to dissolve at least three to ten times the amount of active substance in the tested formulation. Such conditions are commonly recommended in dissolution testing of pharmaceutical dosage forms to maintain a constant concentration gradient and ensure reproducible diffusion-driven release (52). However, the relevance of strict sink conditions in evaluating topical cosmetic systems may be less straightforward. Cosmetic films intended for topical application are exposed to substantially different physiological conditions compared with orally administered dosage forms, since only limited quantities of fluid are available at the skin surface during practical application (53, 54). Consequently, complete sink conditions are unlikely to be achieved *in vivo* (52), and excessively large medium volumes may improve theoretical diffusion conditions while simultaneously reducing the physiological relevance of the experimental model (55).

Because β-arbutin release was completed within approximately 10–15 min under all investigated conditions, the observed differences between release profiles were confined mainly to the first few sampling points. Although the recommendations of the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for mathematical comparison of release profiles were developed for immediate-release oral pharmaceutical dosage forms rather than topical cosmetic films, they indicate that additional mathematical comparison is generally considered unnecessary when more than

85% of the active substance is released within 15 min, because only limited additional information is obtained in such cases (31, 56). In the present study, visual comparison of the initial release behavior was therefore considered sufficient to identify preliminary formulation-dependent trends during method development.

For this reason, the present study did not aim to maximize sink conditions at any cost, but rather to identify hydrodynamic conditions that provide an appropriate balance between diffusion-driven release, analytical reliability, discriminatory ability, and preliminary simulation of realistic cosmetic application conditions. Furthermore, the investigated films were intended to provide local rather than systemic effects, and prolonged maintenance of strict sink conditions was therefore not considered essential for preliminary formulation screening. Similar considerations have previously been emphasized in studies on release testing of topical and dermal formulations, where strict application of classical pharmaceutical dissolution principles may not always be fully suitable for cosmetic systems and locally acting formulations (57–60).

Overall, the results suggest that hydrodynamic conditions influence both the observed release profiles and the analytical applicability of the developed method. Medium volume, apparatus type, agitation mode, and the resulting hydrodynamic conditions collectively affected diffusion behavior, concentration gradients, and achievable analyte concentrations during the experiment. Among the tested conditions, experiments conducted in 100 mL of medium using the shaker apparatus provided the most suitable balance between maintaining the concentration gradient, ensuring reliable analytical quantification, and enabling adequate discriminatory ability for preliminary formulation screening. Under these conditions, formulation-dependent trends were most clearly observed, supporting the suitability of these experimental conditions for further evaluation of β-arbutin release from cosmetic polymeric films and for preliminary screening of formulation-related differences. An additional advantage of the selected conditions was that all measured concentrations remained within the validated working range of the analytical method throughout the experiment, allowing reliable interpretation of the observed formulation-dependent trends.

Influence of Formulation Composition on β-Arbutin Release Under the Selected Optimal Hydrodynamic Conditions

The influence of formulation composition on β-arbutin release was evaluated under the previously selected optimal hydrodynamic conditions: 100 mL of PBS at pH 5.5, using a shaking water bath operated at 50 rpm and 32 ± 0.5 °C. These conditions were chosen because they provided an appropriate balance between maintaining the concentration gradient, ensuring reliable analytical quantification, and providing adequate discriminatory ability for preliminary formulation screening. The corresponding release profiles are shown in Figure 6.

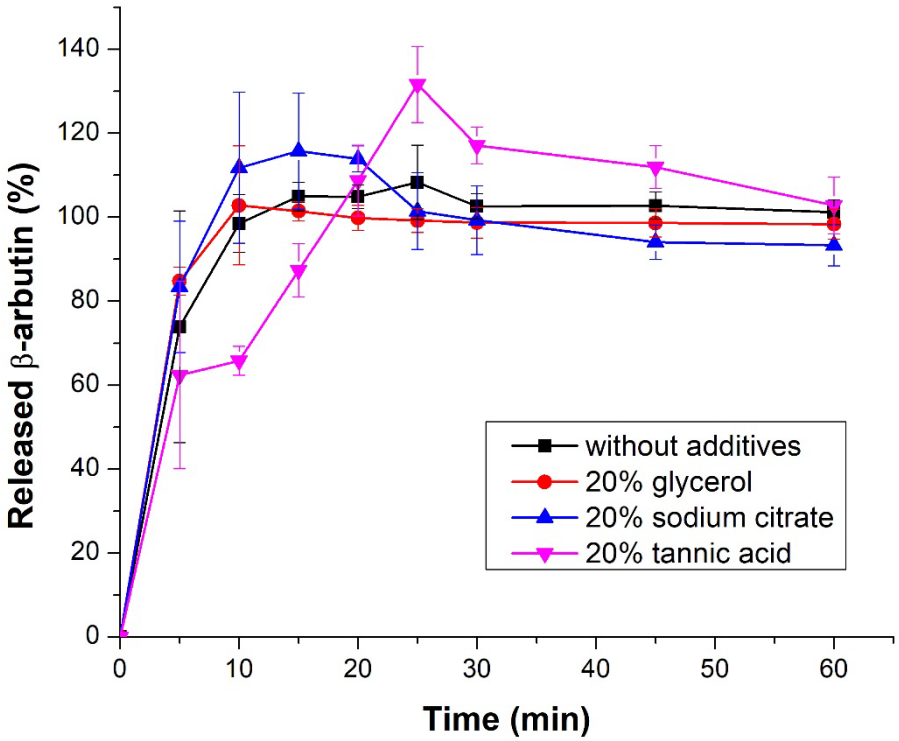

Figure 6. Comparison of β-arbutin release profiles from different chitosan–hyaluronic acid film formulations under the selected optimal hydrodynamic conditions (100 mL of release medium, shaking water bath)

Slika 6. Poređenje profila oslobađanja β-arbutina iz različitih formulacija filmova na bazi hitozana i hijaluronske kiseline u odabranim optimalnim hidrodinamičkim uslovima (100 mL medijuma, vodeno kupatilo sa „šejker“ dodatkom)

The films without additives served as the reference formulation and showed rapid β-arbutin release throughout the experiment. The resulting release profile reflected the intrinsic release characteristics of the chitosan–hyaluronic acid polymer matrix, marked by rapid hydration, swelling (46), and diffusion of the highly water-soluble active substance (39, 43).

The films containing glycerol tended to show slightly higher β-arbutin release values during the initial phase of the experiment compared with the films without additives. Glycerol is a commonly used plasticizer that increases polymer chain mobility, reduces intermolecular interactions, and increases free volume within polymeric systems (16). Consequently, hydration of the matrix and diffusion of dissolved β-arbutin may occur more readily, resulting in a tendency toward faster release. The observed release profile is therefore consistent with the expected plasticizing effect of glycerol on chitosan–hyaluronic acid films, although this interpretation is based on visual comparison of the obtained release profiles.

A similar tendency toward slightly higher release values during the early stages of the experiment was observed for the films containing sodium citrate. Sodium citrate was

incorporated as a mild ionic crosslinking agent to modify the structure of the polymer network. However, under the investigated conditions, the obtained release profile suggests that the degree of ionic crosslinking achieved was insufficient to markedly restrict diffusion of β-arbutin through the hydrated matrix. This observation may be related to the pronounced swelling ability of the chitosan–hyaluronic acid polymer matrix (61) and the high water solubility of β-arbutin (39, 43), both of which favor rapid diffusion processes.

The most distinct release behavior was observed for the films containing tannic acid. Compared with the other investigated formulations, these films exhibited lower release values during the first 10–15 min, followed by a more pronounced increase in β-arbutin release at later sampling points. Rather than indicating uniformly slower release throughout the experiment, the obtained profile suggests the presence of an initial lag phase followed by accelerated release. Such behavior may be associated with stronger interactions between tannic acid and polymer chains (20), resulting in the formation of a more compact polymer network and temporary restriction of diffusion during the early stages of hydration. As swelling progresses and the hydrated matrix becomes more permeable, these diffusional limitations may diminish, leading to a more rapid increase in β-arbutin release.

It should be noted that the observed differences between formulations were evaluated primarily on the basis of visual comparison of release profiles, as formal statistical comparison between formulations was not performed. Therefore, the observed differences should be interpreted as formulation-related trends rather than definitive evidence of superior or inferior release performance. Nevertheless, the selected experimental conditions enabled adequate discriminatory ability for preliminary formulation screening by allowing visualization of formulation-dependent variations in release behavior while maintaining reliable analytical quantification.

In several cases, cumulative release values exceeded 100% of the theoretical β-arbutin content. Since β-arbutin content was not determined experimentally in each individual film specimen prior to release testing, the percentage released was calculated relative to the theoretical β-arbutin content. Together with cumulative experimental variability associated with sample preparation, sampling, medium replacement, and analytical quantification, this may explain deviations from the theoretical maximum value of 100%.

Overall, the results indicate that formulation composition may influence the early-stage release behavior of β-arbutin from chitosan–hyaluronic acid films, although the general release pattern remained similar for all formulations investigated. The selected experimental conditions provided adequate discriminatory ability for preliminary formulation screening by enabling visualization of formulation-dependent trends while maintaining reliable analytical quantification. These findings provide a useful basis for further optimization of film composition and for future studies aimed at establishing more detailed relationships between formulation structure, polymer interactions, and β-arbutin release behavior. Future studies should also investigate membrane-based release systems

to further enhance the physiological relevance of the proposed *in vitro* release method for cosmetic films.

Conclusion

This study successfully developed and validated a UV–Vis spectrophotometric method for quantifying β-arbutin during *in vitro* release studies of chitosan–hyaluronic acid films intended for cosmetic applications. The analytical method demonstrated satisfactory linearity, sensitivity, and reliability within the tested concentration range and was suitable for monitoring β-arbutin release under various experimental conditions. The results indicated that hydrodynamic conditions influence the observed release profiles, analytical reliability, and discriminatory ability of the method. Medium volume, apparatus type, and agitation mode collectively affected concentration gradients, mass transport, and achievable analyte concentrations during the experiment. Although larger medium volumes may help maintain sink conditions, they also caused pronounced dilution of β-arbutin and reduced analytical sensitivity. In contrast, smaller medium volumes allowed higher analyte concentrations, but provided less favorable conditions for maintaining concentration gradients. Among the tested conditions, experiments conducted in 100 mL of PBS at pH 5.5 using a shaking water bath at 50 rpm and 32 ± 0.5 °C provided the most appropriate balance between analytical reliability, discriminatory ability, and relevance for cosmetic applications. Under these conditions, formulation-dependent trends in the early-stage release behavior of β-arbutin were most clearly observed. The formulation additives studied influenced release behavior primarily during the initial release phase, although the overall release pattern remained similar for all formulations. The developed method is therefore a useful tool for preliminary formulation screening and for the optimization of cosmetic polymeric films containing β-arbutin and may contribute to future standardization of *in vitro* release testing for cosmetic film formulations.

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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.

Author Contributions

A.Ć.: Conceptualization, Methodology, Investigation, Formal analysis, Validation, Resources, Visualization, Writing – original draft, Writing – review & editing; **Ž. R.**: Investigation, Data curation, Formal analysis, Resources, Writing – review & editing; **J. M.B.**: Supervision, Resources, Writing – review & editing; **L.P.**: Supervision, Project administration, Resources, Writing – review & editing.

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Razvoj i optimizacija *in vitro* metode za ispitivanje oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline

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Kratak sadržaj

Cilj ovog rada bio je razvoj i optimizacija *in vitro* metode za ispitivanje oslobađanja β-arbutina iz filmova na bazi hitozana i hijaluronske kiseline namenjenih kozmetičkoj primeni, kao i ispitivanje uticaja hidrodinamičkih uslova i sastava formulacije na profil oslobađanja. Filmovi sa β-arbutinom pripremljeni su metodom izlivanja rastvora korišćenjem hitozana i hijaluronske kiseline kao film-formirajućih polimera. Kao aditivi korišćeni su glicerol, natrijum-citrat i taninska kiselina. Ispitivanja brzine oslobađanja sprovedena su u fosfatnom puferu (PBS, pH 5,5) na temperaturi 32 ± 0,5 °C pri brzini mešanja od 50 rpm korišćenjem različitih zapremina medijuma (50–200 mL) i dve vrste aparatura. Koncentracija β-arbutina određivana je validiranom UV–Vis spektrofotometrijskom metodom. Metoda je pokazala odličnu linearnost (R^2 = 0,9994), dok su vrednosti LOD i LOQ iznosile 0,0035 i 0,0105 mg/mL. Hidrodinamički uslovi uticali su na profile oslobađanja, analitičku primenljivost i diskriminatornu sposobnost metode. Kao najpogodniji uslovi izdvojeni su eksperimenti sprovedeni u 100 mL medijuma korišćenjem šejker aparature. Svi ispitivani filmovi pokazali su brzo oslobađanje β-arbutina, dok su aditivi uticali na profil oslobađanja tokom njegove početne faze. Razvijena metoda predstavlja pogodan alat za preliminarnu procenu i poređenje kozmetičkih polimernih filmova sa β-arbutinom.

Ključne reči: β-arbutin, filmovi na bazi hitozana i hijaluronske kiseline, *in vitro* oslobađanje, hidrodinamički uslovi, kozmetičke formulacije