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Table 1 to 4 are available in the Supplementary Files section.

Niosome-Based Supramolecular Biosolvents: Aligning Analytical Efficiency with Green Chemistry Criteria

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Abstract

The intrinsic properties of supramolecular solvents (SUPRAS), which stem primarily from their nanostructured architecture, hold great promise for the development of efficient and innovative sample treatments in analytical chemistry. Aligning these properties with green chemistry criteria is essential to ensure the long-term sustainability of these analytical processes. Here, sorbitan monopalmitate (Span 40) is proposed for the development of niosome-based bioSUPRAS. Coacervation was induced at room temperature using a 1-propanol/water medium and Na₂SO₄, producing tunable hydroalcoholic phases containing 0.11-0.43% (w/w) Span 40 and 0.12-0.57% (w/w) salt. Electron microscopy revealed densely packed, flexible globular aggregates compatible with a niosome-like, vesicle bilayer organization, thereby demonstrating supramolecular structuring at remarkably low amphiphile concentrations. Analytical applicability was demonstrated through the extraction of bioactive compounds from white mulberry leaves. Compared with conventional ethanol- and 1-propanol-based extractions, the bioSUPRAS increased total phenolic, flavonoid, and carotenoid content by 77-118%, 24-59%, and 8-35%, respectively. LC-ESI-MS/MS analysis revealed the simultaneous extraction of structurally diverse phenolics, including glycosylated and non-glycosylated compounds spanning logP values from -1.3 to 2.9, providing a representative phytochemical fingerprint. Finally, assessing the developed bioSUPRAS against the 12 criteria for sustainable solvents demonstrated excellent performance in extracting compounds across a wide polarity range, alongside broad compliance with both green and market requirements.

Keywords: Supramolecular solvents; Bio-based solvents; Green sample preparation; LC-MS/MS; Phenolic compounds; *Morus alba L*

Introduction

Driven by the health and environmental risks of petroleum-derived solvents, developing sustainable alternatives has become a paramount strategic objective. This shift is likewise crucial for fostering innovative applications across multiple chemical fields, including analytical sample preparation [1]. Alternative solvents must satisfy twelve criteria, grouped in three primary categories, to be considered sustainable [2, 3]: *performance*, which should match or exceeds that of conventional options for the intended purpose, *green criteria*, which include efficient recyclability, sustainable synthesis, negligible toxicity, safe biodegradation, robust stability, low flammability and renewability; and *market criteria*, including stable availability, competitive pricing, grade versatility and logistical ease.

Benefiting from unique structural advantages, supramolecular solvents (SUPRAS) have emerged as high-performance alternatives to conventional media in analytical extractions. This capability is especially beneficial when targeting the isolation of analytes across a broad polarity range from both solid and liquid matrices [4]. In such scenarios, conventional solvent selection is heavily governed by analyte polarity. This introduces extraction bias in chemically diverse samples, necessitating the use of solvent mixtures or sequential extractions, which ultimately leads to complex workflows and compromised chemical coverage [5].

SUPRAS are nanostructured liquids obtained from amphiphiles in aqueous or hydro-organic media through self-assembly and coacervation processes [6]. Analogous to nanostructured materials, which exhibit distinct characteristics relative to bulk matter [7], SUPRAS possess properties that differ notably from those of traditional bulk solvents. Thus, amphiphiles self-assemble into ordered nanostructures comprising regions of different polarity and multiple binding sites. These structures provide distinct mechanisms for analyte solubilization, including hydrophobic, hydrogen-bonding, and ionic interactions, among others [4, 9]. This capability allows for the simultaneous extraction of analytes across a broad polarity range, a crucial requirement for simplifying sample preparation in diverse fields such as exposomics, food and environmental monitoring, and the characterization of bioactives [10, 11]. Likewise, SUPRAS nanostructures and properties can be tailored through amphiphile selection and/or the experimental conditions set for coacervation. This versatility has facilitated the design of

SUPRAS with restricted access properties that exert size-exclusion effects on macromolecules, thereby eliminating interferences and enabling the development of matrix-independent methods that integrate analyte extraction and clean-up into a single step [8]. On the other hand, SUPRAS do not constitute continuous phases but are instead composed of micrometer-sized coacervate droplets. This structural feature facilitates mass transfer from the sample to the solvent, thereby accelerating extraction times [4]. Collectively, these intrinsic characteristics of SUPRAS enable the development of efficient, simple, and rapid analytical extraction processes [11].

Regarding green criteria, SUPRAS naturally align with sustainable synthesis, as they are prepared through the spontaneous coacervation of amphiphiles at room temperature, allowing the remaining colloidal solution to be reused for subsequent synthesis. However, despite the growing trend toward developing greener SUPRAS, such as those derived from fatty acids, monolaurin, or rhamnolipids [10, 12, 13], combining high analytical performance (e.g., compatibility with state-of-the-art techniques like mass spectrometry) with full compliance across all seven green criteria [3] remains a significant challenge.

This research aims to contribute to the field by developing niosome-based bioSUPRAS through the coacervation of sorbitan monopalmitate (Span 40). Derived from sorbitol and palmitic acid, Span 40 is a safe, biodegradable, and non-ionic amphiphile widely utilized as a stabilizer and emulsifier in food, cosmetic, and pharmaceutical formulations [14]. Niosomes are vesicular nanocarriers obtained from non-ionic surfactants such as sorbitan esters, polyoxyethylene alkyl ethers or alkyl amides, extensively used for drug delivery [15, 16]. Although their biodegradability and vesicular structure make niosomes highly attractive for exploitation as bioSUPRAS, their conventional formulation remains time-consuming, requires specialized processing equipment, and often demands high-energy inputs [17]. Furthermore, achieving adequate stability typically requires structural bilayer modification using cholesterol as well as surface modification with charged molecules [18].

In this study, several strategies were evaluated for the development of green niosome-based bioSUPRAS. The objective was to achieve full or partial compliance with established green solvent criteria without compromising analytical performance, specifically regarding the capability to extract compounds across a broad polarity range and full compatibility with liquid chromatography-tandem mass spectrometry (LC-

MS/MS). The synthetic strategies investigated herein are detailed below, alongside the chemical composition, physicochemical properties, and nanostructural characterization of the resulting bioSUPRAS. As a proof of concept, these bioSUPRAS were evaluated for their analytical applicability by extracting and analyzing by LC-MS/MS bioactive targets from *Morus alba L.* leaves. This complex biological matrix was specifically chosen for its rich and structurally diverse profile of bioactives, most notably polyphenols and flavonoids [19]. The greenness of the synthesized BioSUPRAS was discussed according to established criteria.

Experimental

Chemicals and reagents

Details on chemicals, reagents, *Morus alba L.* leaves collection and their pretreatment, as well as the apparatus used throughout the study, are provided in **S1** section of the Electronic Supplementary Material (ESM).

BioSUPRAS synthesis

Different agents and conditions were investigated to induce the coacervation of Span 40 in aqueous and hydroorganic (1-propanol–water) media, including temperature variations (20-90 °C), as well as the addition of inorganic salts (potassium sorbate and sodium sulfate) and hydrotropes (sodium benzoate and sodium citrate). BioSUPRAS were successfully obtained by adding aqueous sodium sulfate to solutions of Span 40 in 1-propanol. Phase diagrams to delimit the bioSUPRAS formation region were constructed according to the procedure outlined in **Figure 1**. Specifically, aqueous sodium sulfate (0.14-1.40 M) was added to solutions of Span 40 (0.1-0.3%, w/v) in 1-propanol. The final volumes of 1-propanol and water ranged from 20% to 80% (v/v), with a total volume of 10 mL maintained in 15-mL polypropylene tubes (LGC Standards S.L.U., Barcelona, Spain) (n=3). After gentle mixing, phase separation occurred spontaneously, yielding an upper bioSUPRAS phase and a lower equilibrium solution. The occurrence of coacervation was verified through visual observation, as well as by comparing the water content of the bioSUPRAS phase with that of blank experiments prepared without Span 40.

Physicochemical characterization of the bioSUPRAS

Volume and density. The volume of bioSUPRAS formed under different synthesis conditions was calculated from the height of the separated phase and the tube diameter, measured with a precision digital calliper, assuming cylindrical geometry. Multiple linear regression analysis (Statgraphics Centurion XVI.I software) was applied to the experimental volumes to derive an empirical equation that predicts the bioSUPRAS volume based on the initial concentrations of the components in the synthesis mixture. The density of the bioSUPRAS phase was determined gravimetrically using a known volume and expressed in g/mL. All measurements were performed in triplicate (n=3).

Chemical composition. The contents of water, sodium sulfate, 1-propanol, and Span 40 in the bioSUPRAS phase were determined using complementary analytical methods. Water content was quantified via Karl Fischer coulometric titration. For this purpose, a 50- μ L aliquot of bioSUPRAS was diluted 1:40 (v/v) with methanol in 2-mL microtubes (Safe-Lock, Eppendorf Ibérica, Madrid, Spain), with pure methanol utilized as the blank. Measurements were conducted in triplicate. The non-volatile fraction, which comprises Span 40 and sodium sulfate, was determined by lyophilizing the bioSUPRAS and weighing the resulting residue. Sodium sulfate was quantified by turbidimetry based on BaSO₄ precipitation, following EPA Method 9038. Finally, the individual contents of Span 40 and 1-propanol were estimated by mass balance.

Elucidation of the bioSUPRAS nanostructure. The nanostructure of the bioSUPRAS phase was investigated using transmission electron microscopy (TEM) and cryogenic scanning electron microscopy. For TEM imaging, a 20- μ L aliquot of bioSUPRAS was deposited onto a 300-mesh carbon-coated copper grid for 2 min, blotted, and negatively stained with 2% (w/v) uranyl acetate for 1 min prior to analysis. For cryo-SEM analysis, bioSUPRAS samples were flash-frozen in liquid nitrogen, fractured at $-120\text{ }^{\circ}\text{C}$ under high vacuum (3.2×10^{-6} mbar), sublimated at $-90\text{ }^{\circ}\text{C}$ for 15 min, and analyzed at $-120\text{ }^{\circ}\text{C}$.

Application of bioSUPRAS to the extraction, global parameter determination, and LC-MS/MS quantification of bioactive compounds from *Morus alba* L. leaves.

BioSUPRAS extraction. Powdered white mulberry leaves (0.1 g) were extracted with 1.5 mL of bioSUPRAS in 2-mL microtubes. To enhance sample-extractant contact, two

to three glass beads (3 mm) were added to each tube. The mixtures were then vortex-shaken for 10 min and centrifuged at 8000 x g for 10 min.

Global parameter determination. The bioactive fraction extracted by the bioSUPRAS was characterized by determining the total phenolic (TPC), flavonoid (TFC), and carotenoid (TCC) contents. A detailed description of the experimental procedures is provided in **Section S2** of the ESM.

LC-MS/MS quantitation. Individual bioactive compounds, those for which analytical standards were available, were quantified by LC–MS/MS using electrospray ionization in positive and negative modes (ESI $\pm$) and multiple reaction monitoring (MRM). Chromatographic separation was performed on a Kinetex XB-C18 column (2.6 μ m, 100 $\times$ 3 mm) using ultrapure water containing 0.5% (v/v) formic acid (A) and MeOH (B) at 0.3 mL/min. The gradient consisted of 5-99% B over 30 min, 99-5% B in 1 min, followed by 5% B for 5 min, with an injection volume of 5 μ L. Source conditions were 350 $^{\circ}$ C, 10 L/min gas flow, 40 psi nebulizer pressure, and 4000 V capillary voltage. Compound-specific MRM transitions and detection parameters are provided in **Table S1**.

Results and Discussion

Synthesis of niosome-based bioSUPRAS

Sorbitan monopalmitate (Span 40), which has been widely investigated for niosome preparation [18], was selected as a model for bioSUPRAS development due to its combination of excellent green properties, high potential extraction efficiencies, and compatibility with LC-MS/MS. Its chemical structure is shown in **Figure S1**.

SUPRAS formation involves two consecutive processes: self-assembly and coacervation. Initially, a colloidal solution of three-dimensional aggregates (i.e., micelles, reversed micelles, or vesicles) is formed in aqueous solutions or hydroorganic mixtures above the critical aggregation concentration of the amphiphile. Subsequently, the coacervation process requires these aggregates to grow until their density differs from that of the synthesis solution, leading to phase separation [6]. For nonionic amphiphiles, this is typically achieved by dehydrating polar groups, thereby enabling closer packing of the aggregates [4].

Regarding the formation of the colloidal solution, Span 40 is highly lipophilic with a low hydrophilic-lipophilic balance (HLB) value of 6.7. Consequently, at room temperature, it does not dissolve sufficiently in water to independently reach the required concentration for direct aggregation in a pure aqueous phase. To address this, the formation of a stable colloidal solution was initially investigated by progressively increasing the temperature up to 90 °C. This approach promoted both Span 40 solubilization and the dehydration of its polar groups, subsequently inducing the formation of a second liquid phase and, presumably, coacervation. However, this phase proved unstable upon cooling, leading to the reprecipitation of Span 40 and rendering this strategy unsuitable for room-temperature analytical applications. Alternatively, hydrotropes such as sodium benzoate (0.95-3.50 M) and potassium sorbate (0.85-3.10 M) were investigated to enhance the aqueous solubility of Span 40; however, they were insufficient to produce a stable colloidal solution.

Subsequently, the self-assembly of Span 40 was investigated in hydroorganic mixtures. For this purpose, 1-propanol was selected due to the solubility of the amphiphile in it and its classification as a Class 3 solvent in the US Food and Drug Administration (FDA) Guidance for Industry Q3C [20]. This selection aligns with the low-toxicity requirements established for the development of bioSUPRAS. Because Span 40 dissolves completely at a molecular level in pure 1-propanol, it is unable to independently form a colloidal solution. Therefore, the preparation of a colloidal solution of this amphiphile was evaluated using 1-propanol–water mixtures. Previously, the formation of Span 40 aggregates had been demonstrated in tetrahydrofuran–water and ethanol–water mixtures [21, 22].

Experiments were performed by dissolving 20 mg of Span 40 in 1-propanol, followed by the addition of water to achieve a final volume of 10 mL. At water contents exceeding 30% (v/v), the palmitate tails of Span 40 were inadequately solvated by the medium, leading to precipitation. Within the solubilization region (1-propanol content above 70%), Span 40 was presumed to undergo aggregation. Under these conditions, a highly non-ideal solvation anomaly has been reported for Span 40 in other hydroorganic media [22]. In such cases, the organic solvent and water molecules form a highly structured, mutually associated network that exhibits an enhanced solvating capacity for amphiphilic molecules, thereby stabilizing oligomers or small H-like aggregates.

Once the colloidal solution was obtained, the coacervation of Span 40 was investigated by adding inorganic salts, which were expected to desolvate the polar groups of the amphiphile and consequently promote aggregate growth. This effect is ion-specific and strongly dependent on the particular amphiphilic system; therefore, the behavior of different anions cannot be predicted solely from their position in the Hofmeister series [23, 24]. Sodium citrate and sodium sulfate were evaluated for this purpose, and both successfully induced phase separation at room temperature. Surprisingly, sodium citrate required a minimum concentration of 10% (w/v) to induce coacervation, a substantially higher concentration than that required for sodium sulfate (2% w/v). This occurred even though the water-withdrawing power (or salting-out effect) of citrate is theoretically higher than that of sulfate according to the Hofmeister series.

This apparently anomalous behavior has been previously documented for anions with a mild amphiphilic character, such as citrate, which can shift from a salting-out to a salting-in (hydrotropic) effect when moving from pure water to hydroorganic mixtures [25]. Consequently, citrate can interact favorably with 1-propanol molecules and the hydrophobic or interfacial regions of Span 40 aggregates. This moderate affinity for the organic phase stabilizes the alcohol and the amphiphile within the aqueous environment, meaning a higher citrate concentration must be added to overcome this stabilization and induce phase separation. Furthermore, spatial and steric constraints at the Span 40 aggregate interface may also play a critical role [26]. Sulfate is a small, compact, and highly symmetric tetrahedral ion that can easily approach the dense interfacial region of Span 40 aggregates to withdraw water and screen interactions. In contrast, citrate is a large, bulky polyatomic species. Its steric hindrance restricts how efficiently it can pack tightly near the surfactant headgroups to strip away their closely bound hydration layer, thereby requiring higher bulk concentrations to achieve the same structural dehydration effect. Because sulfate induced more efficient coacervation, this ion was selected for the synthesis of bioSUPRAS.

Pseudo-binary phase diagrams for the quaternary system composed of sulfate, 1-propanol, water, and Span 40 were constructed by varying the concentrations of 1-propanol and sulfate at fixed Span 40 concentrations of 0.1%, 0.2%, and 0.3% (w/v), respectively (**Figures 2A-C**). Importantly, the observation of two immiscible liquid phases cannot alone be considered definitive evidence of bioSUPRAS formation, since sulfate can also induce phase separation in water/1-propanol mixtures through a

conventional salting-out mechanism. The identity of the newly formed phase was therefore assessed by analyzing its water content in systems prepared both in the presence and absence of Span 40.

As **Figure 2** illustrates, the phase diagrams exhibited three distinct regions across the different Span 40 concentrations investigated. The bioSUPRAS region was bounded by two areas where the precipitation of either Span 40 or sodium sulfate occurred. Notably, the SUPRAS region narrowed as the concentration of Span 40 increased. Span 40 concentrations above 0.3% led to precipitation in the quaternary system, most likely because the available 1-propanol was insufficient to maintain the solubilization of the amphiphile.

The minimum sulfate concentration required to promote dehydration of the polar groups and induce coacervation was 0.14 M. Notably, coacervation occurred in 1-propanol–water mixtures where Span 40 was otherwise insoluble (e.g., below 70% 1-propanol). This indicates that sulfate-induced salting-out also took place and that the resulting 1-propanol-rich phase was capable of solubilizing Span 40 at higher water contents in the synthesis solution (e.g., from 40% to 80% v/v). The formation of Span 40 supramolecular structures was initially inferred by comparing the phase water content with that obtained via salting-out in the absence of Span 40. **Table 1** presents representative results for these systems. In all cases, the incorporation of Span 40 led to a significant increase in the water content of the separated phase. This behavior supports the formation of a supramolecular phase, wherein self-assembled Span 40 structures provide polar domains capable of retaining water, rather than a simple 1-propanol-rich phase generated by salting-out.

At 1-propanol proportions above approximately 80% (v/v), phase separation was still observed (Figure 2, dashed line). However, in this region, the separated phases exhibited similarly low water contents regardless of the presence of Span 40, indicating the absence of a supramolecular nanostructure capable of driving additional water incorporation. Consequently, phase separation above 80% (v/v) 1-propanol was attributed predominantly to the salting-out of 1-propanol by Na₂SO₄, rather than to bioSUPRAS formation.

BioSUPRASs volume and density

The volume of bioSUPRAS produced under different synthetic conditions was mainly governed by the concentrations of 1-propanol and Na₂SO₄ in the synthesis mixture. **Table**

2 presents representative results obtained for a total synthesis solution volume of 10 mL. Increasing the proportion of 1-propanol progressively increased the volume of the supramolecular phase, whereas increasing Na₂SO₄ produced the opposite effect.

The dependence of SUPRAS volume on the composition of the synthesis mixture varies considerably among supramolecular systems. In general, the volume of the supramolecular phase increases linearly with amphiphile concentration, whereas its dependence on the proportion of organic solvent can range from linear to exponential. In contrast, the effect of coacervating salts like Na₂SO₄ is more system-dependent, ranging from negligible effects to either increases or decreases in SUPRAS volume depending on the composition of the colloidal solution [4, 27]. Remarkably, the Span 40 concentration had no significant effect on the bioSUPRAS volume within the range investigated here, despite being essential for its formation. This unusual behavior points to a supramolecular organization in which a very small amount of amphiphile can structure a comparatively large solvent volume, an aspect further examined through compositional and nanostructural characterization.

The experimental data (n = 55) were fitted using multiple linear regression to predict the bioSUPRAS volume based on the composition of the synthesis mixture. The Span 40 concentration was not statistically significant ($p = 0.880$) and was therefore excluded from the model. The resulting model is described by Equation 1:

$$\text{(Eq. 1)} \quad V_{\text{bioSUPRAS}} \text{ (mL)} = 2.93 (\pm 0.30) - 1.35 (\pm 0.18) [\text{Na}_2\text{SO}_4, M] + 0.092 (\pm 0.003)[1 - \text{propanol} (\% v/v)]$$

The model showed excellent agreement between predicted and experimental volumes ($R^2 = 0.9942$; **Figure S2**), enabling the volume of the bioSUPRAS to be readily estimated from the synthesis conditions.

Experimental density data are provided in **Table S2**. No significant differences in density were observed among the bioSUPRASs obtained under different conditions, with a mean value of 0.81 ± 0.02 g/mL.

Chemical composition of the BioSUPRASs phase

The chemical composition of the bioSUPRASs obtained under different synthesis conditions is shown in **Table 3**. The supramolecular phases consisted mainly of water and 1-propanol, with contents ranging from approximately 15 to 37% (w/w), and 42-85% (w/w), respectively; whereas Span 40 and Na₂SO₄ represented minor components (< 1% w/w). The composition was highly dependent on the initial proportions of the components in the synthesis mixture, indicating that the properties of the resulting bioSUPRAS can be tuned through the synthesis conditions and, therefore, that these solvents exhibit an environment-responsive behavior. 1-Propanol constituted the major component of the bioSUPRASs, accounting for approximately 42-85% (w/w) of the resulting phase. Its incorporation was generally high, with most values ranging from approximately 80 to 98%, although lower incorporation was observed under some synthesis conditions. The high transfer of 1-propanol to the supramolecular phase is consistent with its significant contribution to the bioSUPRAS volume described above. At constant Na₂SO₄ concentration, increasing the proportion of 1-propanol progressively decreased the water content of the bioSUPRAS (**Figure S3A**). Likewise, at a constant 1-propanol/water ratio, increasing the Na₂SO₄ concentration decreased the content of water incorporated into the supramolecular phase, irrespective of the Span 40 concentration investigated (**Figure S3B**). This behavior is consistent with the proposed coacervation mechanism, since increasing the salt concentration promotes dehydration of the polar groups of the amphiphile and consequently reduces the amount of water retained within the resulting supramolecular phase. This trend is also consistent with the decrease in bioSUPRAS volume observed at increasing Na₂SO₄ concentrations, since lower water incorporation results in a smaller volume of the separated supramolecular phase.

Particularly relevant for analytical applications was the very low content of both Na₂SO₄ and Span 40 in the resulting bioSUPRASs. Na₂SO₄ concentrations ranged from approximately 0.12 to 0.57% (w/w), as only a small fraction of the salt initially used for coacervation was transferred to the supramolecular phase. Na₂SO₄ incorporation was generally below 2%, although values up to 3.6% were observed at the lowest initial salt concentrations. The preferential retention of the salt in the equilibrium solution is analytically advantageous, since it minimizes the salt load of the resulting extractant and, consequently, potential compatibility problems with instrumental techniques.

Elucidation of the bioSUPRAS nanostructure

The nanostructures of bioSUPRAS were investigated using TEM and cryo-SEM. Both techniques confirmed that the new solvent was not a homogeneous molecular liquid, but rather contained a population of discrete supramolecular entities. TEM micrographs (**Figure 3A-B**) revealed numerous individual predominantly spherical aggregates with diameters ranging from 100 to 200 nm. Cryo-SEM provided further insights into the three-dimensional morphology of these supramolecular entities (**Figure 3C**). The fractured BioSUPRAS sample exhibited numerous globular structures with well-preserved, rounded boundaries, indicating high structural integrity and minimal deformation during the cryogenic fixation process. This morphology suggests that the individual entities are composed of curved interfacial structures compatible with bilayer-based aggregates. Furthermore, cryo-SEM confirmed that these vesicles exist as discrete, intact nanostructures in their native, hydrated state. Taken together, the findings from both techniques point toward a vesicle-like organization based on Span 40 bilayers, confirming the formation of niosomes [15].

The proposed structural model is consistent with the chemical composition and volume data obtained for the bioSUPRAS. Span 40 represents less than 0.5% (w/w) of the supramolecular phase, whereas water and 1-propanol constitute most of its mass. In a bilayer-based organization, Span 40 would mainly define the interfaces of the aggregates, with the polar sorbitan moieties exposed toward the hydroalcoholic domains and the palmitate chains associated within the less polar region of the bilayer. Water would be preferentially associated with hydrated polar regions, whereas 1-propanol, owing to its amphiphilic character, could be distributed throughout the hydroalcoholic medium and the interfacial regions (**Fig. 3D**). The interfacial role of Span 40 provides a rationale for its unusually low concentration in the bioSUPRAS, since most of the supramolecular phase volume is occupied by water and 1-propanol rather than by the amphiphile itself.

Potential of bioSUPRAS for extraction and quantification of a broad polarity range of bioactives in *Morus alba* L. leaves.

The analytical applicability of the developed BioSUPRAS was evaluated through a proof-of-concept study. This assessment focused on its ability to extract structurally and physicochemically diverse compounds from a complex matrix while meeting green chemistry requirements, maintaining robust analytical performance, and enabling direct instrumental analysis of the extracts. *Morus alba* L. is widely used for its health-

promoting properties, which have been associated with its high content of bioactive compounds. Since the antioxidant and pharmacological effects of these compounds are closely related to their chemical structure, their characterization is essential to explore and exploit their potential [28]. White mulberry leaves were selected as a model matrix because of their complexity and rich bioactive profile. In addition to major matrix components such as proteins, carbohydrates and pigments, the leaves contain structurally diverse bioactives, particularly polyphenols and flavonoids, spanning a broad range of physicochemical properties [19].

BioSUPRAS extraction. The general procedure for extraction of bioactives is depicted in **Figure S4**. BioSUPRAS composition was first optimized according to the phytochemical profile expected for *M. alba* leaves, mainly comprising polar and medium-polarity bioactives. As discussed above, Na_2SO_4 concentration influences water incorporation into the bioSUPRAS; therefore, a concentration near the lower boundary in the phase diagram (Fig. 2, 0.2 M) was selected to favor more hydrated supramolecular phases while allowing a wider range of 1-propanol proportions to modulate the less polar environment. The effects of 1-propanol (60-80%, v/v) and Span 40 (0.1-0.2%, w/v) were then evaluated using TPC, TFC and TCC as extraction responses (Section S2 in ESM). For this purpose, 0.1 g of white mulberry leaves was extracted with 0.4 mL of each bioSUPRASs. The obtained results are shown in **Figure S5**. TPC and TFC generally decreased with increasing 1-propanol, particularly at 0.1% Span 40, where TPC decreased from 1120 ± 20 to 550 ± 20 mg GAE/100 g DW between 60 and 80% 1-propanol. In contrast, TCC values were considerably lower and showed no clear dependence on bioSUPRAS composition, probably because of the comparatively low carotenoid content of white mulberry leaves. Overall, 60% 1-propanol provided the best extraction performance, particularly for polyphenols and flavonoids. Once the bioSUPRAS composition was selected, the amount of extractant required for efficient recovery of the different bioactive families was evaluated. BioSUPRAS volume was varied from 0.4 to 1.8 mL per 0.1 g of white mulberry leaves (**Table S3**). TFC and TCC remained essentially unaffected throughout the investigated range, whereas TPC progressively increased from 1120 ± 20 mg GAE/100 g DW at 0.4 mL to a maximum of 2030 ± 60 mg GAE/100 g DW at 1.5 mL. Further increases in bioSUPRAS volume provided no improvement. Therefore, 1.5 mL was selected as the optimum volume. Extraction time was evaluated between 1 and 60 min (**Figure S6**). Efficient extraction was already achieved at short times, with 10

min providing the best overall response for the three bioactive families. Longer extraction did not improve recoveries, and 10 min was therefore selected. This rapid equilibration represents an additional advantage of the bioSUPRAS-based extractions in terms of both sample throughput and energy consumption.

The capability of the bioSUPRAS to extract a wide polarity range of bioactive compounds was compared with that of conventional solvents typically used for this purpose, namely 1-propanol and ethanol. The bioSUPRAS provided higher extraction in all cases (**Table S4**), with TPC and TFC increasing by 77-118% and 24-59%, respectively, compared with the conventional solvents. Importantly, the improvement over 1-propanol alone (118%, 59% and 35%, for TPC, TFC and TCC, respectively) supports the contribution of the supramolecular organization rather than that of its major organic component alone. Furthermore, the TPC obtained with the bioSUPRAS (20.5 mg GAE/g DW) was within the range reported for white mulberry leaves, including 12.7-18.9 mg/g using ultrasound-assisted ethanol extraction (50 °C, 60 min) [29], and 12.8-15.5 mg/g with acidified methanol under ultrasound (25 min) [30].

LC-ESI-MS/MS quantification of phenolic compounds. The analytical performance of the bioSUPRAS was further evaluated by the quantification of phenolic acids and flavonoids as representative constituents of white mulberry leaves. **Table 4** shows the calculated concentrations. Ten phenolic compounds were quantified in the extracts, with rutin (82 ± 2 mg/100 g DW), chlorogenic acid (44 ± 2 mg/100 g DW) and neohesperidin (33.3 ± 0.3 mg/100 g DW) as the most abundant, followed by oxyresveratrol (30 ± 2 mg/100 g DW), luteolin (24 ± 2 mg/100 g DW) and fisetin (23.8 ± 0.7 mg/100 g DW). Lower concentrations of taxifolin, piceatannol, phloridzin and morin were also determined. Beyond their individual concentrations, the compounds simultaneously extracted illustrate the versatility of the bioSUPRAS for analytical extraction. The identified analytes comprise different phenolic families and include both glycosylated (e.g., rutin) and non-glycosylated (e.g., luteolin) structures, with molecular weights ranging from approximately 244 to 611 g/mol. Their hydrogen-bonding capacities also differed markedly. Oxyresveratrol and piceatannol contain 4 H-bond donors and 4 acceptors, whereas rutin contains 10 and 16, respectively, with intermediate values represented by compounds such as luteolin and fisetin (4 /6) and taxifolin (5/7). This structural diversity is also reflected in their lipophilicity, with logP values ranging from -1.3 for rutin to 2.9 for piceatannol. Their simultaneous extraction is consistent with the

heterogeneous solvation environments inferred from the bioSUPRAS composition and nanostructure. The hydroalcoholic domains can provide extensive hydrogen-bonding interactions, while the interfacial sorbitan groups and less-polar regions associated with the palmitate chains provide additional interaction environments. Thus, rather than relying on a single solvent polarity, the supramolecular organization offers complementary solubilization sites capable of accommodating structurally diverse analytes. Remarkably, this extraction versatility is achieved with less than 0.5% (w/w) Span 40, indicating that the exceptionally low amphiphile content does not compromise the characteristic multiple-interaction mechanisms of SUPRASs. Several of the determined compounds, including taxifolin, luteolin, oxyresveratrol, phloridzin, fisetin and piceatannol, have been more frequently described in mulberry fruits or twigs than in leaves, where they occur at lower concentrations or are inconsistently detected [29]. Overall, the detection of both major and minor phenolic constituents with diverse structural and physicochemical properties supports the ability of the bioSUPRAS to provide a representative phytochemical fingerprint of white mulberry leaves. Remarkably, the Span 40 present in the bioSUPRAS extracts, which were directly injected into the LC-ESI-MS/MS system, did not interfere with the MS measurement of the bioactives in either positive or negative ionization modes.

Compliance of the niosome-based bioSUPRAS with green solvent criteria

Due to the lack of dedicated metrics, the sustainability of the niosome-based **bioSUPRAS** is evaluated based on its compliance with the twelve criteria established for green solvents [2, 3]. In terms of *performance*, this bioSUPRAS has demonstrated potential advantages over conventional solvents traditionally employed in extraction processes, regarding both scope and efficiency (Table S4). In relation to the seven *green criteria* (efficient recyclability, sustainable synthesis, negligible toxicity, safe biodegradation, robust stability, low flammability and renewability), the synthesis of this bioSUPRAS can be considered sustainable since it is carried out through an energy-saving process that involves spontaneous coacervation at room temperature.

On the other hand, there are four green criteria (toxicity, biodegradability, stability, and flammability) for which the characteristics of the bioSUPRAS are closely related to its individual components (Span 40, 1-propanol, water, and sodium sulfate). Water and sodium sulfate fully comply with these criteria. Span 40 is a European-approved food

additive (E495) that is highly stable, non-hazardous, readily biodegradable, and does not spontaneously ignite. For its part, 1-propanol is classified as a Class 3 solvent in the US Food and Drug Administration (FDA) Guidance for Industry Q3C. It is chemically stable, readily biodegradable, and exhibits low toxic potential to human health. However, its handling requires safety precautions due to its flammability. Consequently, the bioSUPRAS is fully compliant regarding toxicity, biodegradability, and stability, but non-compliant with respect to flammability.

Regarding recyclability, one of the most interesting aspects is the possibility of recovering the ingredients remaining in the equilibrium solution for the successive synthesis of SUPRAS. Based on the data in Table 3, once the synthesis conditions are selected, the incorporation of the different ingredients into the bioSUPRAS can be determined. Therefore, replenishing the depleted ingredients should reduce costs and render the process greener. With respect to the final green criterion, renewability, the synthesis of Span 40 relies entirely on bio-based oleochemical and carbohydrate feedstocks [31]. Renewable 1-propanol can also be obtained through biochemical, chemocatalytic, or electro-synthetic pathways using sustainable feedstocks [32]. Consequently, this bioSUPRAS can be synthesized from renewable ingredients.

In terms of *market criteria* (stable availability, competitive pricing, grade versatility, and logistical ease), all components are already utilized globally for alternative applications; hence, they are considered widely available. Regarding logistical ease, the same regulations that apply to 1-propanol should also be applied to the bioSUPRAS.

Regarding cost, the raw material prices required to produce 1 L of bioSUPRAS can be estimated from the data in Table 3. Considering a bioSUPRAS density of 0.81 kg/L and the average weight percentages (w/w) required for synthesis (7.60% sulfate, 40.84% 1-propanol, 0.16% Span 40, and 51.33% water), the production of 1 L (0.81 kg) of bioSUPRAS requires 0.061 g of sulfate, 39.23 g of 1-propanol, 0.00129 g of Span 40, and 41.57 g of water. All of these reagents are available in various grades (e.g., technical, food, cosmetic, laboratory, and analytical), meaning that bioSUPRAS can be synthesized with tailored grade versatility.

At an industrial manufacturing scale (typically 1 metric ton), the cost for technical-grade sulfate, 1-propanol, and Span 40 ranges from 0.10-0.45 €/kg, 0.77-0.90 €/kg, and 1.70-4.30 €/kg, respectively [33–35]. Consequently, the ingredient costs for preparing 1 L of

technical-grade bioSUPRAS would be 6.1×10^{-6} - 27.4×10^{-6} € for sulfate, 0.030 - 0.035 € for 1-propanol and 2.19×10^{-6} - 5.5×10^{-6} € for Span 40, amounting to a total of approximately 0.030–0.035 €/L. Given the minimal amounts of sulfate and Span 40 required, their low industrial prices, and the fact that sulfate can be almost completely recycled for subsequent synthesis due to its low incorporation rate (average value of 1.5%, Table 3), the overall raw material cost remains remarkably low and is predominantly dictated by the price of 1-propanol.

For laboratory-grade reagents (such as ACS Reagent Grade, Analytical Reagent, etc.) at the same industrial scale (1 metric ton), prices range from 1.20–3.50 €/kg for sulfate, 1.90–3.80 €/kg for 1-propanol, and 4.50–9.50 €/kg for Span 40. Thus, the ingredient costs for 1 L of laboratory-grade bioSUPRAS would be 7.32×10^{-5} - 21.3×10^{-5} € for sulfate, 0.074 - 0.149 € for 1-propanol, and 5.8×10^{-6} - 12.3×10^{-6} € for Span 40, resulting in a total cost of around 0.074–0.149 €/L. Again, this total expenditure is considered low and is driven almost entirely by the cost of 1-propanol.

Conclusions

In this study, a niosome-based bioSUPRAS was synthesized from Span 40 through the combined effects of sodium sulfate, salting-out in a 1-propanol/water mixture, and the dehydration of the amphiphile's polar heads. The developed Span 40 bioSUPRAS contains less than 0.5% (w/w) amphiphile, with the majority of the supramolecular phase consisting of water and 1-propanol organized around vesicle-like aggregates. This unusual composition challenges the typically high amphiphile content of conventional SUPRASs, commonly reported at concentrations in the mg/μL range, and demonstrates that a minor amount of surfactant can structure a comparatively large solvent volume. Furthermore, the relationships established among synthesis conditions, phase composition, volume, and nanostructure provide a robust foundation for tailoring these solvents to specific analytical requirements.

White mulberry leaves provided a challenging proof of concept for this bioSUPRAS. The simultaneous extraction of structurally diverse bioactive compounds underscores the ability of the heterogeneous supramolecular environment to accommodate solutes through multiple types of interactions. Furthermore, the extraction process is simple, rapid, utilizes conventional laboratory equipment, and allows for direct injection into the

chromatographic system. The excellent compatibility of the bioSUPRAS with LC-ESI-MS/MS highlights its broad potential applicability in analytical extraction processes.

The assessment of the green and market criteria for the bioSUPRAS highlighted the paramount importance of ingredient selection during new solvent development, as these criteria are inherently linked to their chemical composition. Consequently, the newly developed bioSUPRAS are fully compliant with several green criteria, including recyclability, sustainable synthesis, negligible toxicity, safe biodegradation, robust stability, and renewability. However, it falls short regarding flammability due to its 1-propanol content. On the other hand, while the solvent fully complies with key market requirements, namely competitive pricing, stable availability, and grade versatility, it presents limitations in logistical ease, which is also dictated by the flammability of 1-propanol

Statements and Declarations

CRedit authorship contribution statement: **APZ:** Formal analysis, Methodology, Investigation, Data curation, Writing - Original Draft. **NCC:** Methodology, Conceptualisation, Writing – review & editing, Supervision. **SR:** Conceptualisation, Writing – review & editing, Supervision, Funding acquisition.

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

Use of Artificial Intelligence (AI) Tools: Not Applicable

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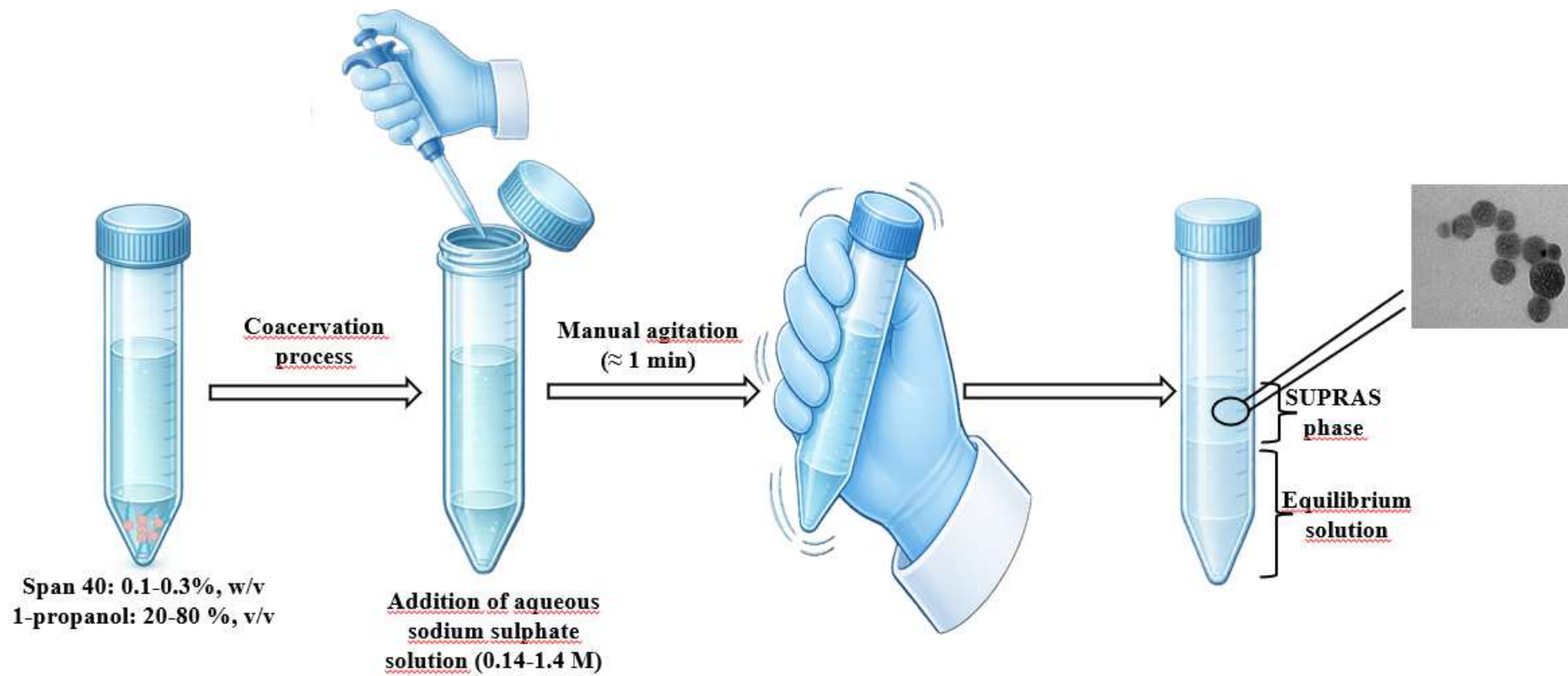


Figure 1. Schematic representation of the synthesis of a Span 40-based bioSUPRAS

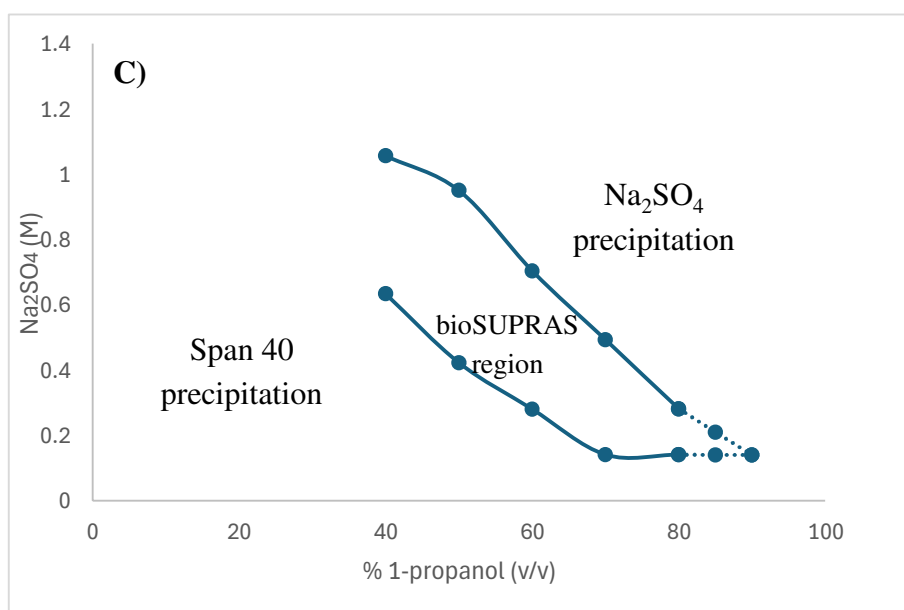
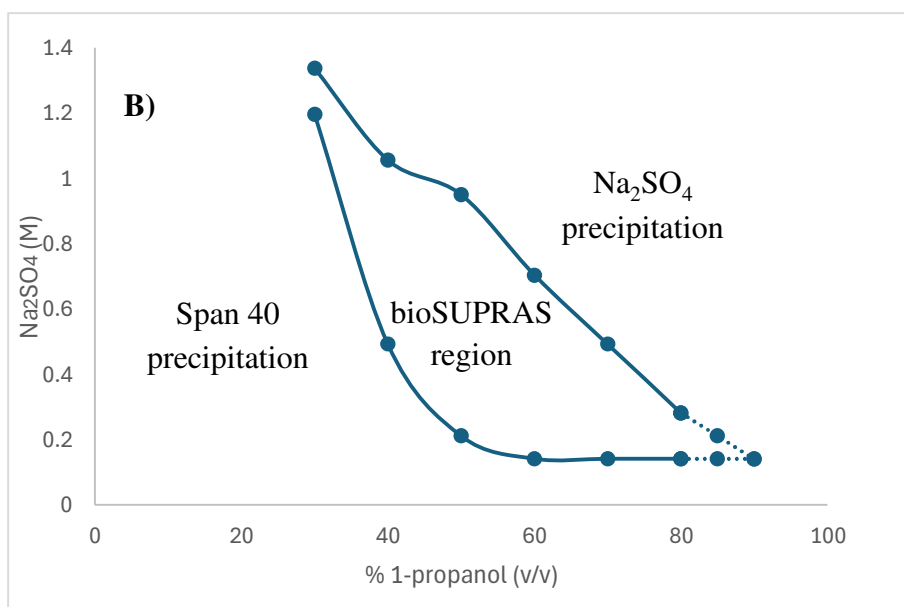
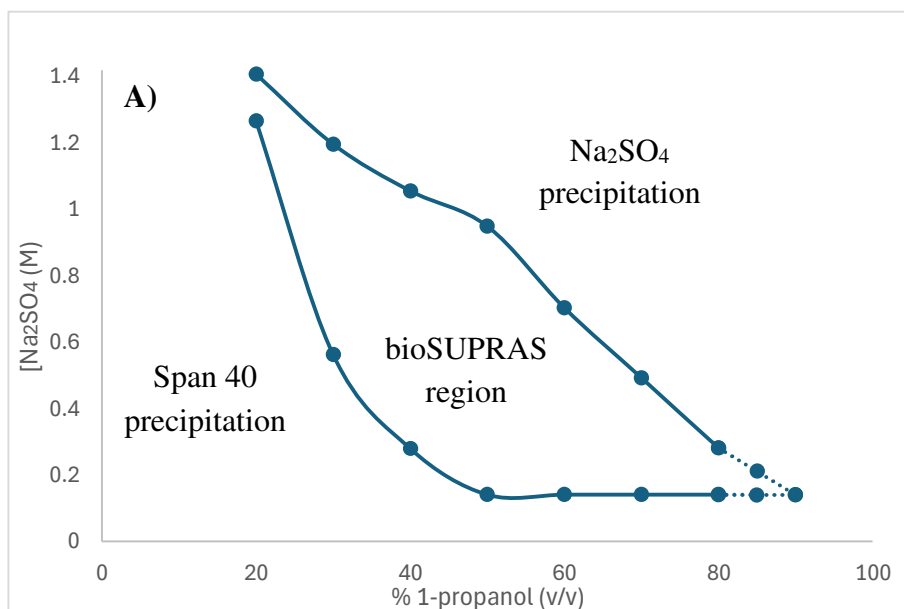


Figure 2. Phase diagrams illustrating the formation of bioSUPRAS systems using Span 40 at concentrations of A) 0.1%, B) 0.2%, and C) 0.3% (w/v).

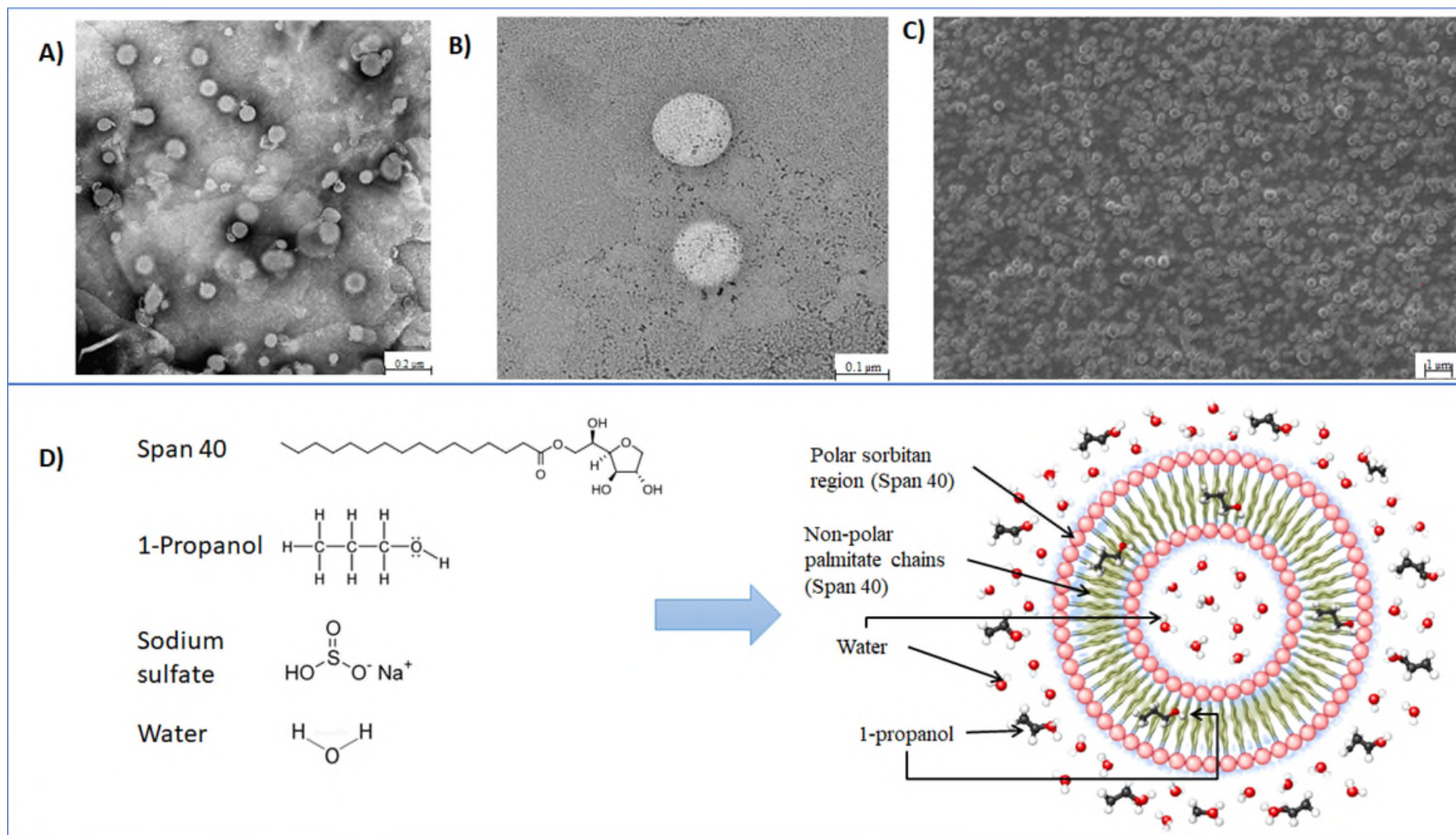


Figure 3. Representative TEM (A,B) and cryo-SEM (C) images of the bioSUPRAS, together with a schematic illustration (D) of the niosomal nanostructures.

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