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Development of a Mouthwash Using Freeze-Drying Technique: An Optimization Study

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Abstract: In recent years, the development of solid cosmetics, as part of sustainable cosmetics strategy, is generating increasing interest. In addition, novel approaches such as Quality by Design concept allowed the development of high-quality products together with a decrease in waste generation. The present study aimed the development of freeze-dried mouthwashes containing *Cetraria islandica* extract using the Quality by Design approach. Based on the results of preliminary experiments, a factorial design with three factors that varied on two levels was developed. As factors, the filler type (sorbitol or mannitol) was chosen as the qualitative factor and the two quantitative factors were: the filler ratio set from 3 to 5% and polymer (methylcellulose) ratio from 0 to 0.5%. After the preparation and the complete characterization of the formulations generated through the experimental design, the effect of the formulation variables on the lyophilized mouthwashes and the interactions between formulation factors were investigated. Finally, an optimal formulation with appropriate mechanical properties that ensure easy manipulation and no material loss when extracted from the package and fast reconstitution was generated.

Keywords: sustainable cosmetics; freeze-drying; natural extracts; mouthwash; design of experiments

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1. Introduction

There is a growing need for performant, safe, and sustainable cosmetic products with a low environmental footprint. In recent years, relevant stakeholders have been working towards developing regulations and strategies to reduce the use of plastic material by the cosmetic industry. As part of the European Strategy for Plastics, the Directive 2019/904 and Implementing Regulation 2020/2151 set out the specifications for marking the products containing plastics. To reduce the quantity of waste generated, Member States of EU should encourage the use of products for multiple uses or provide suitable and more sustainable alternatives for single-use plastic products [1]. In this regard, solid cosmetics have gained importance in the last years due to their multiple advantages, mainly the reduced environmental impact. Solid cosmetics can be more appealing to consumers due to their different appearance and innovative concepts. This novel approach gives rise to new challenges in the design of safe products that will preserve their properties during storage, handling, and transport [2] and in finding alternative approaches to standard manufacturing.

Out of the preparation methods, freeze-drying is employed to preserve the properties of pharmaceutical and cosmetic products [3]. Freeze-drying, also called "lyophilization" or "cryodesiccation" represents a drying technique that involves the removal of the solvent (usually water) by freezing and further evaporation by sublimation upon heating. During the lyophilization process, more than 90% of the containing water is lost. When reaching room temperature, the freeze-dried product remains dry and must be rehydrated before being used. Thus, the water removal prevents bacterial growth and stability issues, but also the need of adding preservatives [4]. The freeze-drying technique allows the development of lyophilized cosmetic compositions that are more stable than traditional products [5] and ensures the long-term stability of the bioactive compounds [3].

The medicinal use of the dried thallus of *Cetraria islandica* (L.) Acharius s.l. from the *Parmeliaceae* family, also known as Island moss or *Lichen islandicus* has been documented thoroughly. The principal phytoconstituents are polysaccharides, 25–50% (lichenan, isolichenan), lichen acids (depsidones fumaroprotocetraric acid, protolichesterinic acid and usnic acid), and minerals [6]. Several in vitro studies indicate the antimicrobial, immunomodulating, anti-inflammatory, antiviral, and antioxidative activity of the *Cetraria islandica* aqueous and ethanolic extracts [6]. The data obtained during in vitro studies are supported by in vivo studies investigating the activation of the reticuloendothelial system, anti-inflammatory, anti-proliferative, and antioxidative effects. Moreover, available clinical data support the efficacy of *Cetraria islandica* extract for the treatment of inflammations of oral mucosa and dryness of the oral cavity [6].

Developed initially by the pharmaceutical industry, the Quality by Design (QbD) concept was adopted lately by the cosmetic industry as a systematic approach for obtaining high-quality products with the desired product performance. The main goals of the QbD paradigm include the design of robust formulations and manufacturing processes to reliably provide the required product quality [7]. Application of QbD principles relies on the use of Design of Experiments (DoE), a basic concept in formulation and optimization which significantly reduces the number of tests, time, and costs [8]. In this work, the application of DoE in the development of freeze-dried mouthwashes has been investigated to select the most appropriate conditions to obtain fast-dissolving structures with appropriate mechanical properties. Preliminary studies were undertaken to investigate the biocompatibility of *Cetraria islandica* extract and the impact of the excipients on the lyophilized structures' properties; further, a two-level full factorial design with three factors was carried out and the obtained results were analyzed to establish the Design Space as the multidimensional combination of inputs that deliver the product to meet the desired attributes. Together, novel strategies such as the freeze-drying technique and QbD have shown their efficiency in the development and optimization of modern cosmetics by generating less waste and allowing greater flexibility.

2. Materials and Methods

2.1. Materials

The hydroalcoholic *Cetraria islandica* extract was obtained through a reflux extraction for 30 min at 60 °C in a 70% (*v*/*v*) aqueous ethanol solution at a solid: solvent ratio of 1:10 (*w*/*v*). After cooling, the sample was filtered, and the supernatant was recovered. Further, the extract was concentrated through rotary evaporation (Hei-VAP Advantage Rotary evaporator HL/G1 (Heidolph, Schwabach, Germany) under reduced pressure at 50 °C and 80 rpm until 40% of the initial mass. The substances used were the following: LMW (low molecular weight) hyaluronic acid and allantoin which were purchased from Elemental (Oradea, Romania), Poloxamer 407 and mannitol from Sigma-Aldrich (Darmstadt, Germany), sorbitol from Fagron (St. Paul, MN, USA) and methylcellulose, Methocel MC 4000 cP (Fluka AG, Buchs, Switzerland).

2.2. Methods

2.2.1. In Vitro Evaluation of the *Cetraria islandica* Extract

Cell Culture

The normal human foreskin fibroblasts (BJ) were cultivated in DMEM (Dulbecco's modified Eagle's medium, Gibco, Paisley, UK) supplemented with 10% FBS (fetal bovine serum, Sigma Aldrich, Steinheim, Germany) and maintained in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. Every 2–3 days the medium was replaced and the cells were subcultured at around 70–80% confluency.

Preparation of Extract Solutions

Working solutions with concentrations ranging from 2.5–100 mg/mL were prepared in DMSO (Riedel-de Haën, Seelze, Germany). These solutions were further diluted in cell culture medium to obtain the tested concentrations ranging from 10 to 400 µg/mL.

Cytotoxicity AssayTrypsinized cells were seeded in 96-well plates and left to attach for 24 h. PBS was used to wash the dead and unattached cells, while the viable cells were incubated with the *Cetraria islandica* extract. After 24 h the cells were washed with PBS and viability was assessed by Alamar Blue assay (Thermo Fisher Scientific, Waltham, MA, USA) a test that measures the cellular ability to convert a non-fluorescent compound such as resazurin to resorufin, a fluorescent compound. The fluorescence intensity was measured at λexcitation = 530/25, λemission = 590/35 following a 4 h exposure to a 200 µM resazurin solution, using a Synergy 2 multi-mode microplate reader (BioTek Instruments Inc., Winooski, VT, USA). DMSO (0.4% in cell culture medium) was used as the negative control.

Antibacterial Activity

The tested microorganisms were: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (ATCC-American Type Culture Collection, Manassas, VA, USA). Out of each bacterial strain grown for 24 h on Nutrient Agar medium [9] a dilution of 0.5 McFarland in sterile saline solution was made.

For the agar-well diffusion method, Petri dishes with Mueller Hinton agar media (Oxoid, Hampshire, UK) were inoculated with each bacterial strain suspension and maintained at 37 °C for 20 min to infiltrate. Then, 5 mm diameter wells were carved in the agar using a sterile cut pipette tip. The wells were then filled with sterile cotton beads and each bead was loaded with 80 µL of *Cetraria islandica* extract. The plate also contained a control well loaded with the solvent used, prepared as an aqueous ethanol solution 70% *(v/v)* and then evaporated at 40% of the initial mass. After 24 h incubation at 37 °C, the hallow formed around the wells was measured, the larger the diameter of the inhibition zone, the higher the sensibility of bacteria to that plant extract [10]. Each experiment was conducted in duplicate, and the mean was calculated.

2.2.2. Preparation of Mouthwash Samples

All samples were prepared similarly: for each mouthwash, two separate 10 mL solutions were prepared and mixed at the end. The hydroalcoholic extract was gravitationally filtered and then transferred to a mortar where a surfactant solution (Poloxamer in 10 mL distilled water) was added dropwise and stirred continuously with a pestle until complete homogenization and defoaming. The second solution was prepared in a Berzelius beaker, by adding all the other ingredients (mannitol/sorbitol, methylcellulose, allantoin, hyaluronic acid, and flavoring agent) in 10 mL of distilled water and stirring until complete dissolution. The two solutions were mixed in a Berzelius beaker and stirred using the VELP Scientifica Mechanic Stirrer (Usmate Velate, Italy) for 10 min at 500 rpm. Further, they were transferred to 2 mL blister pockets.

2.2.3. Freeze-Drying of Mouthwash Samples

The samples were freeze-dried using VirTis Advantage Plus Freeze Dryer (SP Scientific, Gardiner, MT, USA). They were first cooled to −55 °C for 12 h, then subjected to primary drying at −25 °C, 0.2 mbar for 24 h, followed by secondary drying at 20 °C for 12 h. The final freeze-dried products were kept in a desiccator until they were processed further, to prevent hydration and loss of proprieties.

2.2.4. Characterization of Mouthwash Samples

All samples were analyzed at an ambient temperature of 22 ± 5 °C.

Texture Analysis

The texture analysis was performed with the Brookfield CT3 Texture Analyzer (Brookfield Engineering, Middleboro, MA, USA) through compression tests on one freeze-dried tablet at a time. The sample was placed horizontally on the analysis surface and was subjected to a constant force applied by a TA10 probe with a speed of 0.1 mm/s and a load of 10 g until a target distance of 5 mm. For each test, three measurements were performed and the rigidity at two different distances, fracturability, load at target, mechanical work at the first fracture, deformation at the first fracture, mean load were determined using the TexturePro CT Software V 1.9 (Brookfield Engineering, Middleboro, MA, USA). For each parameter, the mean and standard deviation were calculated.

Reconstitution Time

Reconstitution times for each sample were measured by placing a lyophilizate in 10 mL of distilled water followed by manual stirring. The time required for the complete dissolution of the lyophilized tablet was measured with a digital stopwatch. For each test, three experiments were performed, and the mean and standard deviation were determined.

Weight Loss Percentage

To quantify the integrity of the lyophilizates, weight loss for each sample was measured after removing it from the blister pocket. Firstly, the lyophilizate was weighed into the alveolae, then it was removed and weighed separately, and finally after weighing the empty alveolae, we determined the weight difference. For each test, three experiments were performed, and the mean and standard deviation were determined.

Viscosity Measurement of the Reconstituted Mouthwashes

For the reconstituted mouthwashes, the rheological measurements were made using the Brookfield DV-III Ultra (Brookfield Engineering, Middleboro, MA, USA) equipped with LV-1 spindle, at a rotational speed of 200 rpm. For each test, three experiments were performed, and the mean and standard deviation were determined.

Experimental Design

To investigate the effects of the formulation variables on the lyophilized mouthwashes, a DoE was developed, with the possibility to highlight the interactions between factors, using the Modde 12 software (Umetrics, Umeå, Sweden). A full factorial design with three factors that varies on two levels, thus a total of eight runs and three center points, was generated. As factors, a qualitative one was chosen, the filler type (X1) that varied between mannitol or sorbitol, and two quantitative ones: the filler ratio (X2) set from 3 to 5% and methylcellulose (MC) ratio (X3) from 0 to 0.5%. The composition of the formulations is detailed in Table 1. The studied responses derived from the previously described characterization methods: rigidity at 2 (Y1) and 4 mm (Y2), load at target (Y3), fracturability (Y4), first fracture work (Y5), first fracture deformation (Y6), mean load (Y7), the weight loss percent (Y8), reconstitution time (Y9), and viscosity (Y10). For optimizing the final formula, some constraints were applied: weight loss, reconstitution time were

minimized and fracturability (measured as hardness values of initial fractures) and viscosity were maximized.

Table 1. The quantitative and qualitative composition of formulations F1–F11.

	Quantity *w*/*v*%										
Ingredient	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**
Mannitol	3.00	-	7.00	-	3.00	-	7.00	-	5.00	5.00	5.00
Sorbitol	-	3.00	-	7.00	-	3.00	-	7.00	-	-	-
Poloxamer	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Methylcellulose	-	-	-	-	0.50	0.50	0.50	0.50	0.25	0.25	0.25
Allantoin	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Hyaluronic acid	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
Cetraria islandica extract	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Flavoring agent	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

2.2.5. Optimal Mouthwash Formulation

The optimal formula generated was prepared using the same method previously used for the 11 samples (Section 2.2.2). After preparation, the optimal formulation was subjected to freeze-drying process (as described in Section 2.2.3) and then characterized, using the same tests and the same procedures as for the initial 11 samples presented in Section 2.2.4:

- Texture analysis,
- Reconstitution time,
- The weight loss percent,
- Viscosity measurement of the reconstituted mouthwash.

Finally, the theoretical results obtained from the experimental design were interpreted and compared with the practical ones. The residual values were calculated as the difference between the experimental value and the one predicted by the model.

3. Results and Discussion

3.1. Evaluation of the Cetraria Islandica Extract

3.1.1. In Vitro Testing

The *Cetraria islandica* extract was evaluated for a potential cytotoxic effect using a normal cell model, human foreskin fibroblasts following a 24 h exposure. No cytotoxic effects were observed instead, an increase in viability for the concentrations ranging from 75–400 µg/mL was detected (Figure 1). In the case of the higher concentrations tested (300 and 400 µg/mL) the increase in viability was 22–30%, while exposure to 200–150 µg/mL resulted in a 6–9% increase in viability. In the case of intermediary doses (75, 100 µg/mL) an approximately 3% increase was noticed. Our results are in agreement with a study published by Ögmundsdóttir et al. where a small, but consistent increase in viability was observed in fibroblast cultures exposed to protolichesterinic acid, a low-molecular-weight lichen metabolite [11]. The lack of in vitro toxicity encouraged the further preparation of the mouthwash formulations.

3.1.2. Antibacterial Activity

In this study, the antibacterial activity of *Cetraria islandica* extract against three bacterial strains: *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* (Table 2) was investigated. Even these microorganisms are considered less common in the oral microbiota, recent studied suggest their presence in the oral cavity more often than expected [12,13]. The antibacterial activity was investigated by direct contact between the plant extract and the culture medium. After the end of the incubation period, at 37 °C, the inhibition zones (mm) were determined for the tested bacterial strains. It was observed that the results from

the tested samples varied in the size of the diameter of the inhibition zone, depending on the microbial strain tested.

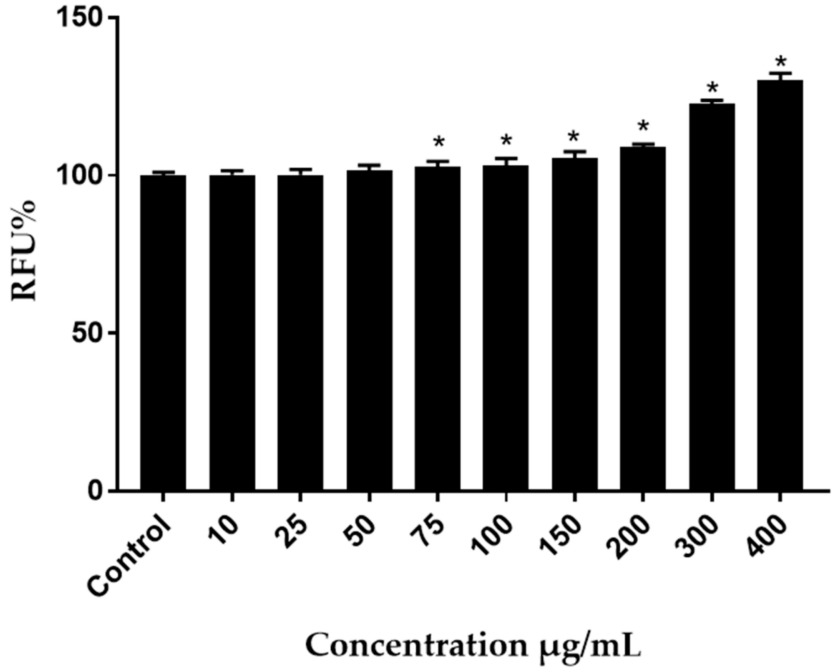

Figure 1. Effects of the *Cetraria islandica* extract on normal human foreskin fibroblasts cells' viability after 24 h exposure. The results are presented as relative mean values ± standard deviations, where the negative control (Control) is 100%. Significant differences in comparison with the negative control (ANOVA + Dunn's; $p < 0.05$) are marked with *. RFU, relative florescence units.

Table 2. The diameter of inhibition zones (mm) for the tested plant extract.

Sample	*Escherichia coli*	*Staphylococcus aureus*	*Enterococcus faecalis*
		(mm)	
Cetraria islandica extract	0	7 ± 0.00	8.5 ± 0.70
Control	0	0	0

Following the antimicrobial test, it was noticed that *Cetraria islandica* extract had the best activity on *Enterococcus faecalis* strain, a slight lower activity on the Gram (+) strain (*Staphylococcus aureus*), while on *Escherichia coli* strains the extract did not exhibit bacterial inhibition. These results are in line with previously published studies where *Cetraria islandica* extract exhibited antibacterial activity against Gram (+) strains, but did not show antibacterial activity against Gram (−) strains tested in the study, including *Escherichia coli* [14].

3.2. Development and Evaluation of the Cetraria islandica Extract Mouthwash

Following a literature study on existing mouthwashes, active ingredients and natural extracts, some initial formulations were proposed. A complex composition was chosen for the mouthwash samples, which comprised both active ingredients and functional compounds that ensure product stability, acceptability and facilitate its use. *Cetraria isladica* extract was chosen for its antioxidant, antimicrobial, anti-inflammatory activity [15–17]. Poloxamer 407 was chosen as it is a mild, nonionic surfactant, suitable for use in oral care products, that does not irritate the mucosa and membranes [18]. Sorbitol and mannitol were also added as cryoprotectants and structure agents for the freeze-dried product [19] and methylcellulose as matrix-forming agent [20]. Allantoin was previously used in oral

care products for its healing, anti-irritant and repairing properties [21], while hyaluronic acid, a key element in the soft periodontal tissues, gingiva, and periodontal ligament can have an anti-inflammatory and tissue healing effect [22]. The flavoring agent was chosen to give a pleasant sensation to the consumer, during and after using the mouthwash and increase product acceptability.

The characterization follows the critical quality attributes of the products. The lyophilizates had to be easily extracted from the blister pockets, without material loss and had to withstand manipulation when reconstituted in water. Moreover, the obtained mouthwash should display appropriate rheology for a palatable product. Therefore, the characterization had to address every step of product use. As it is well known that products obtained by freeze-drying process are porous, with low crushing strength and highly brittle, texture analysis was selected as an adequate method to characterize the mechanical profile of the structures. The weight loss percent of the product was evaluated when extracted from the blister pocket and further, the reconstitution time was measured. Finally, the end product was tested for viscosity.

3.2.1. Experimental Design

In the complex composition of cosmetics, all the ingredients can exert an important impact on the characteristics of the final products. In this study, DoE was used as a strategy to assess the effects of the key ingredients on the quality attributes of the products. DoE allows knowledge and understanding not only of individual effects but can also show how the interaction between the compounds influences their characteristics. In the case of the freeze-dried mouthwashes, the filler needed to reinforce the structures could have an important influence on the mechanical profile, on the dissolution speed and the taste. Therefore, the type of filler (X1) was evaluated, with two options, mannitol and sorbitol. The first quantitative variable, the filler ratio (X2), varied between 3 and 7%. As a hydrophilic polymer is needed to ensure the three-dimensional structure and integrity of a freeze-dried product, methylcellulose ratio (X3) was chosen as a quantitative variable and varied between 0 and 0.5%. After the preparation and the complete characterization of the 11 formulations generated through DoE, the data analysis aimed at regression equations (model equations) for each of the responses as functions of the input variables X1, X2 and X3.

Several steps were taken to study the impact of variables on the results. The Modde software was used to perform data processing, then fitting the model, refining the coefficients of the results, and finally optimizing the input variables to reach an ideal formulation. Ten responses (Y1–Y10) were analyzed; the results of all the tests performed on the 11 formulations, that were used in the DoE analysis, are presented in Table 3.

Each of the responses (Y1–Y10) corresponding to a certain quality attribute was expressed mathematically as an equation that reveals its correlation to the formulation factors (X1–X3):

$$Y = b_0 + b_1X1 + b_2X2 + b_3X3 + b_4X1^2 + b_5X2^2 + b_6X3^2 + b_7X1X2 + b_8X2X3 + b_9X1X3 + b_{10}X1X2X3 \quad (1)$$

where Y is the response, b_0 is the mean response of the 11 formulations and b_i are the regression coefficients. X1–X3 represent the individual effects, X12–X32 are quadratic effects that indicate a non-linear correlation to the response, while X1X2, X3X2, X1X3, and X1X2X3 are interaction effects which show the variations of the responses when two or more factors are changed simultaneously [23].

Table 3. Values for DoE responses from the experimental analysis of the samples.

	Y1	**Y2**	**Y3**	**Y4**	**Y5**	**Y6**	**Y7**	**Y8**	**Y9**	**Y10**
	Rigidity at 2 mm (g)	Rigidity at 4 mm (g)	Load at target (g)	Fracturability (g)	1st Fracture work done (mJ)	1st Fracture deformation (mm)	Mean load (g)	Weight loss %	Reconstitution time	Viscosity (cP)
F1	703.50 ± 140.71	758.67 ± 118.31	1042.50 ± 207.03	738.83 ± 163.25	4.95 ± 2.02	1.44 ± 0.45	487.23 ± 86.40	5.78 ±1.35	46.00 ± 4.58	6.61 ± 0.11
F2	36.30 ± 22.60	262.50 ± 62.60	524.50 ± 190.00	151.17 ± 173.96	1.31 ± 1.81	1.79 ± 1.72	31.73 ± 11.47	39.58 ± 12.76	39.00 ± 5	6.31 ± 0.15
F3	1244.00 ± 49.39	1736.00 ± 35.77	2147.67 ± 167.61	1232.67 ± 109.58	9.63 ± 4.71	1.53 ± 0.37	868.57 ± 26.46	4.93 ± 0.35	51.00 ± 5	6.97 ± 0.09
F4	885.17 ± 161.82	1470.00 ± 223.34	2289.50 ± 389.54	716.00 ± 78.56	2.41 ± 0.65	0.76 ± 0.11	661.10 ± 118.05	48.73±9.50	31.00 ± 2.64	8.28 ± 0.03
F5	860.83 ± 208.11	1844.50 ± 196.44	3020.83 ± 443.61	2322.67 ± 1652.61	41.68 ± 35.45	3.57 ± 2.46	629.13 ± 146.88	3.61±1.27	95.00 ± 6.24	10.30 ± 0.10
F6	625.00 ± 28.70	1046.83 ± 94.40	1555.00 ± 202.21	1555.00 ± 202.21	34.99 ± 2.62	4.99 ± 0.01	409.77 ± 22.61	4.93±0.02	91.00 ± 3	11.53 ± 0.15
F7	997.50 ± 79.38	1831.33 ± 88.97	3634.00 ± 348.84	1839.50 ± 1567.23	26.47 ± 32.20	2.69 ± 2.02	710.83 ± 77.03	2.47±0.27	60.33 ± 8.62	10.33 ± 0.05
F8	1297.17 ± 253.33	1836.83 ± 333.32	3123.83 ± 852.02	1419.33 ± 238.16	8.50 ± 2.64	1.47 ± 0.29	868.73 ± 239.34	7.73±4.67	90.00 ± 4.58	8.57 ± 0.06
F9	1098.17 ± 229.28	1682.67 ± 282.89	2383.50 ± 483.05	1082.50 ± 295.28	8.91 ± 5.48	1.49 ± 0.42	770.27 ± 125.43	3.53±0.24	114.00 ± 3.60	8.94 ± 0.03
F10	1401.30 ± 198.56	2206.83 ± 121.00	3566.33 ± 201.66	1390.33 ± 166.97	13.79 ± 2.41	1.78 ± 0.14	963.90 ± 172.15	1.54±0.82	111.67 ± 3.51	10.47 ± 0.15
F11	1508.50 ± 87.72	2171.67 ± 147.07	3460.67 ± 407.48	1503.83 ± 183.35	12.00 ± 5.55	1.47 ± 0.44	1116.27 ± 97.59	3.48±0.58	103.00 ± 6.24	8.99 ± 0.01

3.2.2. Evaluation of the Quality of Fit

To check whether the regression equations fit the experimental results, several parameters were calculated (Table 4): R^2, the regression coefficient shows the fraction of the response variance explained by the model equation. The regression parameter R^2 had values over 0.9 for first fracture work, second fracture work, reconstitution time and over 0.8 for rigidity at 2 and 4 mm, fracturability, viscosity which shows a good fit of the data to the created model. For the load at target, mean load and the weight loss percentage, the R^2 values ranged between 0.6–0.8. Q^2 shows the quality of the prediction and had values between 0.5 and 0.7 for almost all responses, validating the predictive power of the model.

Table 4. Statistical parameters for ANOVA test and quality of fit.

Parameter	Response	R^2	Q^2	*p*-Value	Lack of Fit	F-Value	Model Validity	Reproducibility
Rigidity 2mm	Y1	0.87	0.56	0.029	0.529	6.65	0.84	0.74
Rigidity 4mm	Y2	0.80	0.62	0.028	0.411	5.94	0.78	0.77
Load at target	Y3	0.79	0.65	0.009	0.677	8.62	0.90	0.61
Fracturability	Y4	0.94	0.72	0.001	0.721	24.43	0.92	0.86
1st fracture work	Y5	0.94	0.64	0.001	0.258	22.28	0.66	0.96
1st fracture deformation	Y6	0.90	0.69	0.004	0.171	13.77	0.56	0.96
Mean load	Y7	0.60	0.31	0.026	0.427	5.96	0.79	0.66
Weight loss percentage	Y8	0.92	0.79	0.002	0.730	13.64	0.92	0.80
Reconstitution time	Y9	0.94	0.73	0.005	0.167	15.06	0.55	0.96
Viscosity	Y10	0.80	0.49	0.027	0.360	6.03	0.74	0.80

R^2—Percent of the variation of the response explained by the model; Q^2—Percent of the variation of the response predicted by the model, F-value—The ratio of the group means and the mean within group variances; Model validity—Validity of the model; Reproducibility—Variation of the response under the identical conditions, compared to the total variation of the response.

The ANOVA test completed the analysis to show if the variation of responses was determined by the input variables or it occurred by chance. A model is considered significant at a confidence level of 95%, therefore if $p < 0.05$. As shown in Table 3, the p-value was below 0.05 for all responses and indicated highly significant models and a non-significant lack of fit. The data analysis and fitting showed that all the selected independent variables (filler type and ratio and the MC ratio) had a significant influence on the responses.

The interpretation of the results relies on the coefficients of the regression equations displayed below as histograms. The way in which the studied factors influence the responses is shown by the + sign for positive influence and a-sign for a negative effect.

3.2.3. The Effect of the Formulation Factors on the Mechanical Profile of the Freeze-Dried Mouthwashes

Mechanical properties of freeze-dried products significantly impact their behavior during processing, packaging, transport, and use [24]. Texture analysis was chosen for mechanical characterization as a sensitive method that provides accurate results even for porous fragile structures [23]. During sample testing, the equipment records load vs. distance profiles out of which certain parameters can be determined (Figure 2).

Rigidity values were measured at two distances, first at 2 mm (Y1) which ranged between 36.33–1508 g and second at 4 mm (Y2) with visibly higher results for each sample and values from 262.50 to 2171.67 g. These measurements indicate that the F2 sample with sorbitol and no polymer has the smallest rigidity, 36.33 g at 2 mm and 262.50 g at 4 mm, while some formulas with mannitol and MC display significantly higher values, such as F5 and F7-F11. These results are also supported by the regression coefficients histograms (Figure 3a,b) which show that the filler ratio is the most influential factor both on the rigidity at 2 mm and 4 mm; however, when measured at 4 mm, mannitol has a significant positive influence. In other words, the outer layer of the freeze-dried structure

can be reinforced by increasing the amount of filler and for a rigid profound structure, the filler of choice should be mannitol.

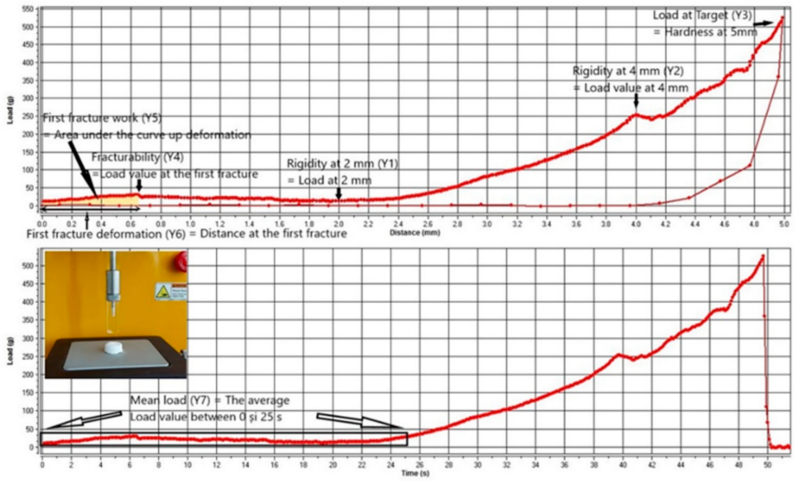

Figure 2. The determination of mechanical characterization responses out of the load vs. distance and load vs. time profiles generated by the texture analysis.

Mannitol, a compound with the structure-forming ability [23] and sweetening properties, was selected as a bulking agent since it favors efficient freeze-drying and the attainment of elegant matrices [25]. It gave freeze-dried compacts with appropriate strength even without matrix-forming polymers and it showed superior mechanical properties when compared to trehalose, and lower when compared to maltodextrin [26].

Load at target (Y3) values were the lowest for F2 (524.50 g), F1 (1042.50 g), and F6 (1555.00 g), while the other formulations had values over 2000 g, with the highest for F7 sample (3634.00 g). The load at target corresponds to the hardness of the sample and corresponds to the resistance of all cumulated layers of product crushed under the texture probe. As expected, it was positively influenced by mannitol in high ratios and by high loads of MC (Figure 3c). Fracturability is an indirect measure of the brittleness of a product. It is recorded as the first load drop on the texture profile. For the fracturability measurements (Y4), the lowest value was obtained for F2 sample, 151.17 g, the most brittle product, compared to the highest one recorded of 2322.67 for F5. The increasing of the MC ratio (X3) conducted to increased resistance to fractures (Y4) (Figure 3d). As previous studies showed, the MC three-dimensional network constitutes the structure of the product that sustains the attached fillers. The presence of high amounts of polymers reinforces the matrices and increases their elasticity [23], which explains the lower brittleness and the high fracturability values.

The other mechanical resistance parameters measured were the mechanical work needed for first fracture (Y5), which showed values within a high range from 1.31 mJ (F2) to 41.68 mJ (F5); the first fracture deformation (Y6), with F5 and F6 having the highest values of 3.57 mm and 4.99 mm; the mean load (Y7) with values of 31.73-1116.27 g, the lowest corresponding to F2 and the highest to F11.

As all the texture parameters, F2 was the weakest formulation, the most fragile, porous, and hard to handle, while F5 and F7 were just the opposite.

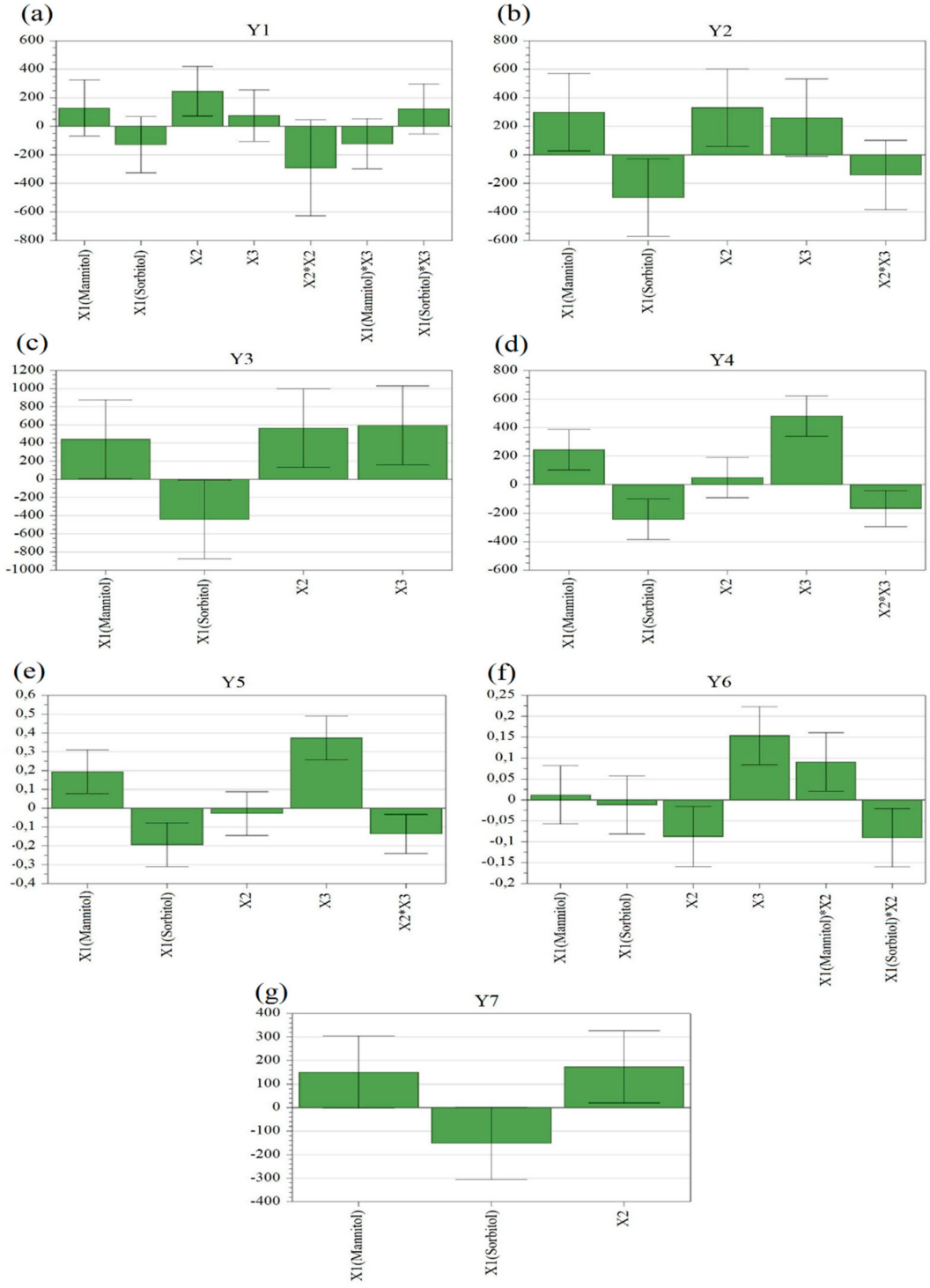

Figure 3. The influences of formulation factors (X1, X2, X3) on the mechanical characteristics of freeze-dried products: (**a**), rigidity at 2 mm (Y1); (**b**), rigidity at 4 mm (Y2), (**c**), load at target (Y3), (**d**), fracturability (Y4), (**e**), 1st fracture work (Y5), (**f**), 1st fracture deformation (Y6), (**g**), mean load (Y7).

3.2.4. The Effect of the Formulation Factors on the Weight Loss Percentage (Y8) of the Freeze-Dried Mouthwashes

The weight loss percentage was calculated to evaluate the consequences of fragile structures on the manipulation and extraction from the blister pockets. It was meant to check if the entire product could be easily removed or a part of it remains stuck into

the alveolae. The weight loss percentage (Y8) was strongly correlated to the mechanical profiles: the highest value was obtained for F2 and F4, formulations with small mechanical resistance, highly fragile samples, not appropriate for packaging and transporting. This behavior is upheld by the small fracturability values of the same formulas. In addition, the weight loss percentages for F7 and F10 were the lowest, in agreement with the fracturability values, which were the highest for these two samples. The filler type (X1) had considerable influence on Y8: the presence of sorbitol determined the embrittlement of the structures and loss of the integrity of the sample (Figure 4a). On the contrary, mannitol had a beneficial impact in maintaining the integrity of the matrices. As expected, the presence of MC in the formulations had a negative effect on the weight loss percentage (Y8), which could be due to the adhesive properties of hydrophilic polymers can contribute to preserving the porous structure and avoid material loss when manipulated.

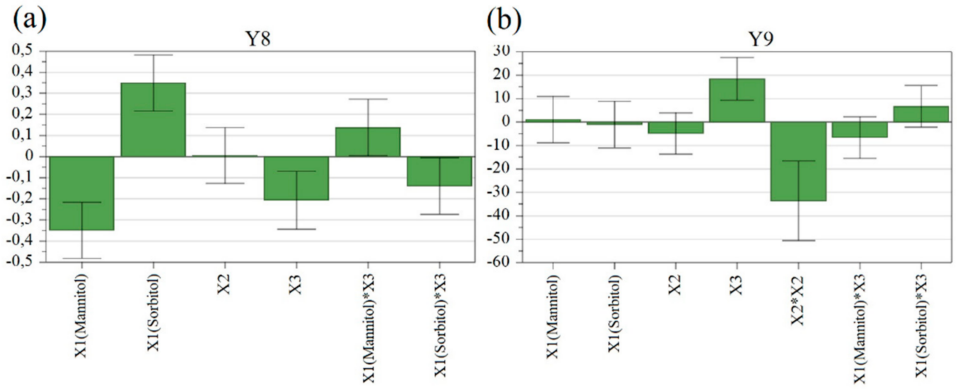

Figure 4. The influences of formulation factors (X1, X2, X3) on (**a**), weight loss percentage (Y8) and (**b**), reconstitution time (Y9).

3.2.5. The Effect of the Formulation Factors on the Reconstitution Time (Y9) of the Freeze-Dried Mouthwashes

Freeze-drying is considered an advantageous method to obtain porous structures which disintegrate rapidly [27]. As shown in Figure 5b, the most important effect on the reconstitution time came from X3, the hydrophilic polymer ratio.

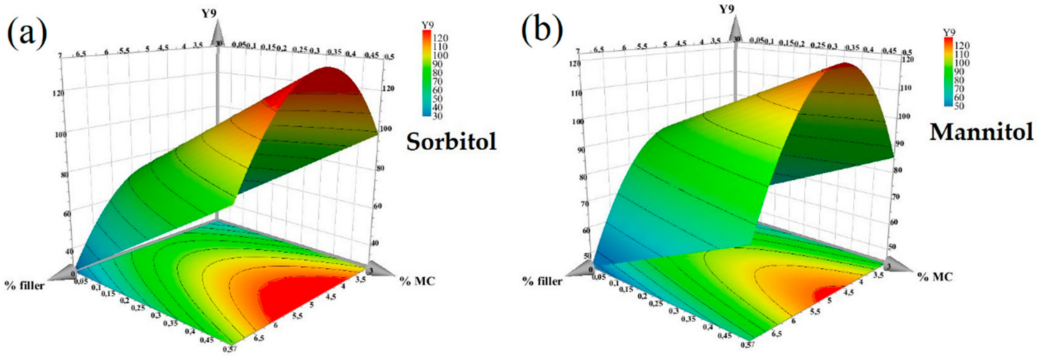

Figure 5. The influences of formulation factors (X1, X2, X3) on the reconstitution time when the filler is (**a**), sorbitol and (**b**), mannitol.

Increasing the amount of MC (X3) led to the formation of dense structures with a slower penetration of the water into the lyophilizate network. It had a growing path along with increasing the filler and MC concentrations, with the highest values of 130.00–114.00 s for F9–F11, which were formulated with 5% filler and 0.25% MC, with one exception, the F7 that showed a small reconstitution time of only 60 s, even if it contains the highest concentrations chosen: 7% filler and 0.5% MC. The nonlinear effect with high magnitude shown on the histogram (Figure 4b) is more evident in the response surfaces of the reconstitution

time displayed in Figure 5. The reconstitution time reaches a maximum inflection point at intermediate filler levels and high ratios of MC, while it decreases when large amounts of filler are associated with high ratios of MC. This effect was observed by other research groups and was explained by the presence of the easily soluble filler that promotes water absorption even in a structure with high loads of hydrophilic polymer [28].

3.2.6. The Effect of the Formulation Factors on the Viscosity (Y10) of the Reconstituted Mouthwashes

For the viscosity of the reconstituted samples (Y10) the range of variation was not very wide. The MC ratio (X3), the viscosity modifying agent, was the only factor with a significant influence on the viscosity values (Y10) (Figure 6). The higher the MC ratio, the higher the viscosity. The lowest values (6.31–8.28 cP) corresponded to the first four formulations with no MC added (F1–F4).

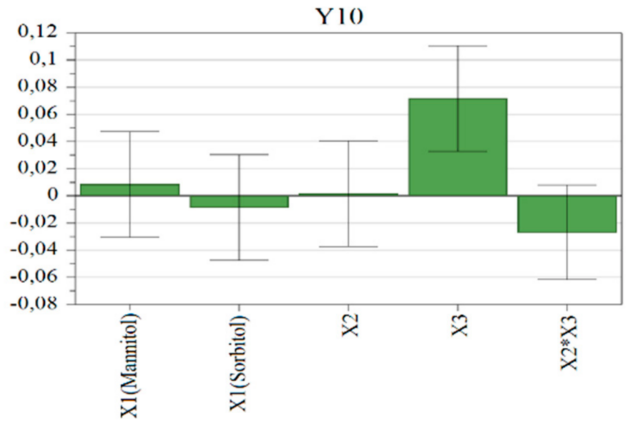

Figure 6. The influences of formulation factors (X1, X2, X3) on the viscosity of the reconstituted mouthwash (Y10).

3.2.7. Optimization of the *Cetraria islandica* Mouthwash Formulation

To obtain an optimal formula in terms of the desired lyophilized characteristics, an optimization process was performed. For that, a set of constraints was applied to the developed models of certain responses and only the formulations that fulfill those conditions with a certain degree of probability were considered. The conditions envisaged the maximization of fracturability, minimization of weight loss percentage, minimization of reconstitution time and maximization of viscosity. Such a formulation would ensure easy manipulation, with no material loss when extracted from the package, fast reconstitution, and a pleasant mouthfeel with good adherence to the oral cavity surface. The composition of the optimal formula generated by the software is presented in Table 5.

Table 5. The quantitative and qualitative composition of the optimal mouthwash formulation.

Factor	Value	Ingredient	Quantity (*w*/*v*%)
Filler Type (X1)	Mannitol	**Mannitol**	**7.00**
% filler (X2)	7	Poloxamer 407	5.00
% MC (X3)	0.5	**Methylcellulose**	**0.50**
	[redacted]	Allantoin	5.00
		Hyaluronic acid	0.125
		Cetraria islandica extract	5.00
		Flavoring agent	0.50

The bold data underlines the factors from the DoE.

For the next step, we prepared the sample according to the generated formula, freeze-dried it, and then analyzed it as the other samples before. The experimental values were

collected using Modde software and, in the end, the predicted values were compared to the experimental ones, as residual values. As shown in Table 6, the values obtained experimentally were close to the values predicted by the software. Therefore, the experimental design showed high reliability and may be used in further studies regarding freeze-dried mouthwashes. This is an innovative product, with no correspondents on the market to be compared with. However, among pharmaceutical products, the oral lyophilizates are quite similar in terms of the production process, product characteristics and use. Literature reports give a hardness minimum limit of 4N for oral lyophilizates and a disintegration time of maximum 180 s, which are both in accordance with the developed freeze-dried mouthwash.

Table 6. Predictions for the optimal formula and experimental results.

Response	Predicted Value(Mean ± σ)	Experimental Value(Mean ± σ)	Residual
Fracturability (g)	1826.85	1951 ± 123	+124.15
Weight loss (%)	2.59	2.39 ± 0.91	−0.20
Reconstitution time (s)	74.71	80 ± 6.56	+5.29
Viscosity (cP)	9.84	8.42 ± 0.28	−1.42

σ = standard deviation.

Up to this point, the study demonstrated that freeze-dried mouthwashes with appropriate characteristics in both solid state and as reconstituted solutions are feasible and that the DoE strategy enabled the optimization of products. *Cetraria islandica* extract was successfully incorporated for its mentioned applications such as mouth and throat anti-irritant, antimicrobial, or moisturizing and soothing effects [29]. Besides the previously described roles of *Cetraria islandica* extracts, recent studies reported the ability of its polysaccharides to induce keratinocyte differentiation with important implications in wound healing [30]. Our results revealed the lack of toxicity of extracts and an antibacterial activity on *S. aureus* and *E. faecalis* which supported the hypothesis of oral care product preparation. As freeze-drying is a state-of-the-art method to dry or concentrate extracts without altering their biological properties, and its use was reported for lichen species, we assumed that the lyophilized mouthwash formulations would preserve the effects of extracts [16]. Future studies envisage the biological evaluation of freeze-dried formulations and in vivo studies in healthy volunteers.

4. Conclusions

The current work describes the application of the QbD approach in the development of freeze-dried mouthwashes. To obtain an optimal formulation of freeze-dried mouthwash, both qualitative and quantitative factors that may impact the main characteristics of the final product were investigated. A full factorial design with three factors and two levels was conceived and eleven mouthwash samples were prepared and analyzed. The results revealed the positive influence of mannitol and methylcellulose to reinforce the structure of matrices and the positive effect of methylcellulose in preserving the integrity of the samples and avoiding material loss when manipulated. Finally, an optimal formula was generated and characterized showing the good predictive capacity of the statistical model. Although the freeze-dried mouthwash formulations with *Cetraria islandica* extract were not yet tested for their in vitro or in vivo efficacy, data regarding freeze-drying process leads to optimistic assumptions. This study offers the methodology for the preparation and optimization of solid mouthwashes loaded with herbal extracts, which could contribute to the development of a whole new area of the oral care industry that addresses the needs of consumers looking for stable, eco-friendly, light weight, easily transportable products.

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