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Natural Deep Eutectic Solvents as a Novel Bio-Based Matrix for Ready-to-Use Natural Antioxidants-Enriched Ingredients: Extraction and Formulation Optimization

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Abstract: The escalating consumer demand for sustainable cosmetic ingredients poses distinct challenges, particularly concerning their stability within the final formulation. Although natural resources offer a pool of antioxidant molecules with diverse structures and polarities, achieving stabilization combined with a comprehensive antioxidant profile often proves incompatible with practical preformulation considerations. Notably, Calendula, which is rich in both polar (glycosylated flavonoids) and nonpolar (carotenoids) antioxidants, is a standout candidate. Nevertheless, the market lacks an ingredient embodying this diversity, primarily due to the limited polarity range of available usable solvents. Natural deep eutectic solvents (NaDESs) emerge as a promising solution. This study explores NaDES technology with the goal of developing a unique Calendula extract enriched in both polarities of antioxidants, a composition that is unattainable with traditional solvents. A screening of 12 NaDESs with varying polarities highlighted a NaDES based on betaine and glycerol as particularly effective, outperforming ethanol. Leveraging response surface methodology, an optimal mechanical stirring procedure for extraction was identified. The resulting extract showed a total flavonoid content of 45.42 ± 0.85 mg eq rutin/g of biomass and a total carotenoid content of 383.54 ± 4.73 µg/g biomass. It was then incorporated into a sustainable cream (1% and 10%wt) using an innovative mixing technology. The resulting creams demonstrated stability over 90 days, with no significant deviations in pH or rheological properties compared to the control, and a droplet size that was inferior to 10 µm. This study lays the foundation for pioneering natural antioxidant cocktail-loaded ingredients that are suitable for eco-friendly cosmetic formulations, substantiating the viability of integrating environmentally friendly ingredient-based solvents.

Keywords: natural deep eutectic solvents; antioxidant; formulation; *Calendula officinalis*

Citation: Boudesocque-Delaye, L.; Ardeza, I.M.; Verger, A.; Grard, R.; Théry-Koné, I.; Perse, X.; Munnier, E. Natural Deep Eutectic Solvents as a Novel Bio-Based Matrix for Ready-to-Use Natural Antioxidants-Enriched Ingredients: Extraction and Formulation Optimization. *Cosmetics* **2024**, *11*, 17. https://doi.org/10.3390/cosmetics11010017

Academic Editor: Othmane Merah

Received: 20 December 2023
Revised: 19 January 2024
Accepted: 22 January 2024
Published: 25 January 2024

1. Introduction

Natural antioxidants, a significant category of bioactive compounds, are currently the focus of extensive research, particularly in the realm of skincare and cosmetics. Natural antioxidants play a crucial role in various essential processes in skin aging, such as preventing inflammation and UV damage or regenerating cells. Botanical extracts provide a wide range of antioxidants, from nonpolar carotenoids and curcumin to highly polar flavonoids or tannins [1–4]. The growing demand for natural active ingredients in cosmetics has revalued plant extracts as a relevant source of antioxidants. These ingredients can be utilized as active agents in antiaging skincare or sun protection products, as well as functional ingredients for preserving cosmetics [2,3].

In many cases, a single plant contains a number of antioxidant compounds, typically a mixture of polar and nonpolar metabolites, that contribute to a complete antioxidant

spectrum. *Calendula officinalis* L. It exemplifies a diverse antioxidant composition and has become a popular source of ingredients in cosmetics, particularly ones dedicated to sensitive skin, due to its antioxidant and anti-inflammatory properties [5,6]. In fact, Calendula flowers are well known for their significant flavonoid content, such as isoquercitrin or narcissin, and carotenoids, including β-carotene or lutein [5,6]. Despite many commercially available ingredients containing Calendula flower extracts, none fully represent the plant's antioxidant spectrum. Indeed, conventional solvents or supercritical fluids that are currently used in Calendula flower extraction are only able to target one class of antioxidant at a time, either flavonoids or carotenoids. Moreover, as is customary with plant extracts, the preformulation stage can be complex, given the limited available options, such as powders that are absorbed on maltodextrin or liquid ethanolic or glycerinic ingredients [1,7].

Recently, the emergence of natural deep eutectic solvents (NaDESs) in the cosmetic market introduces a sustainable alternative (NaDESs) [8]. These bio-inspired solvents were first described at the beginning of the 2010s by Dai et al. [9] and are now considered the third biological liquid in plants, alongside water and lipids. NaDESs are produced using abundant natural molecules such as sugars, amino acids, organic acids, polyols, etc. [9–12]. Initially, DESs and NaDESs were described as mixtures that exhibit a lower melting point than their pure components taken separately, which is attributed to hydrogen bonding, van der Waals, and/or electrostatic interactions. However, a more precise definition based on thermodynamic considerations is now widely acknowledged: a DES refers to mixtures of pure compounds displaying significant negative deviation from ideality, owing to their eutectic point temperature being considerably lower than that of the ideal mixture [13]. From an environmental standpoint, these alternative solvents are an intriguing and appealing substitute for conventional organic solvents due to their natural origin, low toxicity, and high biodegradability [8–15]. However, although the eco-toxicity of NaDESs is generally low for vertebrates, recent research has highlighted their eutrophication effect on some algal species. This should be taken into account when considering their impact on aquatic ecosystems [16]. DESs and NaDESs find application in various fields of interest, including the substitution of organic solvents in organic synthesis [17–20], CO_2 capture [21], cryopreservation [22], and biomass pretreatment [15]. NaDESs, with their enhanced solubilization power and tunable selectivity, prove advantageous in extraction processes, particularly for stabilizing fragile metabolites [10–15]. The cosmetics industry was the first to explore and exploit these solvents for producing original ingredients [23–33].

An underutilized aspect of NaDESs is their ability to generate a distinctive phytochemical profile by combining polar and nonpolar metabolites. Recent studies, specifically those concentrating on the valorization of Spirulina, have indicated that various NaDES formulations, incorporating a blend of glycerin and carbohydrates or amino acids ± water, exhibited a phytochemical profile encompassing polar phycobiliproteins to nonpolar free fatty acids or carotenoids [34,35]. A recent study by Gan et al. [36] reported the simultaneous extraction of crocin and geniposide from gardenia fruits using ultrasound-assisted extraction (UAE) and a NaDES based on choline chloride and organic acid or polyol. Another example is the simultaneous extraction of ascorbic acid, phlorotannin, and fucoxanthin from *Fucus vesiculosus* using two lactic acid-based NaDESs. The use of NaDESs increases the stability of both polyphenols and fucoxanthin, as evidenced by their retention when stored for 360 days at room temperature in the dark [37]. Given these results, there is a promising opportunity for the cosmetic industry to utilize NaDESs in creating ingredients with a comprehensive antioxidant spectrum. Extracts that are enriched in both flavonoids such as rutin and isoquercitrin and carotenoids such as lutein and beta-carotene could be produced from Calendula using NaDESs.

Even if some research teams choose to purify active molecules from the NaDES [38], we consider NaDES-based extracts to be a new type of active cosmetic ingredients. Integrating a NaDES-based extract directly in the final formulation allows us to maintain the sustainability of the NaDES technology [23,39]. Hence, all raw materials must adhere to the prevailing regulations in the areas of production and marketing. For instance, any NaDES

that is based on choline chloride should be excluded to align with European and Chinese cosmetic regulations. Additionally, a significant limitation arises due to the lack of data on the compatibility of NaDESs and NaDES-based ingredients in the final formulation. Substituting a conventional extract with a NaDES-based extract, which is rich in hydrogen-bonding components, may alter the rheology and/or stability of the end product through interactions with the formula's ingredients. Concerning the most prevalent cosmetic form, creams, the primary challenge in achieving a natural profile is replacing petroleum-based surfactants with environmentally friendlier yet more delicate alternatives. Introducing a viscous matrix, such as a NaDES extract, could necessitate adjustments to mixing protocols to preserve the product's stability. A recent study by Jamaleddine et al. demonstrated that the inclusion of a Proline/Glycerol NaDES-based tomato extract in a moisturizing cream was feasible, but the impact of the NaDES on the properties or the stability of the product was not described [40]. More recently, Rocha et al. enriched two commercially available creams with NaDES-based extracts, noting diverse effects on the characteristics and stability of the final mixture [41]. It is noteworthy that, in this specific case, the NaDES-based extract was not part of the manufacturing process. In another study, Santos et al. introduced a NaDES-based antioxidant extract into a nanoemulsion, focusing solely on the antioxidant activity without addressing the stability of or potential alterations to the product [42]. Despite these examples in the cosmetic field, research on the formulation of NaDES-based extracts in emulsions, particularly concerning stability issues, remains limited.

This study introduces an innovative method employing NaDESs to extract a comprehensive antioxidant spectrum from Calendula flowers. After extraction, the optimized extract was incorporated into a sustainable cosmetic cream. The extraction process underwent optimization through the use of the design of experiments and response surface methodology (RSM) to explore eco-extraction processes: ultrasonic-assisted extraction (UAE) and the innovative dual asymmetric centrifugation (DAC) process. The optimized extract was incorporated into a cosmetic cream through a specifically designed manufacturing process utilizing the innovative DAC method. This methodology allowed for the rapid preparation of creams containing 1%wt or 10%wt of the Calendula antioxidant extract without the need for a heating phase. The ensuing properties concerning stability and rheology were documented.

2. Materials and Methods

2.1. Chemicals

Betaine 98% anhydrous, Glycerol 99% extra pure, nonanoic acid 98%, decanoic acid 97%, octanoic acid 99%, proline 99%, potassium acetate 98%, and aluminium chloride 6.0 N titrated solution were purchased from ThermoScientific Chemicals (Kandel, Germany). Lauric acid 99%, levulinic acid 98%, 1,3-propanediol, trifluoroacetic acid, and ammonium acetate were purchased from Acros Organics (Geel, Belgium). Lactic acid 90% was purchased from Alfa Aesar (Haverhill, MA, USA). Hexane, acetone, methanol, ethanol 96%, and analytical-grade acetonitrile were purchased from CarloErba (Val de Reuil, France). Water was purified using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Rutin was purchased from Extrasynthèse (Genay, France). Castor oil was purchased from Aroma-Zone (Paris, France). Glycerol (>99%), Benzyl alcohol, and Dehydroacetic acid were obtained from Fisher Scientific (Illkirch, France). Xanthan gum was obtained from COOPER (Melun, France). Gattefossé (Saint-Priest, France) provided medium-chain triglycerides (Labrafac Lipophile® 1379). Sclerotium gum (Amigum®) was a kind gift from Alban Muller (Fontenay-sur-Eure, France). Hydrogenated lecithin (Lipoid P75-3®) was a kind gift from Lipoid Kosmetik (Steinhausen, Switzerland).

2.2. Biomass

Calendula officinalis L. flowers powder sample (particle size 100–200 µm) was kindly provided by PMA 28 (Varize, France).

2.3. NaDES Preparation

NaDESs were prepared by mixing appropriate ratio of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) (see Table 1). The mixture was heated at 50 °C (nonpolar) or 80 °C (polar) and stirred until a colorless liquid was obtained.

Table 1. Composition and references of NaDESs used in this study.

	NaDES	Component 1	Component 2	Component 3	Molar Ratio	Water Amount (% *w*/*w*)
Nonpolar	C8:C10	Octanoic acid	Decanoic acid		3:1	
	C9:C12	Nonanoic acid	Lauric acid		3:1	
	C9:C10:C12	Nonanoic acid	Decanoic acid	Lauric acid	3:2:1	
	C8:Lac	Octanoic acid	Lactic acid		5:1	1
Polar	Pro:Gly	Proline	Glycerol		1:3	
	Pro:Lev	Proline	Levulinic acid		2:1:	20
	Bet:Gly	Betaine	Glycerol		1:8	20
	Bet:Lev	Betaine	Levulinic acid		1:1	
	Lev:Gly	Levulinic acid	Glycerol		1:1	
	Prop:Lev	1,3-propanediol	Levulinic acid		1:1	
	Prop:Gly	1,3-propanediol	Glycerol		1:1	

2.4. Calendula Extraction

2.4.1. NaDES Screening

Conventional solvent (ethanol (EtOH), hexane/acetone (6:4, *v*/*v*), castor oil): First, 5 g of biomass was extracted using 20 mL of solvent for 60 min using an ultrasonic bath RK-100H (Bandelin electronic, Berlin, Germany) at 30 °C, according to Wils et al. [27,35]. Resulting extracts were then filtered and evaporated to dryness (EtOH and hexane/acetone).

NaDES: a total of 100 mg of biomass was extracted by 2 g of NaDES for 60 min using an ultrasonic bath RK-100H (Bandelin electronic, Berlin, Germany) at 30 °C, according to Wils and al. [34,43]. Resulting extracts were then filtered and collected.

2.4.2. Process Optimization Using the Design of Experiment Approach

Box Behnken design was adopted for parameter optimization of flavonoids and carotenoid extraction. Two extraction processes were explored: ultrasound-assisted extraction (UAE) and mechanical extraction using a Hauschield Speedmixer® DAC 150 (MP2E solutions, Fontenay-aux-Roses, France). For UAE, the parameter ranges were fixed based on conducted preliminary experiments: the extraction time (15–60 min); biomass/NaDES ratio (2–10%); and the temperature (30 °C–60 °C). For mechanical extraction, the parameter ranges were fixed based on conducted preliminary experiments: the extraction time (15–60 min); biomass/NaDES ratio (2–10%); and the rotation speed (500–3000 rpm).

A total of 17 experiments were conducted in this study to optimize carotenoid and flavonoid extraction parameters. The analysis of variance (ANOVA) was conducted for the validation of the theoretical accounts of the optimization process.

The estimation of optimum conditions was achieved through a second-order polynomial equation. The generalized form describes the relationship between the responses and the parameters as shown below:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} {x_i}^2 + \sum_{i \leq 1 \leq j}^{n} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

In the aforementioned equation, Y is the response, while β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, interaction, and quadratic, respectively. x_i and x_j represent the independent variables and the number of independent parameters (n = 3).

The analyses were conducted using Design-Expert 13 (Stat-Ease, Minneapolis, MN, USA). The model adequacy was assessed based on the obtained coefficient of multiple determination (R^2), coefficient of variance (CV), and *p*-values for the model and lack of fit testing.

2.4.3. Extraction Scale-Up

First, 1.620 g of total *C. officinalis* biomass was extracted using 27 g of Bet:Gly (1:8, mol/mol, 20% Water) using Hauschield Speedmixer DAC 150.1 CM148 (MP2E solutions, France) at 1500 rpm for 30 min. Extract was then centrifuged for 10 min at 8000 rpm (Rotanta 460R, Hettich, Kirchlengern, Germany), and resulting supernatants were filtered through 56 µm sieves.

2.5. Extract Analysis

2.5.1. Total Carotenoid Content (TCC)

TCC was adapted from the method of Wang et al. 2005 [44]. First, 100 mg of *C. officinalis* L. NaDES extract was diluted with 0.80 mL of methanol. Then, 290 µL of diluted solution was directly analyzed using UV–vis microplate spectrophotometer reader (Multiskan GO, Thermo Fisher Scientific, Montigny-le-Bretonneux, France). NaDES diluted in methanol was used as control. Total carotenoid content (TCC) was then calculated using the absorbance at 450 as shown in Equation (2).

$$TCC = (4 \times OD_{450}) \times FD \quad (2)$$

where TCC (µg/mL) is the carotenoids concentration, FD is the dilution factor, and OD_{450} is the extract's optical density at 450 nm.

2.5.2. Total Flavonoid Content (TFC)

Quantitative estimation of TFC was carried out by the chloride colorimetric method adapted from Chang et al., 2002, with a slight modification [45]. A 0.5 mg/mL rutin solution was prepared and diluted to prepare for the calibration, ranging from 0.005 to 0.5 mg/mL (Supplementary Material Supporting information). First, 500 µL of standards, or 500 mg NaDES extract was mixed with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride solution, and 0.1 mL of 1 M potassium acetate. After incubation at room temperature for 30 min, sheltered from light, the absorbance of the reaction mixture was measured at 415 nm using UV–vis microplate spectrophotometer reader (Multiskan GO, Thermo Fisher Scientific, SAS). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in control. Results were expressed in mg of rutin equivalents (REs) per gram of NaDES and biomass.

2.5.3. High-Performance Liquid Chromatography (HPLC)

Ethanol(EtOH), C8:Lac, and Bet:Gly extracts were analyzed using HPLC to obtain carotenoids' and flavonoids' profile.

Carotenoids: Analysis was performed according to Wils et al. [43].

Flavonoids: EtOH extract was concentrated under pressure to dryness and then resuspended in MeOH. C8:Lac and Bet:Gly were diluted in MeOH before injection. The samples were analyzed on a Dionex U3000RS HPLC chain equipped with a diode array (Thermo Fisher Scientific SAS). Then, 5 µL of each sample were injected into a column (Accucore aQ 150 mm × 3 mm × 2.6 µm), accompanied by a precolumn (13 mm × 0.3 mm) (Thermo Fisher Scientific SAS). The flow rate was set at 1 mL/min, and the column temperature was maintained at 40 °C. Mobile phases were as follows: water with 0.1% trifluoroacetic acid and B acetonitrile. The gradient was set as follows: initial solvent B

content was 0%, raised to 15% in 2.49 min, then 60% in 9.51 min, and finally, to 100% in 3.31 min and maintained for 2 min.

2.6. Cream Formulation

A COSMOS (COSMetic Organic and Natural Standard)-certifiable chassis cream formula was selected to be able to measure the formulability of the optimized Bet:Gly Calendula extract (BGCE). Either 1% (wt) or 10% (wt) of a BGCE were introduced in formulas. They were replaced by an equivalent weight of glycerol in the control formulations. This formula is reported in Table 2. Creams were produced at room temperature with a DAC 150.1 CM148 mixer (Hauschild SpeedMixer; Hamm, Germany). First, the aqueous phase was prepared by placing the components in a Speedmixer® mixing cup (depending on the sample: glycerol, water, BGCE, and the mix of thickeners), and then mixed for 4 min at 1800 rpm. Then, the emulsion was made by adding directly in the same cup, on top of the aqueous phase, the components of the oily phase (medium-chain triglycerides, the preservative, and the emulsifier) and mixing for 2 min at 3500 rpm. For the cream containing 10% of BGCE, it was necessary to add the Hauschield pulverizing ring set directly into the mixing cup. Each assay was performed in triplicate (n = 3). The creams were stored in a climatic chamber (Memmert, Büchenbach, Germany) at 40 °C with 75% humidity for 90 days. The pH of the creams was measured at D1 and D90 with a suitable pH electrode after a period of stabilization of 20 min.

Table 2. Quantitative composition of the control and Bet:Gly Calendula extract-enriched creams.

		%wt in the Analyzed Samples	
Ingredients	**Function**	**Control**	**Calendula Creams**
BGCE (%wt)	Active	0	1 or 10
Xanthan gum (%wt)	Thickener	0.25	0.25
Sclerotium gum (%wt)	Thickener	0.75	0.75
Glycerol (%wt)	Humectant	10	9 or 0
Hydrogenated lecithin (%wt)	Emulsifier	3	3
Caprylic/capric triglycerides (%wt)	Emollient	25	25
Dehydroacetic acid/ benzyl alcohol mix (%wt)	Antimicrobial agent	1	1
Water (%wt)	Solvent	q.s 100	

2.7. Static Multiple Light Scattering-Based Stability Analysis

Static multiple light scattering-based based stability analyses were conducted with the Turbiscan® instrument (Formulaction, Toulouse, France). TSI (Turbiscan® Stability Index), a value calculated by the Turbisoft lab 3.0.2.0 software, gives an idea of the stability of the emulsion. The droplet size was also calculated using the software. The analysis was carried out 24 h after the preparation of each sample and at D90 in a 4 mL vial.

2.8. Rheology

Rheological characterization was conducted using a Kinexus pro+ rheometer (Netzsch, Selb, Germany) at a temperature of 25 °C. All measurements were performed in triplicate, with a fresh sample loaded for each run. After loading, samples were allowed to relax and acclimatize for a minimum of 2 min prior to measurement.

To assess the flow properties of creams, continuous shear flow tests were conducted using a sanded cone–plate geometry (4° angle, 40 mm diameter). Apparent viscosity values were recorded by subjecting the samples to increasing shear rates ranging from 0.1 to 1000 s^{-1} for a duration of 150 s.

The deformation sweep experiment was performed at a frequency of 1 Hz and a strain ranging from 0.1% to 100%. The frequency sweep experiment was carried out at

a strain of 1% belonging to the linear regime, and the frequency varied from 50 Hz to 0.1 Hz. Viscoelastic modulus results and relaxation times were obtained individually for each replicate using the Rspace 2.0.2193.1 software, supplied with the rheometer and then averaged using R 4.1.1 software.

2.9. Statistical Analysis

TCC and TCC titration: Results are expressed as mean values ± standard deviation (SD). The Tukey test associated with a one-way ANOVA was used for statistical analysis using the GraphPad Prism 7.0 software.

Rheology: Results are expressed as mean values ± standard deviation (SD). The Mann–Whitney test was used for statistical analysis using the GraphPad Prism 7.0 software.

3. Results and Discussions

3.1. NaDES Screening

To identify the most suitable NaDES matrices for extracting and formulating a comprehensive spectrum of antioxidants from Calendula, an initial screening employing ultrasonic-assisted extraction (UAE) was carried out. The selection of NaDES raw materials was conducted meticulously, considering their acceptance and properties as cosmetic ingredients, in alignment with European and Chinese cosmetic regulations. From a shortlist of eleven NaDES options (Table 1), we compiled a diverse polarity range, ranging from the highly polar betain/glycerol mixture to a nonpolar fatty acid-based NaDES, drawing inspiration from prior research on carotenoid extraction from Spirulina [34,35]. Organic acids, sugars, and amino acids have been identified as compounds of interest for the design of broad-spectrum polarity NaDESs, and thus, most systems are based on these raw materials [35–37].

Each extract underwent UV-Vis spectroscopy analysis to determine the total carotenoid content (TCC) and total flavonoid content (TFC). Reference extractions using EtOH, hexane/acetone, and castor oil as a comparative solvent were also conducted. Figure 1 and Table S1 provide a summary of all data.

In terms of flavonoid content, all polar NaDESs showed a high flavonoid rate, with the Bet:Gly (1:8, mol/mol) + 20% water NaDES providing the highest extraction rate in comparison to EtOH. This result is consistent with previous findings by Caprin et al. [16] that amino acid (or sugar)-based NaDESs, glycerol, and water are optimal solvents for the extraction of flavonoids from Calendula flowers. It is clear that fatty acid-based NaDESs are inadequate for this purpose. Interestingly, NaDESs that are derived from polyols, organic acids, or amino acids are ineffective in extracting flavonoids. These findings emphasize the crucial role of water in the flavonoid extraction process.

In terms of carotenoids, none of the NaDESs that were screened showed better extraction performance than the reference hexane/acetone (6:4, v/v) mixture. Among the tested NaDESs, the most effective included C8:Lac (5:1, mol/mol) and Bet:Gly (1:8, mol/mol), with the addition of 20% water. Unexpectedly, NaDESs based on fatty acids, despite being the least polar, did not exhibit the highest efficiency and showed no significant difference ($p > 0.05$). Only the NaDESs that were based on C8, combined with a specific organic acid—lactic acid—displayed a notable affinity for carotenoids.

The strong affinity of Bet:Gly for carotenoids was also consistent with previous data that highlighted the broad polarity range of molecules that could be extracted from spirulina with polar NaDESs based on glycerol and sugar (or amino acids) [34,35].

C8:Lac and Bet:Gly were found to be the best choice for the simultaneous extraction of flavonoids and carotenoids from Calendula flowers. HPLC analysis was performed on both extracts and compared to the EtOH extract, which served as a relevant reference for cosmetic ingredients. The EtOH extract contained low levels of carotenoids, specifically xanthophylls (retention time between 4 and 5.5 min). The carotenoid profiles of C8:Lac and Bet:Gly were similar, dominated by beta-carotene (Figure 2). In terms of flavonoid content, EtOH and Bet:Gly contain two major flavonosides (rutin and narcissin) and a

smaller amount of isoquercitrin. C8:Lac, on the other hand, contains only a small amount of isoquercitrin, which is expected due to its lower polarity as a NaDES.

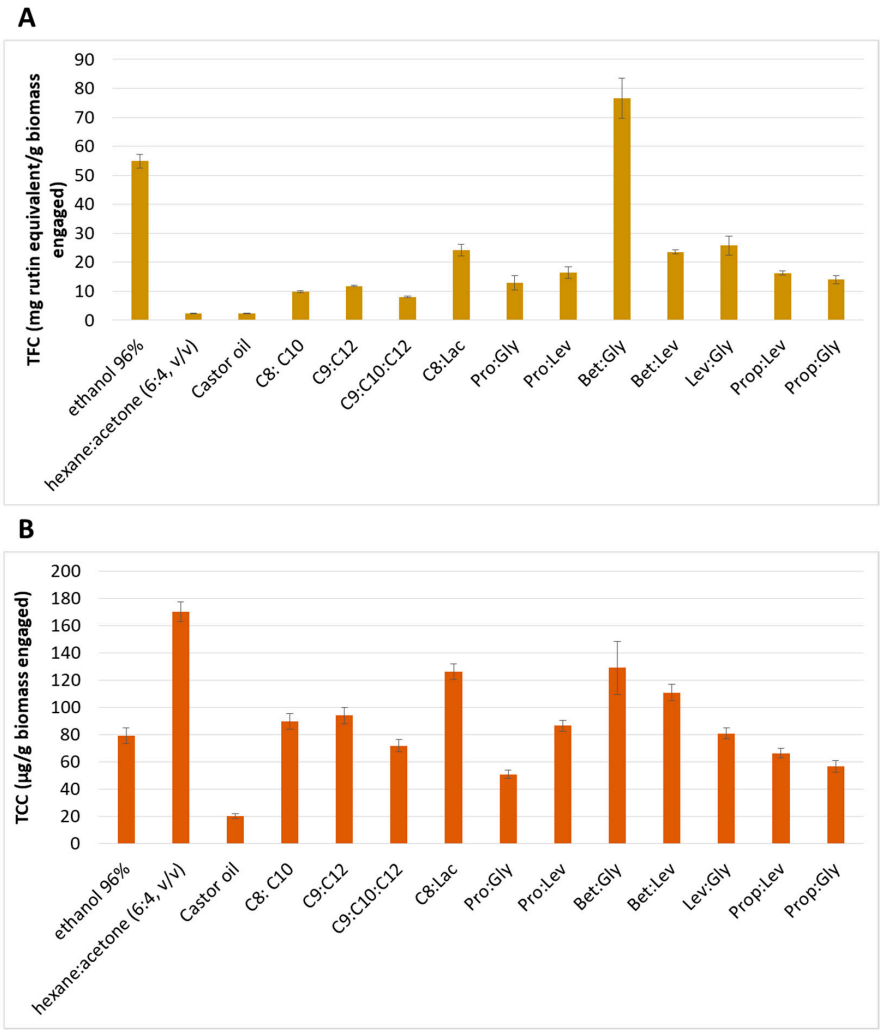

Figure 1. TFC (**A**) and TCC (**B**) of extracts from preliminary screening of NaDESs.

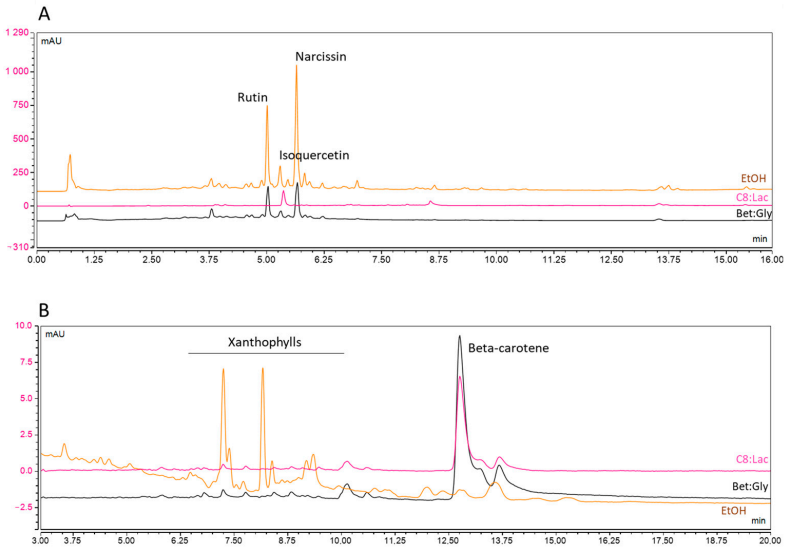

Figure 2. HPLC profiles of EtOH (orange), C8:Lac (pink), and Bet:Gly (black) calendula extract. Flavonoids (**A**) at 280 nm and carotenoids (**B**) at 450 nm, according to Section 2.5.

As the main objective of this study is to generate a comprehensive antioxidant spectrum of Calendula extracts and then to include it in an oil-in-water (O/W) cream up to 10%wt, we chose the hydrophilic Bet:Gly for extraction optimization

3.2. Extraction Optimization Using the Design of Experiment Approach

As Bet:Gly is recognized as a highly viscous solvent, two extraction processes were investigated: a conventional ultrasonic-assisted extraction (UAE) and an innovative mechanical extraction by means of dual asymmetric centrifugation (DAC). The DAC process could overcome the low mass transfer during the extraction process thanks to an intensive mixing of the NaDES with biomass. This process has already been successfully used for mechanochemistry with NaDESs [17], which requires a high mixing capacity. Here, it was applied for the first time to extract biomass with NaDESs.

Both procedures were optimized utilizing TFC (mg equivalent rutin/g biomass) and TCC (µg/g of biomass) as distinguishing criteria, with a particular emphasis on the TCC. A Box–Behnken design with five center points was performed for each process. In order to minimize energy consumption, the process time was used as a discriminator for prediction refinement.

3.2.1. Ultrasound-Assisted Extraction (UAE)

All data are summarized in Table 3 and Figure 3.

Table 3. Experimental plan used for the optimization of UAE and the dosages of TFC (mg rutin equivalent/g of biomass used) and TCC (µg/g of biomass used).

Run	Solid/Liquid Ratio (%)	Temperature (°C)	Time (min)	TCC (µg/g)	TFC (mg eq Rutin/g)
1	6	60	60	211.9	21.5
2	6	45	37.5	255.3	20.7
3	6	30	60	265.0	22.3
4	6	30	15	281.5	23.0
5	10	45	15	223.7	12.9
6	6	60	15	281.2	24.4
7	6	45	37.5	282.5	20.7
8	2	60	37.5	135.8	12.5
9	10	30	37.5	255.9	18.8
10	2	45	15	141.0	12.8
11	2	30	37.5	89.9	9.2
12	10	60	37.5	177.9	11.6
13	6	45	37.5	274.3	24.7
14	2	45	60	126.4	11.8
15	6	45	37.5	414.8	25.1
16	6	45	37.5	367.9	24.9
17	10	45	60	212.8	19.7

Two runs were ignored for the statistical analysis (runs 15 and 16), which were unexpectedly higher than the other center points for TCC optimization.

Considering TCC or TFC, the most critical factor appeared to be the solid/liquid ratio, as time and temperature appeared to be nonsignificant. The R^2 of the two RSMs were in good agreement between the adjusted and predicted value (less than 0.2 of difference) (see Table S2). The TCC and TFC could then be reliably predicted by the following equations:

$$\begin{aligned}\text{TCC (µg/g biomass)} = {} & -99.75259 + 105.21046 \text{ solid/liquid ratio} + 2.38496 \\ & \text{Temperature} - 0.680000 \text{ Time} - 0.516241 \text{ solid/liquid ratio} \times \text{Temperature} + \\ & 0.010278 \text{ solid/liquid ratio} \times \text{Time} - 5.88146 \text{ solid/liquid ratio}^2\end{aligned} \quad (3)$$

$$\begin{aligned}\text{TFC (mg eq rutin/g biomass)} = &-7.28764 + 8.69634\ \text{Solid/liquid ratio} + \\ &0.234605\ \text{Temperature} - 0.118983\ \text{Time} - 0.043623\ \text{Solid/liquid ratio} \times \\ &\text{Temperature} + 0.021898\ \text{Solid/liquid ratio} \times \text{Time} - 0.586024\ \text{Solid/liquid ratio}^2\end{aligned} \quad (4)$$

The optimized conditions that were predicted to maximize both TCC and TFC were found at a 6.0% ratio, 30 °C, and 15 min. The confirmation run performed at these operating parameters led to an extract with a TCC of 256.96 ± 6.35 µg/g biomass and a TFC of 26.30 ± 1.33 mg eq rutin/g of biomass.

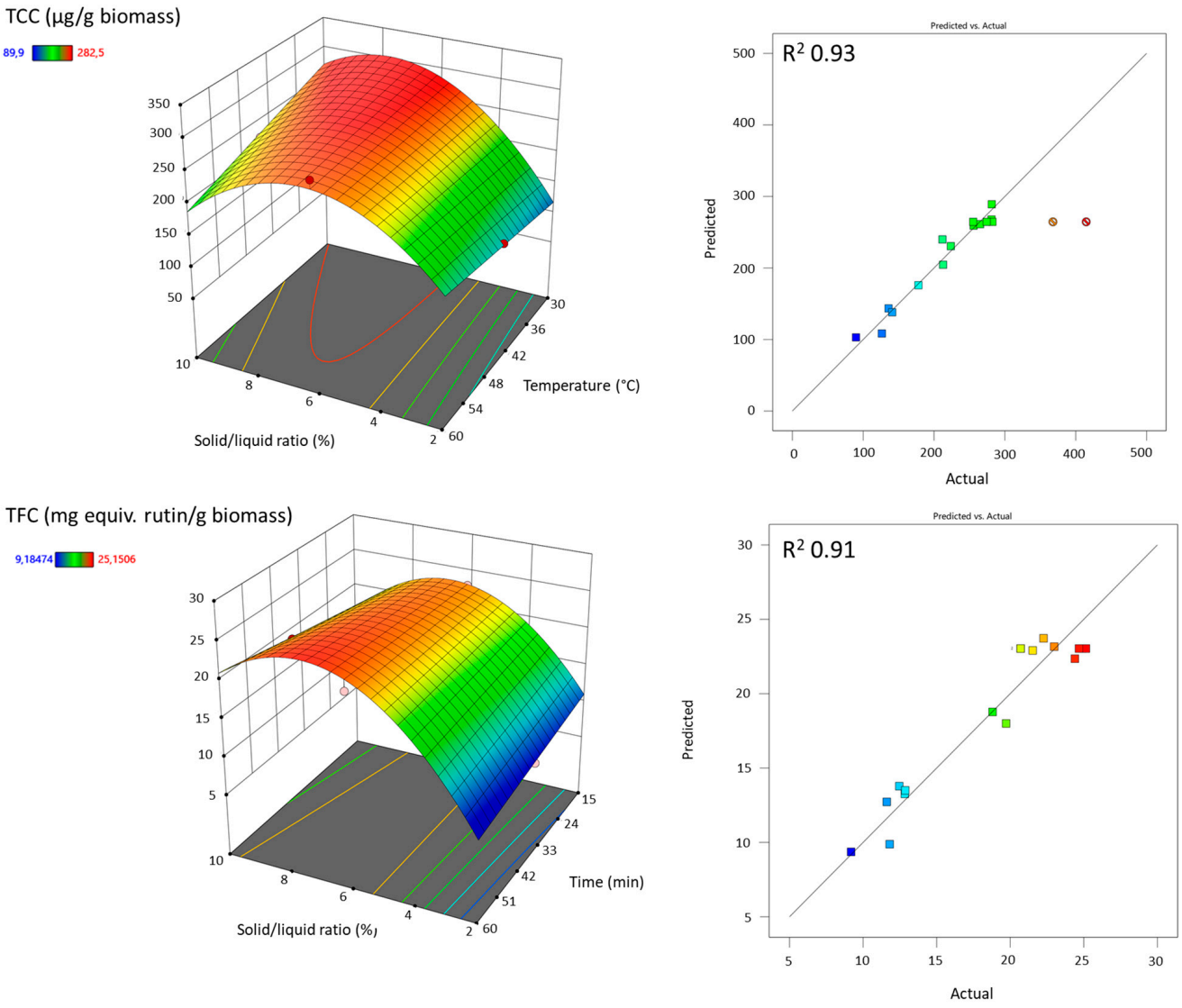

Figure 3. RSM and predicted vs. actual curves of UAE optimization using TCC (on **top**) and TFC (at **bottom**) as discrimination factors.

3.2.2. Dual Asymmetric Centrifugation (DAC)

All data are summarized in Table 4 and Figure 4.

Table 4. Experimental plan used for the optimization of DAC and the dosages of TFC (mg rutin equivalent/g of biomass used) and TCC (µg/g of biomass used).

Run	Solid/Liquid Ratio (%)	Time (min)	Speed (rpm)	TCC (µg/g)	TFC (mg eq rutin/g)
1	2	37.5	3500	238.6	18.6
2	6	37.5	2000	340.3	30.2
3	6	60	500	384.7	29.0
4	10	37.5	500	167.1	34.9
5	2	60	2000	240.5	24.9
6	10	60	2000	173.3	32.7
7	2	15	2000	240.3	18.8
8	10	15	2000	169.1	30.9
9	6	37.5	2000	387.3	38.5
10	6	37.5	2000	301.4	38.8
11	2	37.5	500	188.8	20.5
12	6	15	3500	316.3	37.7
13	6	37.5	2000	318.9	41.7
14	10	37.5	3500	132.1	34.7
15	6	37.5	2000	303.9	41.1
16	6	60	3500	302.1	32.6
17	6	15	500	192.0	33.9

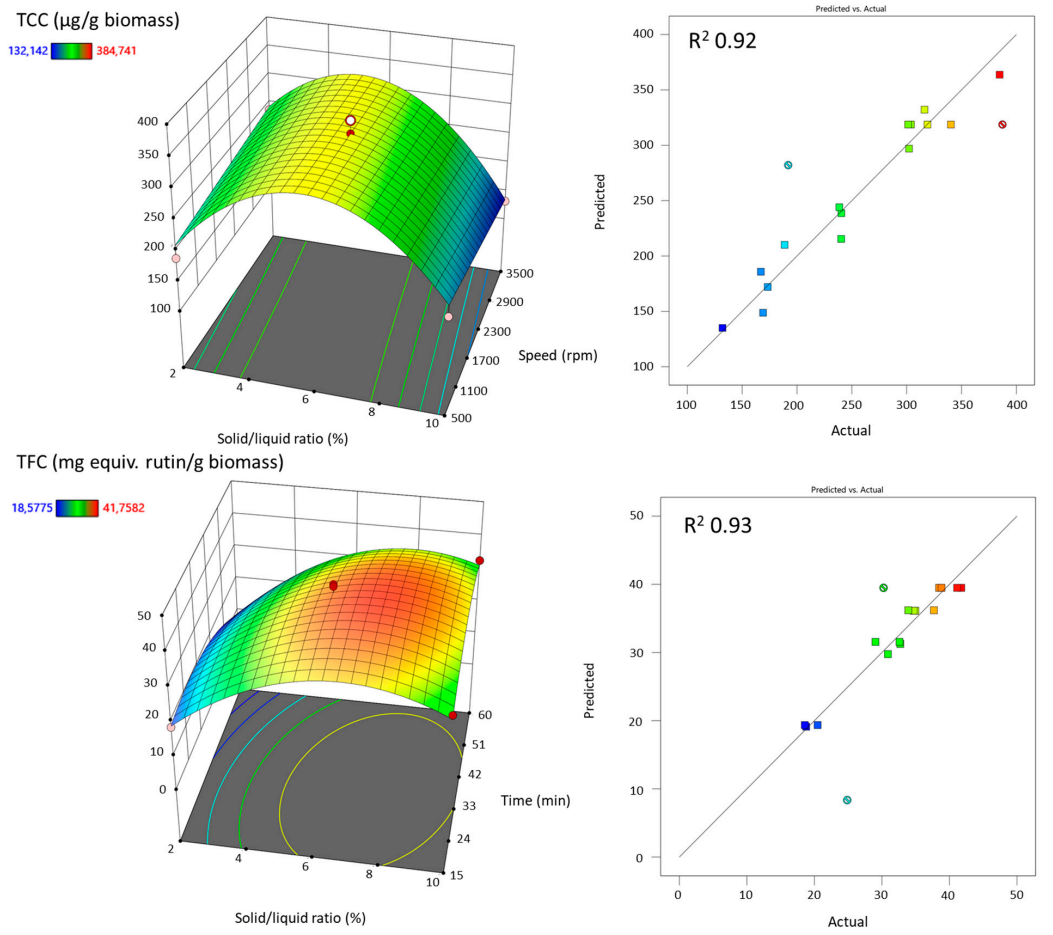

Figure 4. RSM and predicted vs. actual curves of DAC optimization using TCC (on **top**) and TFC (at **bottom**) as discrimination factors.

Two runs were ignored for statistical fitting after data analysis: runs 9 and 17 for TCC; and runs 2 and 5 for TFC.

Considering carotenoids, the most critical factor appeared to be the solid/liquid ratio and the rotation speed, whereas for the flavonoids, the solid/liquid ratio and time were the most significant factors. The R^2 of the two RSMs were in good agreement between the adjusted and predicted value (less than 0.2 of difference) (see Table S3). The TCC and TFC could then be reliably predicted by the following equations:

$$\begin{aligned}\text{TCC } (\mu g/g \text{ biomass}) = &-33.60941 + 92.48333 \text{ solid/liquid ratio} + 2.24627 \text{ Time} \\ &+ 0.050855 - 0.003535 \text{ Speed solid/liquid ratio} \times \text{Speed} - 0.000865 \text{ Speed} \times \\ &\text{Time} - 7.81179 \text{ solid/liquid ratio}^2\end{aligned} \quad (5)$$

$$\begin{aligned}\text{TFC (mg eq rutin/g biomass)} = &-3.58311 + 9.62988 \text{ Solid/liquid ratio} + \\ &0.524081 \text{ Time} + 0.034061 \text{ Solid/liquid ratio} \times \text{Time} - 0.734255 \text{ Solid/liquid} \\ &\text{ratio}^2 - 0.011086 \text{ Time}^2\end{aligned} \quad (6)$$

The optimized conditions that maximized both TCC and TFC were determined to be a 6.0% ratio, 1500 rpm, and 30 min. A confirmation run conducted under these operating parameters resulted in an extract with a TCC of 383.54 ± 4.73 µg/g biomass and a TFC of 45.42 ± 0.85 mg eq rutin/g of biomass. The DAC process yielded higher recoveries of both carotenoids and flavonoids compared to the regular UAE process. As is typically observed with viscous solvents, an increase in mixing forces led to an improved mass transfer. The DAC process proved to be an interesting candidate for other applications.

An alternative hypothesis suggests that the DAC process, operating at room temperature, prevents damage to flavonoids and carotenoids, leading to a higher recovery of antioxidant molecules. In contrast, the conventional process using a NaDES as the extraction solvent typically involves elevated temperatures ranging from 50 to 75 °C [14,15]. Increasing the temperature is commonly employed to address viscosity issues; however, the DAC process's vigorous mixing facilitates mass transfer at room temperature. This represents a significant stride towards a more sustainable process, aligning with the eco-extraction principle [46]. Another notable advantage of this process is its shortened extraction time requirement, in contrast to the conventional NaDES processes that are described with UAE or stirring and heating, which can extend beyond 3 h [47].

The optimized set of parameters was used to select the DAC process, resulting in the production of 27 g of Bet:Gly Calendula extract (BGCE) containing 2.74 mg of rutin equiv and 23.1 µg of carotenoids/g BGCE. The goal of this was to analyze its effect in a cosmetic formulation.

3.3. Cosmetic Formulation of Bet:Gly Calendula Extract

3.3.1. Cream Preparation

The manufacturing process plays a pivotal role in the investigation of stable emulsions, as it significantly influences the size of the dispersed phase droplets. Conventional approaches involve using a mechanical stirring tool coupled with high temperature to provide the necessary energy for fragmentation, especially when dealing with a viscous dispersed phase. In the study by Jamaleddine et al., the NaDES extract was introduced into the aqueous phase before emulsification, with both phases heated to 80 °C [40]. In certain food-related studies, emulsification in the presence of a NaDES in the aqueous phase has been reported without a temperature increase, achieved by using an ultra-turrax® homogenizer at high speed for several minutes, a process known to elevate the sample temperature due to friction [38,48]. On the other hand, Rocha et al. and Barros Santos et al. added a NaDES at room temperature but after the emulsification process [41,42]. Here, the full antioxidant spectrum BGCE was introduced in an O/W cream using the DAC process as the formulation process according to Section 2.6. DAC technology was used to prepare the creams in a very short amount of time (6 min) and at room temperature to preserve the activity of the extracted antioxidants. Although DAC processes have already been described in the literature for the preparation of emulsions in the pharmaceutical or in the food fields [49,50], they are not described in the literature on the preparation of a

cosmetic cream yet. BGCE was introduced at 1%, a common concentration for an active ingredient or the preservation of a cosmetic formula, and 10%, a high concentration, to test the robustness of the formula and of the manufacturing process. The concentration of flavonoids (TFC) and carotenoids (TCC) in 100 g of 1 and 10% BGCE cream were 2.74 mg (TFC) and 23.1 µg (TCC) and 27.4 mg (TFC) and 231.0 µg (TCC), respectively. These concentrations are consistent with those that are typically found in studies on antioxidant creams based on natural extracts of flavonoids or carotenoids [51,52]. The samples were then subjected to accelerating ageing for 90 days (40 °C, 75% humidity, dark). The samples were cream-yellow (control and 1%wt extract) to cream-orange (10%wt extract) (See Figure S1). None of the creams showed any visible sign of destabilization over time, such as creaming or oil release. It is crucial to note that while the protocol that was utilized for preparing the control samples was applied to create the cream containing 1%wt Calendula extract, adjustments were necessary when incorporating 10%wt extract. The shear rate proved inadequate for achieving cream stability for 90 days, presumably due to the heightened viscosity of the aqueous phase that was enriched with 10%wt of BGCE. To ensure stability over 90 days under accelerated aging conditions, specific steel rings, originally designed for pulverization, were introduced during the emulsification step without altering the duration or temperature of the process.

3.3.2. Cream pH Measurements

The impact of the introduction of BGCE in the cream was evaluated over a 90-day period (Table 5). The initial pH of the control cream, devoid of Calendula extract, was determined to be 5.3 ± 0.2, and was stable in time (5.4 ± 0.1 by day 90). The introduction of the extract at 1 or 10%wt did not significantly alter the pH of the preparation (5.4 ± 0.1 for the 1%wt extract cream and 5.3 ± 0.1 for the 10%wt extract cream). At day 90, the three batches of cream exhibited a similar pH, of 5.0 ± 0.3 for the 1%wt extract cream and 5.8 ± 0.1 for the 10%wt extract cream. Despite slight variations, the statistical analysis revealed no significant differences between the pH values of the different batches at each time point, suggesting that the inclusion of BGCE, even at a high concentration, did not exert a significant impact on the pH stability of the creams over the 90-day period (Table 5). Moreover, all the measured pH values are compatible with a cutaneous use.

Table 5. pH of creams with or without BGCE. Data represent the mean ± SD.

	D1	D90
Control	5.4 ± 0.2	5.4 ± 0.1
1% BGCE Cream	5.4 ± 0.1	5.0 ± 0.3
10% BGCE Cream	5.3 ± 0.1	5.8 ± 0.1

3.3.3. Static Multiple Light Scattering-Based Stability Analysis

Static multiple light scattering measurements were conducted at two crucial time points: Day 1 (D1) and Day 90 (D90). Results are presented in Table 6 in terms of TSI and of estimated droplet size. The control cream, free of Calendula extract, exhibited a TSI value of 0.83 ± 0.21 at D1 and a droplet size of 9.3 ± 0.2 nm, indicating an initially stable formulation [53]. For the creams containing 1 and 10%wt BGCE, the TSI values observed at day one were also very close to 1 (1.23 ± 0.23 and 0.97 ± 0.35, respectively), and the droplet size was also inferior to 10 µm, showing a low susceptibility to creaming or oil release. By D90, the TSI and droplet size of all creams remained close to the initial values, signifying only an early stage of destabilization after the 90-day period. These findings, coupled with a visual assessment at D90, indicate that the incorporation of the NaDES-based extract into the formula did not compromise its stability. Notably, no studies addressing the integration of Bet:Gly NaDES in the aqueous phase of O/W emulsions have been reported to date. However, in the context of other DESs or NaDESs introduced into the aqueous phase of emulsions, comparable droplet sizes were observed upon measurement. For instance, Liu

et al. incorporated various percentages of choline chloride-based DESs in emulsions and obtained droplet sizes ranging from 8 to 24 µm [38]. Regarding the stability of emulsions containing NaDESs under accelerated aging conditions, no research reports were found. Nevertheless, Pontes et al. [48] incorporated NaDESs into the aqueous phase of an emulsion at different concentrations, resulting in stable systems. Intriguingly, they demonstrated that the emulsion containing a NaDES appeared to be more stable than the control (with water), although the aspect of time was not explicitly addressed. They attributed this enhanced stability to the nature of choline chloride, which exists as a quaternary ammonium in the formula [48].

Table 6. Turbiscan Index Stability (TSI) and droplet diameters of creams with or without Bet:Gly -based Calendula extract in NaDES. Data represent the mean ± SD.

	TSI		**Droplet Diameter (µm)**	
	D1	**D90**	**D1**	**D90**
Control	0.83 ± 0.21	0.90 ± 0.10	9.3 ± 0.2	9.7 ± 0.2
1% BGCE Cream	1.23 ± 0.23	0.83 ± 0.15	9.2 ± 0.2	10.2 ± 0.2
10% BGCE Cream	0.97 ± 0.35	1.00 ± 0.44	7.3 ± 0.3	8.6 ± 0.2

3.3.4. Rheology

The rheological flow analysis of creams incorporating BGCE aimed to elucidate the formulations' flow behavior on D1 and D30. The shear rate-dependent viscosity profiles revealed a consistent shear-thinning characteristic across all samples (showed in Figure 5). As the creams underwent a 30-day storage period, the rheological profiles retained their shear-thinning nature. While variations in viscosity were observed (viscosity values of interest are summarized in Table 7), the statistical analysis revealed no significant flow differences between the creams at D30, emphasizing the formulation's capacity to maintain consistent rheological properties over time.

Table 7. Value of viscosity (in Pa.s) obtained at different shear rates (in s^{-1}) during the flow test of creams studied.

Creams	**η (0.01)**	**η (0.1)**	**η (1)**	**η (10)**	**η (100)**	**η (1000)**
Control D1	1430.0 ± 37.8	221.1 ± 23.0	29.1 ± 0.7	4.1 ± 0.0	0.7 ± 0.0	0.1 ± 0.0
Control D30	1072.5 ± 124.2	165.8 ± 19.8	21.9 ± 0.6	3.1 ± 0.1	0.5 ± 0.0	0.1 ± 0.0
1% BGCE D1	2236.3 ± 61.2	311.6 ± 7.3	40.6 ± 1.2	5.6 ± 0.1	0.9 ± 0.0	0.2 ± 0.0
1% BGCE D30	1945.6 ± 53.3	271.1 ± 6.3	35.3 ± 1.1	4.9 ± 0.1	0.8 ± 0.0	0.1 ± 0.0
10% BGCE D1	3084.0 ± 49.5	446.5 ± 19.9	55.8 ± 2.7	7.2 ± 0.6	1.2 ± 0.1	0.3 ± 0.0
10% BGCE D30	1694.7 ± 87.8	287.9 ± 10.8	37.2 ± 1.0	4.8 ± 0.1	0.7 ± 0.0	0.1 ± 0.0

On Day 1, the control cream had viscosity values ranging from 1430.0 Pa.s at the lowest shear rate (0.01 s^{-1}) to 0.1 Pa.s at the highest shear rate (1000 s^{-1}). Interestingly, the inclusion of BGCE at different concentrations increased the overall formulation viscosity, particularly in the low-shear viscosity zone (close to resting viscosity). A factor 1.6 (2236.3 Pa.s) increase can be seen for creams containing 1% BGCE, and a factor 2.2 (3084.0 Pa.s) increase can be seen for those containing 10% BGCE. The increase in viscosity at rest is probably due to the presence of a NaDES but also to the components that were extracted from Calendula [41]. In the shear zone corresponding to the spreading zone of the cosmetics (between 100 s^{-1} and 1000 s^{-1}) [54], the values normalize to be statistically indistinguishable. The presence of the extract will not alter the product conditions of use for the consumer. After 30 days, a decrease in viscosity values was observed for the different points of interest, both for controls and for formulations containing 1 or 10% BGCE. The viscosity loss is most significant at the lowest shear value (0.01 s^{-1}), with a loss of 25% for the control cream and losses of 13 and 45% for the creams with 1% and 10% extract,

respectively. However, this reduction in viscosity is not statistically significant for the rheological shearing zones corresponding to the formulation's area of cosmetic use. The uniformity in rheological characteristics is especially significant for cosmetic formulations, as it indicates that the incorporation of BGCE, even at elevated concentrations, does not undermine the desired flow behavior of the creams. These results validate the stability and uniformity of the formulations, highlighting the well-balanced interaction among BGCE, thickeners (xanthan gum and sclerotium gum), emulsifiers (hydrogenated lecithin), and other ingredients.

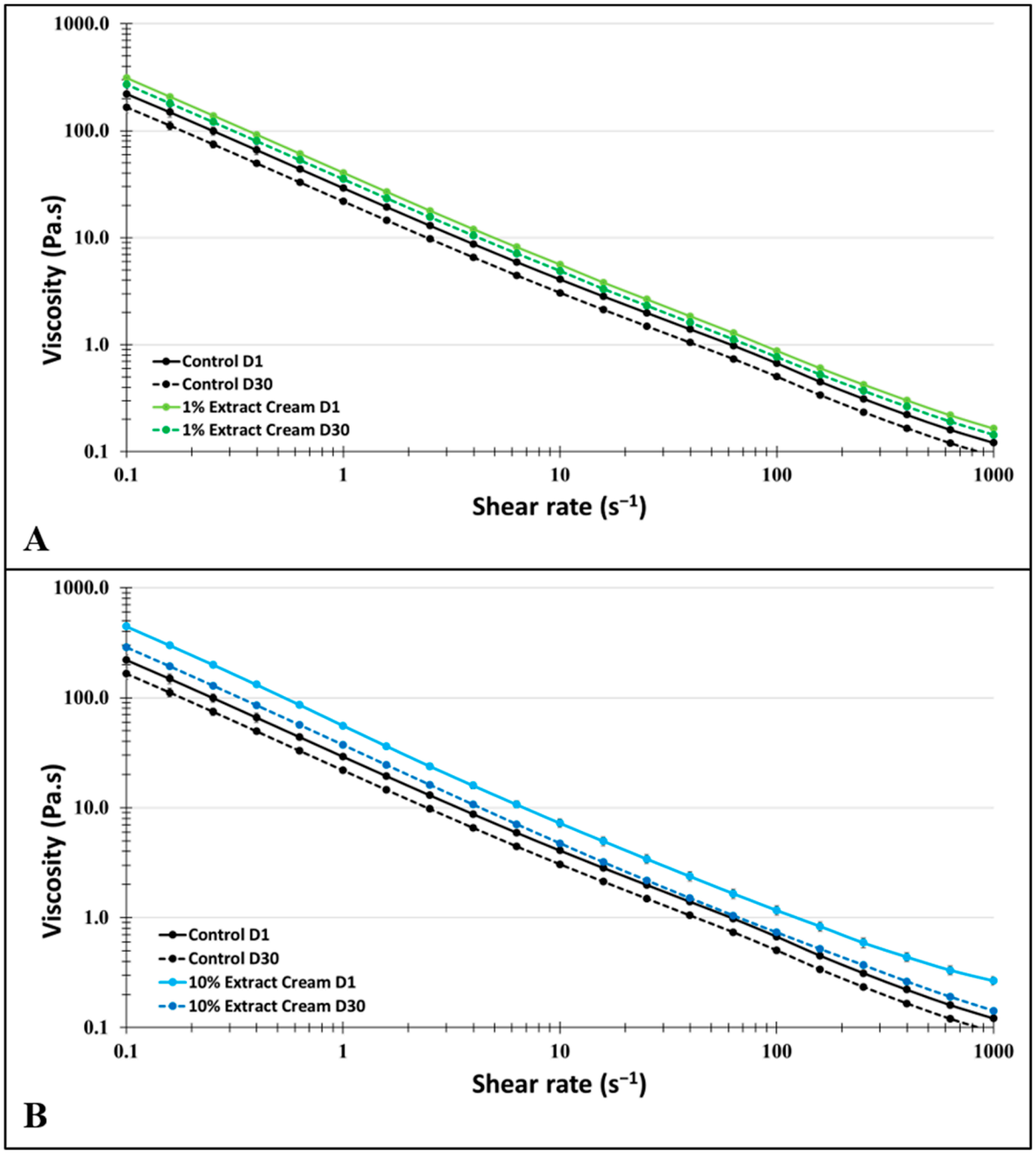

Figure 5. Flow properties of creams without BGCE in black, with (**A**) 1%wt (in green solid line), and (**B**) 10%wt (in blue solid line) of BGCE in NaDES. Flow properties of D30 samples are shown in dotted line.

Strain sweep measurements were conducted for a further study of the viscoelastic properties of the creams. These experiments provided crucial insights into the creams' mechanical properties that remain independent of the applied strain amplitude, relying solely on time or frequency. Table 8 presents a comprehensive overview of the rheological data obtained from the strain sweep tests, further illustrating the variations. The rheological properties of creams, including the storage (elastic) modulus (G′) and the loss (viscous) modulus (G″), were analyzed through amplitude sweep measurements on D1 and D30. The obtained data allow for an in-depth exploration of the viscoelastic behavior of the creams, providing valuable insights into their structural and stability characteristics.

Table 8. Rheological data (means ± SD) obtained from the strain sweep tests. The values of G′ and G″ correspond to the linear viscoelastic region.

	D1		D30	
	G′ (Pa)	**G″ (Pa)**	**G′ (Pa)**	**G″ (Pa)**
Control	188.1 ± 3.1	36.0 ± 3.2	215.7 ± 7.0	40.6 ± 5.6
1% BGCE Cream	260.2 ± 7.9	47.1 ± 8.0	226.3 ± 4.3	41.0 ± 3.0
10% BGCE Cream	389.6 ± 10.4	68.2 ± 3.8	228.7 ± 7.3	50.6 ± 4.3

On Day 1, the control cream exhibited a storage modulus (G′) of 188.1 ± 3.1 Pa and a loss modulus (G″) of 36.0 ± 3.2 Pa. These values reflect a predominance of elastic behavior over viscous behavior, indicating that the cream has a relatively solid structure. With the addition of 1 or 10% BGCE, the rheological behavior remains identical, with a predominance of the storage modulus. However, an increase in values is observed for the two concentrations, which is directly correlated with the increases in formulation viscosity that was already observed in the flow experiments. Increases in G′ and G″ moduli are of the order of a factor of 1.3 for the 1% extract formulation and a factor of 2 for the 10% formulation. This increase in the values of G′ and G″ can be advantageous for cosmetic formulations by contributing to stability, resistance to deformation, and so on [55–57]. This immediate increase in G′ and G″ with the concentration of the NaDES was also described by Liu et al., working with a chloride-based DES introduced in emulsions that were stabilized by proteins [38]. The hypothesis in our case is that an interaction in the type of supplementary hydrogen bonds appears between the NaDES components and the thickening polysaccharides that are present in the formula. Indeed, the fact that the viscosity of the three formulas is very similar in the high-shearing zone indicates that the new chemical bonds that are implicated in the increase in the viscosity at rest are easily broken by shearing.

The frequency sweep analysis objectives were to investigate the dynamic behavior of the creams and explore potential crossover points between the storage modulus and the loss modulus, as well as to identify any characteristic relaxation times. The results revealed a consistent absence of crossover points between G′ and G″ across the frequency range for all samples, including the control cream and those with 1% and 10% BGCE. This absence suggests that the creams maintain a predominantly elastic behavior, where the storage modulus remains higher than the loss modulus at all tested frequencies. The lack of a crossover point implies that the creams do not undergo a viscoelastic transition.

The absence of clear relaxation peaks suggests that the creams do not exhibit pronounced internal structural rearrangements or relaxation processes within the tested frequency range. This finding is consistent with the overall stable rheological behavior that was observed in the amplitude sweep measurements and shear-thinning analyses. The combination of favorable flow properties and structural stability positions these formulations as promising candidates for cosmetic use, providing insights for further refinement and optimization in cosmetic product development. In particular, BGCE-loaded creams exhibit viscosity values and G′ and G″ values that remain superior to the initial viscosity of the control cream after 30 days, whereas the spreading properties are very similar.

These promising results tend to prove that in future studies, the BGCE property of being a viscosity booster in final products containing gums could be explored.

4. Conclusions

This study represents a significant milestone in achieving a comprehensive spectrum of antioxidant metabolites from Calendula through the application of a NaDES formulation based on Betaine and Glycerol. The optimization process unveiled the effectiveness of the DAC technology, enhancing the carotenoid content, with improved mixing and smoother operational conditions. This breakthrough lays the foundation for crafting distinctive blends of natural antioxidant compounds using NaDESs as extraction matrices. However, it is crucial to acknowledge that the use of a NaDES as an alternative solvent may result in the extraction of undesirable metabolites, necessitating a comprehensive chemical profile and toxicological evaluation for full safety consideration.

The successful integration of a full-spectrum extract into a COSMOS-certifiable cream formula was accomplished through a specialized manufacturing process employing the same DAC technology. This innovative approach facilitated rapid emulsification without the need for heating, avoiding excessive temperature elevation compared to conventional methods utilizing mechanical homogenizers. At a 1%wt inclusion rate, the extract seamlessly integrated into the cream, demonstrating a minimal impact on the color and spreading properties, with only a slight observed thickening at rest. Scaling up to a 10%wt inclusion, the manufacturing process was efficiently modified using a ring set that was initially designed for pulverization. While a more pronounced thickening and coloration were noted, the differences from the control cream were not statistically significant when considering spreading.

In summary, our study successfully developed a ready-to-use NaDES-based Calendula extract as part of a sustainable process for cosmetic cream creation. Notably, the viscosity values at rest for the extract-loaded formulation after 30 days remained higher than the initial viscosity of the control cream, while the spreading properties remained comparable. These promising findings suggest that Bet:Gly Calendula extract possesses multifunctional potential in cosmetic products, contingent on its concentration of use.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cosmetics11010017/s1: Table S1: TFC and TCC results and statistical analysis of NaDES screening Table S2: Statistical data for UAE DoE; Table S3: Statistical data for DAC DoE. Figure S1: Pictures of the prepared BGCE-enriched creams.

Author Contributions: Conceptualization, L.B.-D. and E.M.; methodology, L.B.-D., E.M. and A.V.; investigation, I.M.A., I.T.-K., X.P., R.G. and A.V.; data curation, L.B.-D.; writing—original draft preparation, A.V. and L.B.-D.; writing—review and editing, E.M. and L.B.-D.; project administration, E.M. and L.B.-D.; funding acquisition, E.M. and L.B.-D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by le Conseil Régional Centre Val de Loire (ARD Cosmetosciences program, grant number CVL 2020-00141275).

Data Availability Statement: Data will be made available on request.

Acknowledgments: The authors thank MP2E Solution and Lipoid Kosmetik for their technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

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