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The Chemistry of Sporopollenin Ektexine and Endexine Layers Isolated from Sunflower Pollen through an Ionic Liquid-Mediated Process

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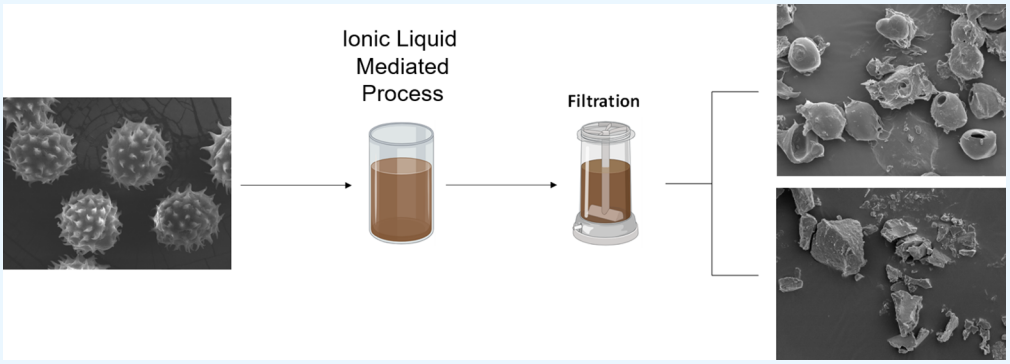

ABSTRACT: Sporopollenin is a plant polymer present in the exine of the pollen grains that comprises two layers: the endexine and the ektexine. It possesses remarkable mechanical, thermal, and chemical stability and is also highly recalcitrant to hydrolysis. The chemical backbone of sporopollenin mostly consists of a polyhydroxylated aliphatic component and polyketide-derived aliphatic α-pyrone elements. Recent works have provided important insights into its molecular structure, yet due to the extreme inertness of the polymer, outstanding questions still exist. In this work, we produced and characterized sporopollenin enriched materials obtained from dewaxed sunflower pollen using conventional acetolysis and two ionic liquid solvents or combinations of both. Microscopic (SEM) and spectroscopic analyses (mostly NMR) showed that either method alone could render sporopollenin enriched fractions. Only the acetolyzed materials showed an increase in acetate content. Ionic liquids used alone led to the isolation of naked spore capsules containing only the endexine layer, suggesting that the ektexine layer could be solubilized by the ionic liquid. On the contrary, the acetolyzed sporopollenin capsules could not be further modified by the ionic liquid treatment, preserving the two exine layers and an echinate surface. Our results suggest that the acetolysis altered the surface hydrophobicity of sporopollenin due to the introduction of acetate. The ionic liquid process led to the isolation of either exine layer, with both showing virtually the same chemistry.

INTRODUCTION

A pollen grain is a microspore that encapsulates the mature/immature male gametophyte of seed plants.[1] They exist in a variety of dispersal units, shapes, polarities, apertures, ornamentations, and pollen wall's variations and present uniformity within the same plant species. Taken as an example, sunflower (*Helianthus annuus*) pollen grains are spheroids of ∼30 μm, with echini (i.e., spikes) and perforate surface (Figure 1) and tricolporate (i.e., three germinal furrows).[2] The inner and outer cell wall layers are named intine and exine, respectively.[1,3,4] The exine is mainly composed of sporopollenin, and comprises an inner, endexine, and an outer, ektexine, section.[3] The intine is composed of cellulose, hemicellulose, pectin, and hydrolytic and hydrophobic proteins. Sporopollenin is the protective capsule of the pollen, shielding it against biotic and abiotic stresses.[1,5,6] It is considered the toughest natural biopolymer,[7] exhibiting remarkable mechanical, thermal and chemical stability.[7,8] Exine sporopollenin capsules derived from sunflower pollen grains have been utilized for encapsulating specific molecules owing to their mechanical and chemical integrity, large empty cavity, uniform size distribution, and lack of allergens,[9−13] besides large production volumes: ∼55 million metric tons of seed volume in 2019/2020.[14]

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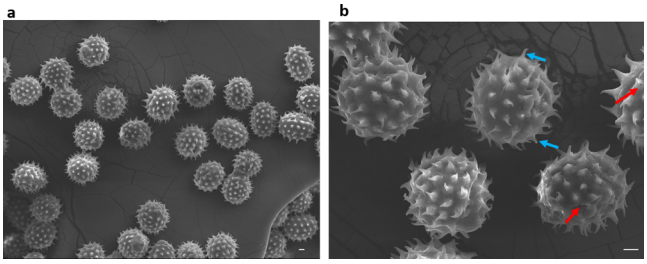

Figure 1. Morphological characterization of pollen. SEM images of defatted sunflower (*Helianthus annuus*) pollen from Pharmallerga (Czech Republic) (a,b). The blue and red arrows mark the echini (i.e., spikes) and the perforations, respectively. The scale bars, at the bottom of the micrographs, correspond to 10 μm.

Sporopollenin molecular structure remains partially unresolved due to its extreme inertness, which hinders its solubilization.[8] As a result, the recovered polymer is often modified or degraded.[8] The removal of the intine is using chemical or enzyme-based processes. The last, using, for example, cellulases, β-xylanase, pectinases, and proteases, results in isolated sporopollenin capsules,[15,16] preserving most of the spectroscopic characteristics of the natural pollen.[16] Usually, the dissolution of the intine relies on alkaline-lysis (e.g., with KOH)[11,17] or acidolysis methods (e.g., with orthophosphoric acid)[9,17] conducted at high temperatures, alone or in combination.[17,18] Acetolysis is frequently used for isolating sporopollenin capsules and does not require the prior removal of the pollen coating.[16,19] Studies focusing on various pollen sources and analytical methods have proposed different structures for sporopollenin. However, there is the general consensus that sporopollenin primarily comprises a polyhydroxylated aliphatic component and polyketide-derived aliphatic α-pyrone elements.[8] While the backbone of sporopollenin may be conserved across plant groups, there are distinct mechanisms by which phenolic-derived elements assemble these units.[8] For instance, sporopollenin extracted from *Pinus rigida* pollen consists mainly of aliphatic-polyketide-derived poly(vinyl alcohol) units and 7-*O*-*p*-coumaroylated C16 aliphatic units, cross-linked via a distinctive dioxane moiety featuring an acetal.[20] In this study, various forms of sporopollenin's constituents, insoluble, hydrolyzable (upon ethanethiol-based thioacidolysis), and acetolyzed, were analyzed using solid state nuclear magnetic resonance (ssNMR) and high-performance liquid chromatography coupled with ultraviolet detection and mass spectrometry (HPLC-UV-MS). In contrast, a different structure was proposed for *Lycopodium clavatum* sporopollenin, obtained by refluxing pollen with acetone, potassium hydroxide, and orthophosphoric acid. ssNMR analysis highlighted a macrocyclic oligomer/polymer backbone composed of polyhydroxylated tetraketide-*like* monomeric units (α-pyrone ring plus hydroxylated aliphatic chain), and a poly(hydroxy acid) network with glycerol as a core component.[21] The absence of aromaticity[21] contrasts with that reported for sporopollenin of *P. rigida*[20] and *L. clavatum*.[22] In another study focusing on *Pinus ponderosa* sporopollenin, the enzymatically isolated sporopollenin retained the pollen' spectral features, suggesting a copolymer of aliphatic lipids and *trans*-4-hydroxycinnamic acid.[16] Acidolysis and acetolysis treatments partially removed the aromatic components while maintaining exine morphology, indicating that *trans*-4-hydroxycinnamic acid and the other phenolic compounds are not major structural elements.

Ionic liquids, by definition, are salts that remain fluid at temperatures equal or below 100 °C.[23] Typically, the cation consists of an organic structure with low symmetry,[24] while the anion is generally a weakly basic compound, either inorganic or organic, with a diffuse or protected negative charge.[24] These solvents have the ability to dissolve biopolymers that are poorly soluble in organic solvents, such as cellulose, lignocellulose, chitin, and keratin.[25] Previous studies also demonstrated their potential to isolate the plant polyesters suberin[26,27] and cutin,[27,28] while preserving their polymeric structure. Not surprisingly, ionic liquids have been used to isolate sporopollenin capsules in several studies,[19,29−31] resulting in complete dissolution of pollen or extraction of empty capsules.

In this work, we produced and characterized sporopollenin enriched materials obtained from dewaxed sunflower pollen using conventional acetolysis and two ionic liquid solvents or combined approaches. Using mostly microscopic and spectroscopic methods, we have comprehensively characterized the ensuing polymeric materials corresponding to various forms of sporopollenin's constituents, insoluble, soluble, and acetolyzed. The potential of ionic liquids for separating ektexine's sporopollenin from endexine's sporopollenin is discussed in detail.

MATERIALS AND METHODS

Chemical Reagents. Hexanoic acid (99%) and deuterium oxide (100.0 atom % D) were obtained from Acros Organics. Dimethyl sulfoxide (≥99%), diethyl ether (≥99.5%), acetic acid (glacial), ethanol (absolute, ≥ 99.8%), and dichloromethane (≥99.8%) were purchased from Fisher Chemical. Cholinium bicarbonate (~80% in H_2O), sulfuric acid (95.0−97.0%), and dimethyl sulfoxide-d_6 (deuteration degree min 99.8%) were purchased from Sigma-Aldrich. 1-Butyl-3-methylimidazolium chloride (99%, BMIMCl) was obtained from IoLiTec. Acetic anhydride was obtained from ITW Reagents. Cholinium hexanoate (ChHex) was synthesized by dropwise addition of hexanoic acid to cholinium bicarbonate (1:1), as previous reported;[32] purity was confirmed through ^{1}H NMR using deuterium oxide as the solvent in an Avance II + 400 MHz spectrometer (Bruker Biospin).

Sporopollenin Isolation. Sunflower (*Helianthus annuus*) pollen (defatted with acetone) was purchased from Pharmallerga (Czech Republic). It was stored at 4 °C until used.

Acetolysis. Acetolysis was applied as reported before,[19] with some modifications: 5 g of pollen was reacted with 13.5 mL of acetic anhydride and 1.5 mL of sulfuric acid (stirring, 15 min, 160 °C); the reaction was cooled down to room temperature, filtrated to remove the solubilized constituents, and the solid residue sequentially washed with solvents (15 mL of acetic acid, 20 mL of ethanol, 30 mL of distilled water, and 15 mL of dichloromethane), then with an excess of water, and finally lyophilized (Labconco) and weighed. The acetolyzed sporopollenin was stored at room temperature for further use.

Ionic Liquid Isolation. The ionic liquid isolation protocol was adapted from those used for the isolation of the plant polyesters suberin[27] and cutin.[27] Briefly, pollen (acetolyzed or not) was mixed with either ionic liquid (1:10 w/w): BMIMCl and ChHex (100 °C, with stirring, 30 min or 2 h); the reaction stopped by addition of DMSO (1:80, w/v); the solubilized constituents were removed by filtration (0.45 μm nylon

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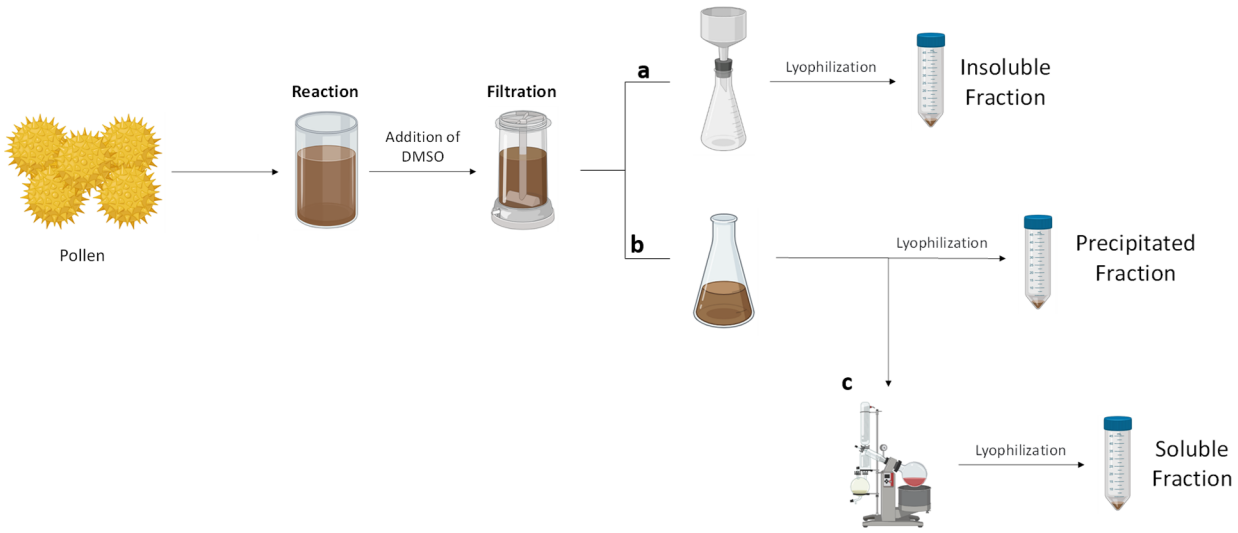

Figure 2. Schematics of the workflow used to recover sporopollenin enriched materials. Schematic representation of the experimental workflow used for the extraction of insoluble (a), precipitated (b), and soluble (c) fractions from sunflower pollen using an ionic liquid, namely BMIMCl and ChHex. The reactions run at 100 °C, with stirring, for 30 min or 2 h. The same workflow was applied to acetolyzed sunflower pollen.

membrane; overnight, 2 bar, room temperature, with stirring); and the solid residue was washed in excess of water. The obtained solid material corresponds to the insoluble fraction (IF), whereas the filtrate rendered a precipitated fraction (PF) that was precipitated overnight in water and recovered by centrifugation (1 L water per g of pollen; 18,566*g*, 30 min at 4 °C) and a soluble fraction (SF) recovered by drying (rotary evaporator) the nonprecipitated supernatant followed by its centrifugation (8514*g*, 1 h at 20 °C). All fractions were lyophilized, weighed, and stored at room temperature until used. The workflow is schematically represented in Figure 2.

Microscopy Analysis. Scanning electron microscopy (SEM; JEOL JSM-7001F microscope) was used to analyze the morphology of the lyophilized samples.

Cryogenic Grinding Process. All sporopollenin samples were subjected to cryogenic milling using a RETSCH Cryomill equipped with two 5 mL stainless steel grinding jars and two stainless steel grinding balls in each jar, at −196 °C (liquid nitrogen) using 200 milling cycles as follows: 3 min of precooling followed by nine milling cycles, each cycle consisting of 3 min of milling at 30 Hz, followed by 0.5 min of intermediate cooling at 5 Hz.

Fourier-Transform Infrared (FTIR). The infrared spectra of the fractions were obtained with an FTIR spectrometer accessory, Bruker IFS 66/S, using the KBr pellet method. Briefly, 4.7 to 7.6 mg of each sample and 100 mg of dried KBr were crushed and pressed together to form pellets. All spectra were recorded in absorbance mode in the range of 4000−400 cm^{-1} with 4 cm^{-1} resolution and accumulating 64 scans analyzed by OPUS software.

QUANTITATIVE ANALYSES OF TOTAL CARBOHYDRATE CONTENT

To evaluate the sugar moiety content of all of the insoluble sporopollenin materials, the samples were subjected to acid hydrolysis (1 M H_2SO_4 in methanol) for 4 h at 90 °C. The hydrolyzable sugars were recovered in the supernatant through centrifugation (18,514*g*, 4 °C, 20 min), and the pH was neutralized using 5 M NaOH in water. All samples were dried under a flux of nitrogen at room temperature. Quantification of carbohydrates in the dried hydrolysates was performed using the total carbohydrate assay kit from Sigma-Aldrich according to the manufacturer's instructions. The samples were analyzed in triplicate.

Elemental Analysis. Determination of C/H/N was done using an Elemental analyzer Thermo Finnigan-CE Instruments Flash EA 1112 CHNS series. For CHN analysis, 1 to 2 mg of each sample was weighed in tin capsules, in triplicate. Internal quality control was performed using methionine as the control standard.

Nuclear Magnetic Resonance (NMR). NMR spectra of each sample were recorded by using an Avance III 800 CRYO spectrometer (Bruker Biospin, Rheinstetten, Germany). All NMR spectra (^1H, $^1H-^1H$ COSY, $^1H-^{13}C$ HSQC, and $^1H-^{13}C$ HMBC) were acquired at 60 °C, using 5 mm-diameter NMR tubes: 2.1 mg to 6.5 mg of sample in 500 μL of DMSO-d_6. Quantitative ^{31}P NMR of different material was also performed using an Avance III 500 (Bruker Biospin, Rheinstetten, Germany).[33] For quantitative ^1H NMR, 10 μL of benzene solution (1.60 M) was added as internal standard, and 30° radio frequency pulses of 8.1 μs, relaxation delay of 1 s, acquisition time of 2.04 s, and spectral resolution of 0.245 Hz were used. MestReNova, Version 11.04−18998 (Mestrelab Research) was used to process the raw data acquired.

RESULTS AND DISCUSSION

Acetolyzed Sporopollenin Isolation. Sporopollenin capsule production requires (i) removal of the pollen coating (defatting step), (ii) full dissolution of the intine, (iii) removal of the nonsporopollenin components of the exine, and (iv) release of the cytoplasmic content. In this study, the used sunflower pollen was already defatted with acetone, which removes up to 8% of the mass of the sunflower pollen grains but not all surface components.[34] We chose acetolysis as the archetypal process to isolate sporopollenin from the defatted sunflower pollen grains (afterward defined solely as pollen) since it ensures rapid, efficient removal of all nonsporopollenin materials from pollen.[3,35] The obtained acetolyzed pollen is a dark brown powder, accounting for 37.0 ± 5.4% of the initial pollen mass. Significantly lower recovery yields of sporopolle-

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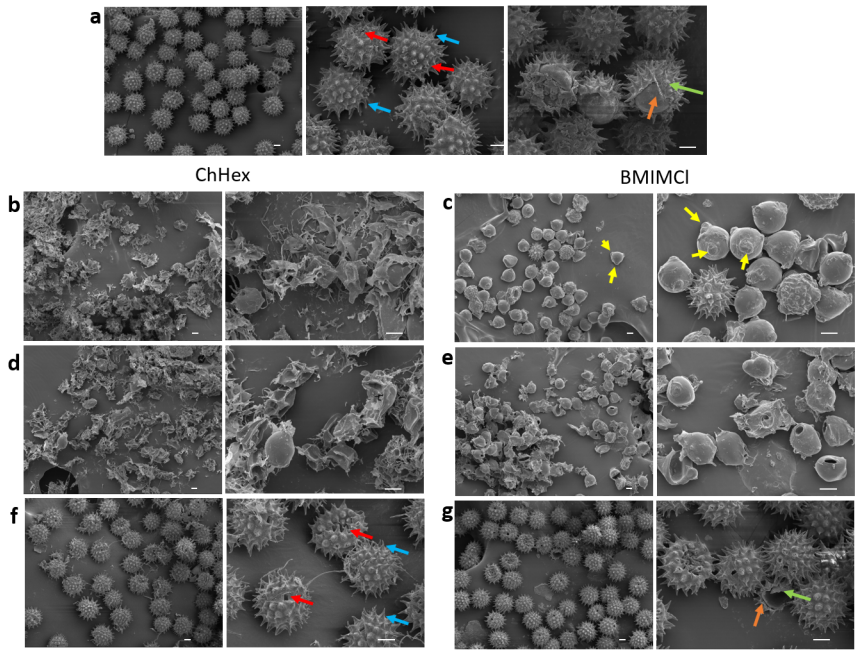

Figure 3. Morphological characterization of sporopollenin enriched materials. SEM micrographs of sporopollenin enriched materials, i.e., insoluble fractions, obtained from sunflower pollen through acetolysis (a) or an ionic liquid-mediated reaction (b−d; c−e), and from acetolyzed sunflower pollen using an ionic liquid-mediated reaction (f, g). 30 min (b, c, f, g) and 2 h (d, e) reactions were used. The blue and red arrows mark the echini and the perforations, respectively (visible in all acetolyzed samples: (a), (f), (g)); the yellow arrow marks the colpori in the intine layer next to the cytoplasm (note the tricolporate morphology in (c)); the orange and green arrows mark the ektexine (outer) and endexine (inner) sections of the exine layer, respectively, both visible in (g). All scale bars at the bottom of the micrographs correspond to 10 μm.

nin hollow capsules obtained from pollen have been reported using acidolysis (5 h, orthophosphoric acid),[34] enzymatic digestion (cellulase and β-glucosidase),[29] and an ionic liquid (40% tetrabutylphosphonium hydroxide in water),[29] corresponding to only 5.5% to 12.5% of the initial pollen mass, suggestive of partial dissolution/degradation of the polymer.

The pollen grains are spheroidal with an echinate surface (Figure 1a,b). The three colpori are not visible because these aperture regions are sunken when the pollen is dry and may be masked by the pollen coating as well. The acetolyzed sporopollenin (Figure 3a) preserves the spheroidal shape and echinate surface. However, perforations in the exine, particularly around the base of the echini, are visible, consistent with the efficient removal of the pollen coating via acetolysis. Some capsules lost the echini and others show a cracked bilayer exine surface, outside, the ektexine (stratified, with cavities and echini), and inside, the endexine (smooth appearance) (Figure 3a). These two distinct layers have been previously identified in sunflower pollen capsules after acidolysis alone[36] or combined with alkaline lysis.[11] The exine is resistant to acetolysis but not the intine.

Ionic Liquid-Based Sporopollenin Isolation. The selected ionic liquids were 1-butyl-3-methylimidazolium chloride (BMIMCl) and cholinium hexanoate (ChHex). BMIMCl dissolves cellulose[37] and sporopollenin,[19] and ChHex catalyzes mildly the hydrolysis of acylglycerol ester bonds[27,28] but not of primary and secondary aliphatic ester bonds[27] and ester-aromatics.[27] The last has been used before for the extraction of plant polyesters from different sources mostly due to its unique catalytic capabilities,[38] low toxicity (lower than its corresponding sodium salt), and biodegradability.[32] The ionic liquid processes rendered three fractions: insoluble (the solid material, likely the major sporopollenin enriched fraction), precipitated, and soluble (Figure 2). The last two correspond to the ionic liquid's solubilized pollen constituents. This differs from the previous studies on sporopollenin isolation using ionic liquids, where only the solid material was analyzed.[19,29−31]

ChHex-mediated reactions led to recovery of a solid material corresponding to 7−8% of the initial pollen mass, regardless of the reaction time, whereas BMIMCl-mediated reactions yielded greater amounts of solid material, namely 24% and 34% of the initial pollen mass for the 2 h and 30 min reactions, respectively. The precipitated fractions were ≤0.3% for all of the reactions. The soluble fractions were 22−26% of the initial pollen mass in the BMIMCl reactions but vestigial in those with ChHex. These results suggest that either ionic liquid could partially solubilize sporopollenin. BMIMCl (90 min at 160 °C) completely solubilized the sporopollenin from *Populus deltoides* pollen.[19]

The morphologies of the solid materials obtained with either ChHex reactions were similar: mostly amorphous aggregates and a few spheroidal capsules with a smooth surface and open aperture (Figure 3b,d). The morphologies of the solid materials recovered after the two BMIMCl reactions are similar, but the reaction time had a great impact (Figure 3c,e). After 30 min, there was a mix of capsules with and without echini, all having visible colpori but without any aperture, hence likely not empty (Figure 3c); whereas after 2 h, there were mostly naked capsules without any colpori and with an open aperture mixed with amorphous aggregates (Figure 3e). The capsules having an open aperture apparently comprise only the endexine layer (Figure 3e). The SEM results are suggestive that both ionic liquids could mediate the removal of the colpori and of the cytoplasmatic components, and the dissolution of both exine layers: endexine and ektexine,

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regardless of distinct reactions efficiencies. The isolation of empty exine capsules carrying only the endexine layer and not the ektexine is therefore apparently possible. This is distinct from that reported before for sporopollenin enriched materials isolated with ionic liquids, where the pollen grains preserved the two-layered exine morphology, either modified[19,30] or unaltered.[29,31] The dissolution of the ektexine has been reported before using 2-aminoethanol, 3-aminopropanol, and 2,2′,2″-nitrilotriethanol.[39]

Sporopollenin Isolation Combining Acetolysis and Ionic Liquids. The ionic liquid ability to solubilize/hydrolyze the acetolyzed sporopollenin was evaluated, selecting the 30 min reactions with ChHex and BMIMCl. The solid materials recovered corresponded to 59% and 72% of the initial acetolyzed sporopollenin mass for ChHex and BMIMCl, respectively. Only the second reaction rendered a precipitated fraction, weighing 8% of the initial mass. The solubilizing capacity of either ionic liquid was greatly hindered by the acetolysis pretreatment compared to the pollen. This result indicates that the acetolysis process increased the resistance of the capsules to either ionic liquid-mediated process. SEM imaging shows that the obtained solid materials (Figure 3f,g) are mostly similar to those of the acetolyzed pollen (Figure 3a). The isolated capsules show the two-layered exine and the echini preserved as well as some open apertures. The precipitated fraction obtained after the 30 min reaction with BMIMCl shows irregular shapes of different sizes resulting from the aggregation of the dissolved materials after lyophilization (Figure S1). These results further confirm that the acetolysis process modified ektexine chemistry, increasing its resistance to the action of the ionic liquids. Previously, it was demonstrated that acetolysis treatment also led to the insolubility of the *Thypha angustifolia* exine in 2-aminoethanol.[40]

Fourier-Transform Infrared (FTIR) Spectroscopy. FTIR was used to analyze the chemistry of pollen and all sporopollenin solid materials (Figure 4, Figure S2, and Table S1). The bands assigned to the stretching (ν) vibrations, asymmetrical (ν_{asym}) and symmetrical (ν_{sym}), of the CH_2 groups in aliphatics (~2926 cm^{-1}, 2855 cm^{-1}), both associated with lipids and sporopollenin,[16,41] and the signal attributed to the carbonyl ν(C═O) of ester groups in lipids (1742 cm^{-1})[16] is visible in all samples. The pollen spectral bands associated with either proteins (ν(C═O) in amide I, 1651 cm^{-1}; δ(NH) + ν(CN) in amide II (1547 cm^{-1})[16] and with polysaccharides (~1078 and 1038 cm^{-1}),[16] are absent in acetolyzed samples as reported before[16] but visible in solid materials obtained with the ionic liquids from pollen. These results suggest an incomplete removal of proteins and polysaccharides by either ionic liquid under the tested conditions. The presence of polysaccharide moieties was confirmed by quantifying the hydrolyzable carbohydrates present in each insoluble sporopollenin material (Table 1). The results show polysaccharides' levels in the ionic liquids' extracted samples ≤0.7 ng·mg^{-1}, in the acetolyzed samples ~0.24 ng·mg^{-1}, and in the samples subjected to both process ≤0.16 ng·mg^{-1}. The 2-fold decrease in the amounts of polysaccharides in the acetolyzed samples subjected to either ionic liquid is consistent with their capacity to remove polysaccharides. The analysis of the C/H/N relative abundances of all sporopollenin's insoluble materials (Table 1) supports the higher relative abundance of N in the ionic liquid extracted materials compared to the acetolyzed ones. The insoluble materials subjected to both processes show higher N levels compared to those subjected only to acetolysis. This result suggests minor contamination with either ionic liquid. As none of the NMR analyses (see below) could detect the signals assigned to either ionic liquid, this minor contamination can likely be eliminated through washing.

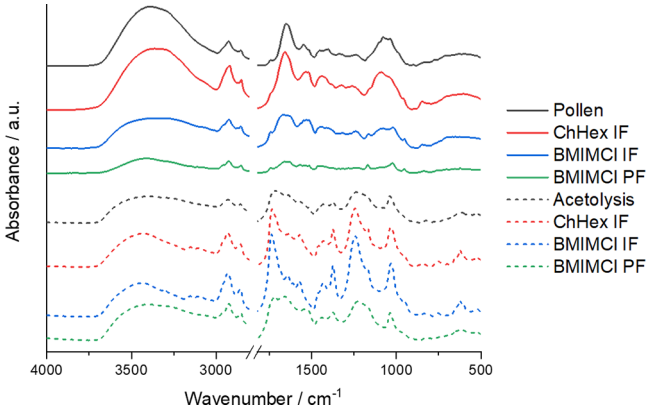

Figure 4. FTIR spectra of sunflower pollen (continuous lines) and acetolyzed sunflower pollen (dash lines), and their corresponding fractions isolated with either ionic liquid (30 min reaction): insoluble (IF) and precipitated (PF).

Signals assigned to $\delta(CH_2)$ in aliphatics associated with lipids (1452 cm^{-1}, 1402 cm^{-1}),[16] δ(OH) in alcohols (~1337 cm^{-1}), and ν(CO) in esters (1240 cm^{-1})[16] are visible in all spectra, except in acetolyzed samples. In all acetolyzed samples, a band at ~1715 cm^{-1}, representing the overlap between the ν(C═O) of carboxyl[16] and of acetate esters,[16] is visible. Moreover, the bands assigned to the ν(C═C) in alkenyl (1645 cm^{-1}),[16] $\delta(CH_2)$ and $\delta(CH_3)$ (1470 cm^{-1}, 1420 cm^{-1}),[41] and ν(CO) in acetate esters (~1236 cm^{-1})[16,19] are only visible in acetolyzed samples. A broad band between 3300 and 3500 cm^{-1}, attributed to the ν(OH),[16,19] is reduced in the acetolyzed pollen as previously reported.[16,19] Some bands have been assigned to aromatic ring structures in sporopollenin,[16,41] namely 3013 cm^{-1}, 1157 cm^{-1}, and 847 cm^{-1}. The second is visible in all samples; the last is absent in only the pollen, and the first is visible only in the solid material obtained after 2 h with the BMIM-based ionic liquid. The band assigned to the $\delta_{sym}(CH_3)$ (~1387 cm^{-1})[16] is visible in all samples except in pollen; it likely derives from hydrolytic action in the case of ionic liquids' processed samples and from the introduction of acetate esters in acetolyzed samples.[16] Some signals remain unassigned, e.g., 3304 cm^{-1}, 3151 cm^{-1}, 3109 cm^{-1}, 1572 cm^{-1}, 1038 cm^{-1}, 960 cm^{-1}, and 899 cm^{-1}.

The FTIR results suggest that the acetolysis removed the nonsporopollenin constituents of the pollen more efficiently than either ionic liquid under the conditions tested. Four signals found in solid materials isolated with the ionic liquids from pollen were absent in the pollen (one is assigned to aromatic rings), but three of them are also visible in the acetolyzed samples. Finally, the acetolyzed samples show spectral signals associated with the introduction of acetate esters.

Nuclear Magnetic Resonance (NMR). All solid materials were subjected to cryogenic milling to facilitated their solubilization in DMSO-d_6, as applied before in other plant biopolymers,[27,28] essential to attain high NMR spectral resolution. The assignments of the spectra were performed through a combination of 2D spectra (^{1}H−^{1}H COSY, ^{1}H−^{13}C

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Table 1. Quantification of Non-Sporopollenin Constituents in All the Extracted Insoluble Materials, Specifically Elemental C/H/N Analysis Total Hydrolysable Polysaccharides (n = 3)

Starting material	Solid materials enriched in sporopollenin (insoluble fractions)	Elemental analysis (%)			Hydrolyzable polysaccharides ($ng \cdot mg^{-1}$)
		C	H	N	
pollen	untreated	47.1 ± 0.04	7.5 ± 0.1	4.7 ± 0.09	2.65 ± 0.701
	+BMIMCl (30 min)	48.8 ± 0.2	7.6 ± 0.1	7.7 ± 0.1	0.64 ± 0.502
	+BMIMCl (2 h)	50.9 ± 0.22	7.5 ± 0.26	7.8 ± 0.1	0.69 ± 0.46
	+ChHex (30 min)	49.2 ± 0.08	7.5 ± 0.08	5.1 ± 0.2	0.43 ± 0.063
	+ChHex (2 h)	49.4 ± 0.18	7.6 ± 0.08	4.4 ± 0.08	0.52 ± 0.064
	+acetolyzed	52.0 ± 0.04	6.4 ± 0.03	2.5 ± 0.09	0.24 ± 0.037
acetolyzed pollen	+BMIMCl (30 min)	54.4 ± 0.33	6.4 ± 0.08	2.9 ± 0.07	0.14 ± 0.031
	+ChHex (30 min)	52.1 ± 0.36	7.1 ± 0.06	3.0 ± 0.04	0.16 ± 0.141

HSQC, and $^{1}H-^{13}C$ HMBC), guided by previous spectral information on sporopollenin: ^{1}H full spectra plus COSY of the aromatic region,[15,42] and the HSQC of the aromatic region,[22] complemented by assignments done for other complex poly aliphatic/aromatic biopolymers.[26−28] The spectral analysis of pollen is shown in Figure 5a, depicting both the ^{1}H NMR (I) and $^{1}H-^{13}C$ HSQC NMR spectra (II), where the aliphatics (III) and CH/CH_2-X aliphatics (IV) regions are identified. Unsaturated fatty acids are also present. The aliphatics region is rich in CH_3 and CH_2 groups, with visible signals related to (i) allylic CH_2 at a ^{13}C shift of 26.39 ppm and ^{1}H shift of 1.97 ppm; (ii) $\alpha(C{=}O)$ at a ^{13}C shift of 33.00 ppm and ^{1}H shifts of 2.27 and 2.17 ppm (attributed to esters); and (iii) acetate at a ^{13}C shift of 20.71 ppm and a ^{1}H shift of 1.90 ppm. In the CH/CH_2-X aliphatics region, the assigned signals correspond to (iv) primary aliphatic esters (PAE-α) at a ^{13}C shift of 62.18 ppm and a ^{1}H shift of 4.00 ppm; and (v) acetal at a ^{13}C shift of 101.63 ppm and a ^{1}H shift of 4.92 ppm. Acetal has been described as one of the major interunit linkages in pine sporopollenin.[20]

The full NMR ^{1}H and $^{1}H-^{13}C$ HSQC spectra of the solid material isolated after 30 min with BMIMCl are depicted in Figure 5b, detailing the same three main regions shown for the spectral analysis of pollen. The aliphatic (III) and CH/CH_2-X aliphatic (IV) regions show the signals previously assigned in the pollen spectrum. The only difference is the presence of the 1,2,3-triacylglycerol (1,2,3-TAG) configuration ($CH^{1,3}$ signals at ^{13}C shifts of 61.51 and 61.41 ppm and ^{1}H shifts of 4.26 and 4.13 ppm, and CH^{2} signal at ^{13}C shift of 68.41 ppm and a ^{1}H shift of 5.18 ppm). The corresponding soluble fraction shows the signal of linoleic acid (aliphatics region) with a ^{13}C shift of 24.63 ppm and a ^{1}H shift of 2.78 ppm (Figure S3). The spectrum of the solid material obtained after the 2 h reaction with the BMIMCl is also similar to that of the pollen (Figure S4a), except for the presence of the linoleic signal, also visible in the precipitated (Figure S4b) and soluble fractions (Figure S4c). Note that in the fractions derived from the filtrate, the sporopollenin's archetypal signals in the aliphatics region (CH_2 and CH_3 groups, allylic CH_2 and $\alpha(C{=}O)$ esters) and in the CH/CH_2-X aliphatics region (1,2,3-TAG, PAE-α and acetal) are all visible. This result indicates that this ionic liquid partially solubilized sporopollenin, consistent with the idea that the ektexine layer of the exine was removed. The solid material isolated from pollen after 30 min of reaction with BMIMCl (Figure 5b) is virtually identical to that obtained with the ChHex reaction at the same reaction time (Figure S5a). The spectrum of the solid material attained with the 2 h reaction with ChHex presents very few signals, and only in the aliphatics (II) and CH/CH_2-X aliphatics regions (III) corresponding to the acetate, the $\alpha(C{=}O)$ esters and very few CH_2 and CH_3 groups, mostly reflecting its low solubility (Figure S5b). Moreover, together with the SEM imaging (Figure 3E), this suggests that ChHex efficiently solubilized sporopollenin.

Finally, in the spectral analysis of the acetolyzed pollen (Figure 6a), the signal corresponding to unsaturated fatty acids is not visible, but the remaining signals visible in the aliphatics (III) and the CH/CH_2-X aliphatics regions (IV) are the same found in the pollen (Figure 4a). The acetate signal is apparently more intense than that in the pollen. The spectra of the solid materials obtained from the acetolyzed pollen with the BMIMCl (Figure 6b) and ChHex (Figure S6b), 30 min reactions, do not present the previous assigned signals of unsaturated fatty acids (both ionic liquids): few signals assigned to CH_3 and CH_2 groups (both), two (BMIMCl) or a single (ChHex) $\alpha(C{=}O)$ esters signal, an intense acetate signal (both), and few (BMIMCl) or none (ChHex) signals from aromatics. Moreover, the spectrum of the precipitated fraction derived from the acetolyzed pollen with BMIMCl (30 min) is similar to that of pollen, acetolyzed or not, except for the presence of the signals corresponding to linoleic acid, 1,2,3-TAG and PAE-α and for the absence of an acetal signal (Figure S6a). The absence of acetal, despite the high solubility of this sample, suggests enrichment in partially degraded sporopollenin constituents.

Quantification of free OH content and acetate in pollen, acetolyzed pollen, and both corresponding solid materials obtained with a 30 min reaction with BMIMCl, were quantified by ^{31}P NMR (through a phosphorylation reaction) and ^{1}H NMR (using an internal standard method) (Table 2). The obtained results confirm the introduction of significant amounts of acetate in either acetolyzed solid material. The proportion of OH aliphatics and free acids is suggestive that no major hydrolyzes occurred. The decrease of OH aliphatics after acetolysis- or BMIMCl-mediated reaction compared with that of untreated pollen reflects the removal of free lipidic compounds. Further reduction of OH aliphatics in the solid material obtained from the acetolyzed material with BMIMCl is noticed. Finally, none of the treatments modified significantly the amount of OH aromatics, consistent with previous reports on the molecular structure of sporopollenin.[16,20,22]

The NMR analyses (Figure 5) showed the presence of PAE and acetal signals in the pollen (Table 2). After the ionic liquid-mediated reaction, the only glycerol configuration detected was 1,2,3-TAG, seen due to the polymer increased

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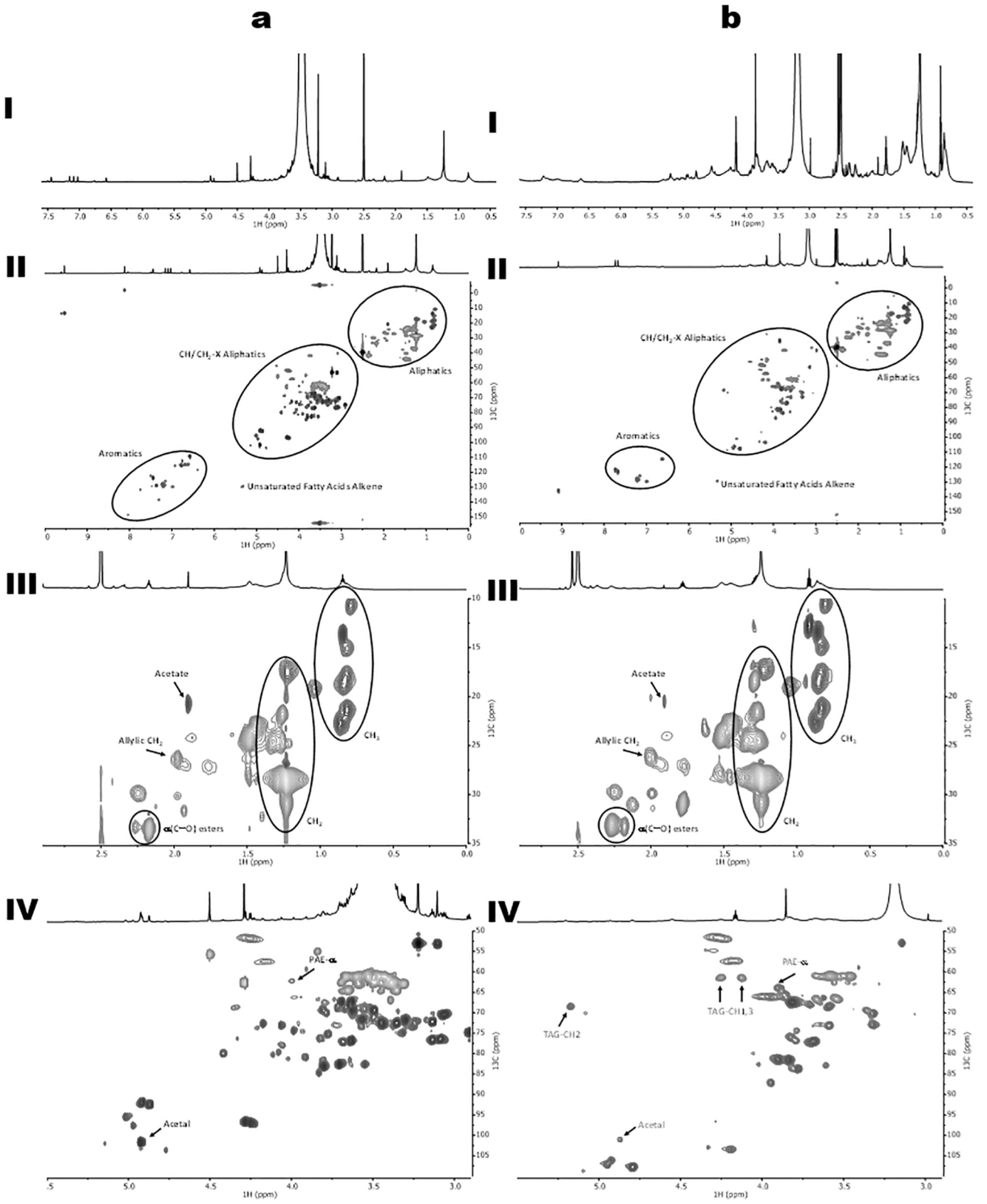

Figure 5. Wide-ranging NMR spectral characterization of sunflower pollen directly dissolved in DMSO-d_6 (a), and of sporopollenin enriched solid material extracted from pollen with BMIMCl for 30 min (b). The ^{1}H NMR (I); the HSQC full spectrum (II) and the regions corresponding to aliphatics (III), CH/CH$_2$−X aliphatics (IV) are shown. Some correlations (unlabeled) are uncertain or unidentified.

solubility. Based on the NMR analyses and quantifications, the cleavage of PAE is considered unlikely, consistent with previous studies showing that these bonds are not efficiently cleaved by either ChHex and BMIMCl.[28,38] Moreover, the data

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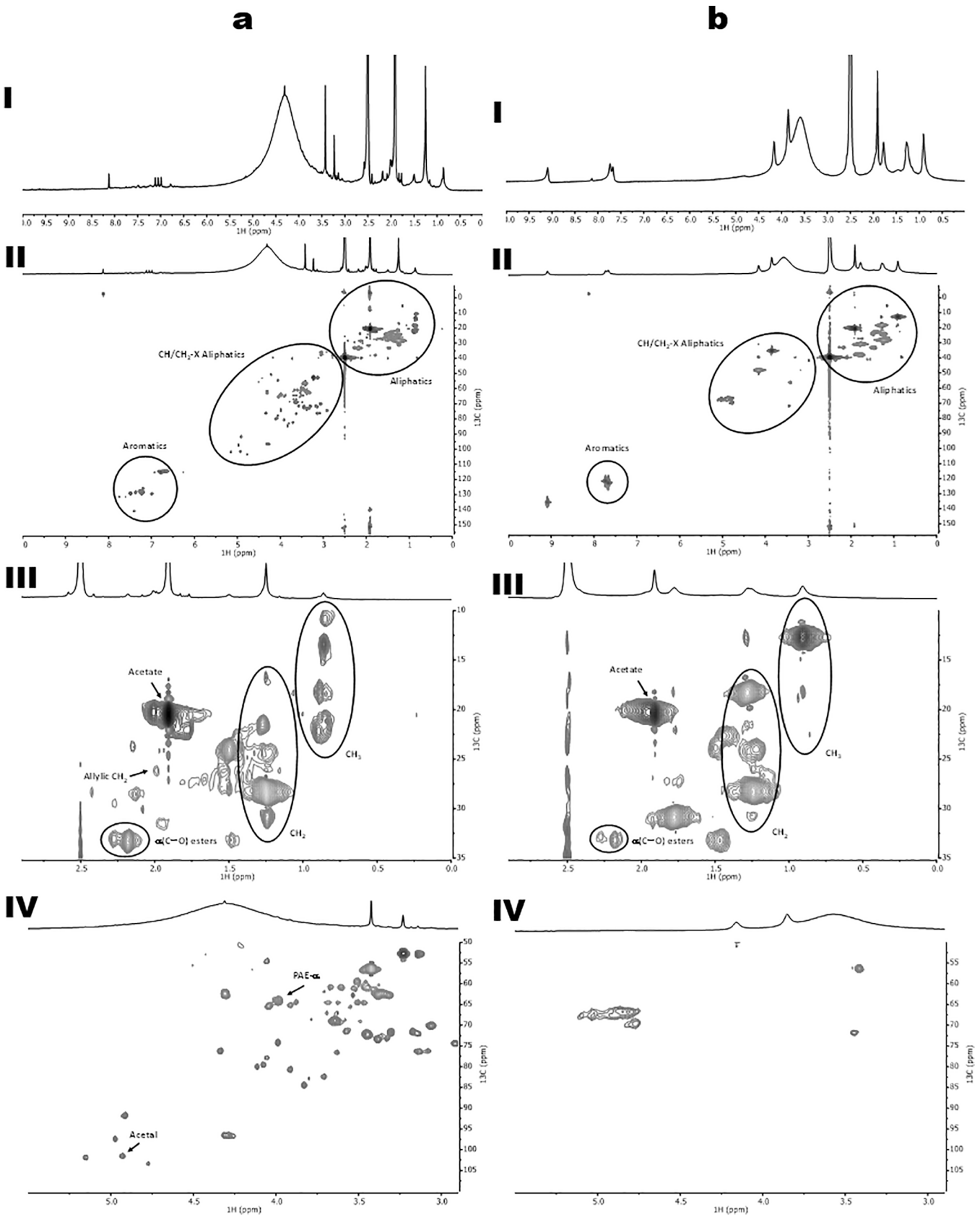

Figure 6. Wide-ranging NMR spectral characterization of sporopollenin in acetolyzed sunflower pollen directly dissolved in DMSO-d_6 (a), and in the solid material extracted from acetolyzed pollen by BMIMCl for 30 min (b). The ^{1}H NMR (I); the HSQC full spectrum (II); regions corresponding to aliphatics (III); CH/CH_2−X aliphatics (IV). Some correlations (unlabeled) are uncertain or unidentified.

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Table 2. NMR Quantifications of Pollen and Acetolyzed Pollen, and Both Corresponding Insoluble Fractions Obtained after a 30 min Reaction with BMIMCl[a]

		^{31}P NMR (mmol·g^{-1})			
Starting material	Solid materials enriched in sporopollenin (insoluble fractions)	OH aliphatics	OH aromatics	Free acids	Acetate (mmol·g^{-1})
pollen	untreated	3.32 ± 1.00	0.07 ± 0.02	0.23 ± 0.07	0.17
	+BMIMCl (30 min)	0.65 ± 0.05	0.10 ± 0.01	0.16 ± 0.04	0.09
	+acetolyzed	0.42 ± 0.16	0.05 ± 0.02	0.86 ± 0.22	2.40
acetolyzed pollen	+BMIMCl (30 min)	0.05 ± 0.02	0.01 ± 0.01	0.30 ± 0.10	1.53

[a]Free acids and hydroxyl groups were quantified by ^{31}P NMR, whereas acetate was quantified by ^{1}H NMR using an internal standard (n = 3).

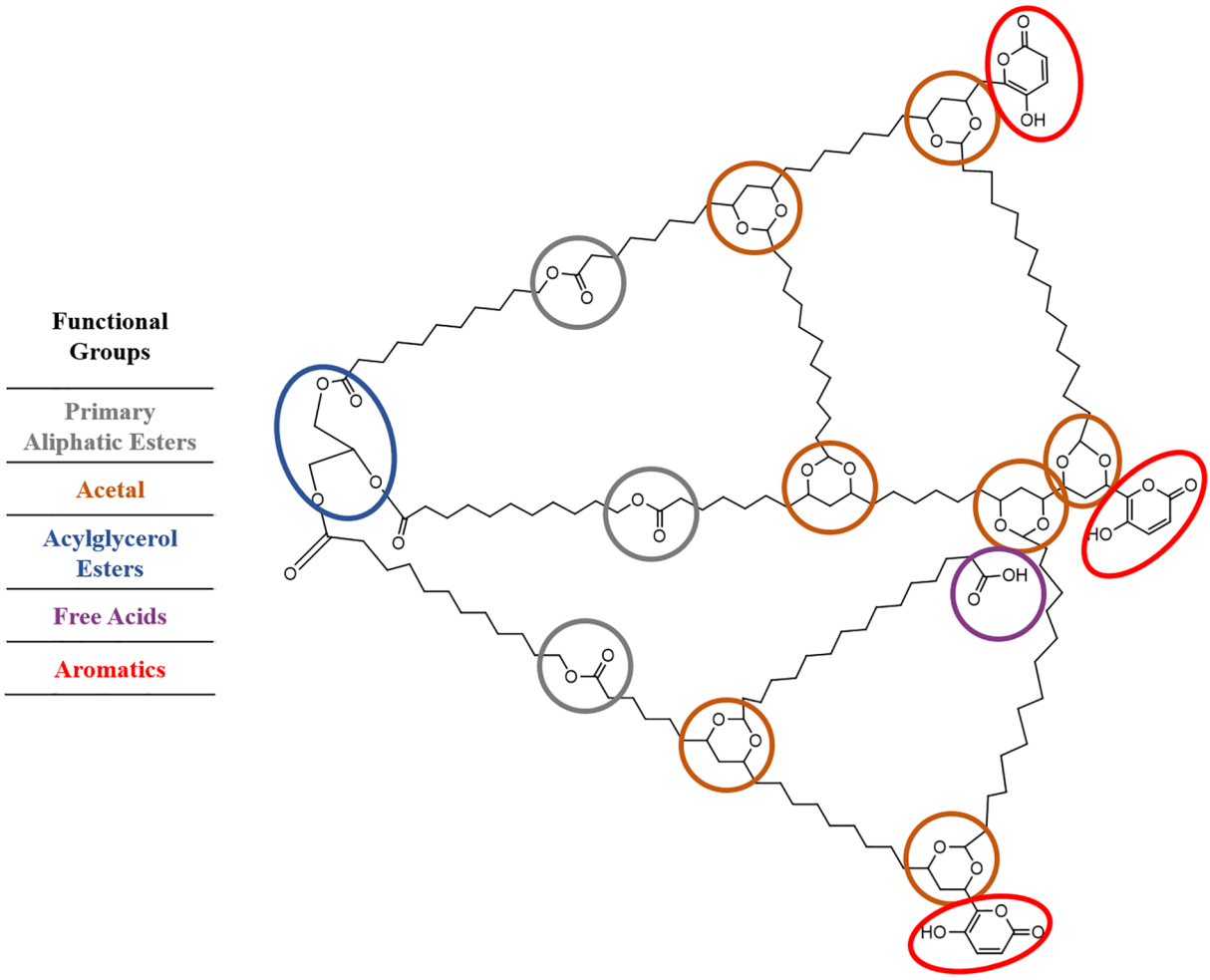

Figure 7. Schematic representation of the main midchain and end-chain functionalities (highlighted in different colors) of the sporopollenin' ektexine and endexine layers.

suggest that BMIMCl mediated the cleavage of acetals to a minor extent. Collectively, the results indicate that the acetolysis modifies sporopollenin, increasing the exine resistance to the ionic liquid treatment (mostly of the ektexine), possibly due to the introduction of acetate as quantified by the NMR (Table 2), and seen in the FTIR (presence of acetate ester bands plus reduction of the hydroxyl groups band).

CONCLUSIONS

In this study, materials enriched with sporopollenin were obtained through acetolysis, ionic liquid mediated reactions, or their combination and subjected to a comprehensive morphological and chemical analysis. Cryogenic milling enabled the solubilization of the materials in DMSO-d_6, facilitating their molecular structure analysis via wide-ranging NMR. Our data confirm that sporopollenin primarily comprises a polyhydroxylated aliphatic component and polyketide-derived aliphatic α-pyrone elements (schematic representation in Figure 7). Both tested ionic liquids could solubilize sporopollenin, consistent with previous reports.[19,29−31]

The solid materials isolated with either ionic liquid consisted of empty capsules (or their fragments) comprising apparently only endexine. This indicates that both ionic liquids could dissolve the intine and also the ektexine layer of the exine. Specifically, BMIMCl led to the isolation of empty, naked pollen endexine capsules (Figure 3c,e). All of the obtained

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materials were subjected to NMR analysis (Figure 5 and 6), confirming the presence of aromatics, primary aliphatic esters, and acetals in the polymer. The presence of acetal signal in some precipitated and soluble fractions suggests that this ionic liquid did hydrolyze to a minor extent sporopollenin. The observation of 1,2,3-triacylglycerol as the only glycerol configuration, appearing solely after ionic liquid treatment, indicates the enhanced solubility of the polymer. Acetolysis, typically considered a conventional isolation method for sporopollenin, modified its chemistry, resulting in increased resistance to either ionic liquid mediated-treatment compared to untreated pollen. This modification likely involves the introduction of acetate into the polymer, as reported before,[16] consistent with the spectroscopic analyses conducted herein (Figures 4 and 5a).

The isolation of sporopollenin with ionic liquids alone or combined with conventional methods, such as acetolysis, offers novel pathways to obtain sporopollenin samples with varying degrees of polymeric preservation and purity. Further optimization is necessary, although BMIMCl demonstrated superior potential for isolating endexine capsules. Cleaning the obtained solid materials from any residual polysaccharides and/or proteins can be achieved by controlling the reaction time or introducing another catalytic step with hydrolyzing enzymes, as successfully applied in other studies.[19,30,31] The ionic liquid capacity to mediate the isolation of either exine layer having similar chemistries creates an opportunity to improve our understanding of sporopollenin structural chemistry, which is valuable for solving fundamental unknowns in plant biology and further its biotechnological applications.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data is available in the manuscript. Supplementary Figures and Tables are provided to support the results presented on this manuscript. The NMR data has been deposited in the Figshare with the accession number 10.6084/m9.figshare.25975828 and 10.6084/m9.figshare.25975366.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06524.

SEM micrographs, FTIR spectra, assignment of the main functional groups present and their ensuing fractions after extraction, wide-ranging NMR spectral characterizations, presence or absence of particular NMR signals (PDF)

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Author Contributions

±AB and VGC are equally contributing authors. CSP supervised the project and the interpretation of data; CSP prepared the final version of the manuscript. All authors have made substantial contributions to the acquisition, analysis and interpretation of data and contributed to the drafting of the manuscript: LA, AB, and VC: sample preparation and analyses; LA, preparation of the initial draft of the manuscript. All authors read and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Evert, R. F.; Eichhorn, S. E. *Raven Biology of Plants*, 8th ed.; W. H. Freeman and Company Publishers, 2013.

(2) Halbritter, H.; Heigl, H.; Svojtka, N. Helianthus Annuus. *PalDat - A palynological database*, 2020. https://www.paldat.org/pub/Helianthus_annuus/304619.

(3) Halbritter, H.; Ulrich, S.; Grímsson, F.; Weber, M.; Zetter, R.; Hesse, M.; Buchner, R.; Svojtka, M.; Frosch-Radivo, A. *Illustrated Pollen Terminology*, 2nd ed.; Springer International Publishing, 2018. DOI: 10.1007/978-3-319-71365-6_1.

(4) Ariizumi, T.; Toriyama, K. Genetic Regulation of Sporopollenin Synthesis and Pollen Exine Development. *Annual review of plant biology* **2011**, *62* (1), 437−460.

(5) Wellman, C. H. Origin, Function and Development of the Spore Wall in Early Land Plants. *Evolution of Plant Physiology* **2004**, No. 1, 43−63.

(6) Quilichini, T. D.; Grienenberger, E.; Douglas, C. J. The Biosynthesis, Composition and Assembly of the Outer Pollen Wall: A Tough Case to Crack. *Phytochemistry* **2015**, *113*, 170−182.

(7) Mackenzie, G.; Boa, A. N.; Diego-Taboada, A.; Atkin, S. L.; Sathyapalan, T. Sporopollenin, The Least Known Yet Toughest Natural Biopolymer. *Frontiers in Materials* **2015**, *2* (66), 1−5.

(8) Grienenberger, E.; Quilichini, T. D. The Toughest Material in the Plant Kingdom: An Update on Sporopollenin. *Frontiers in Plant Science* **2021**, *12*, 1−9.

(9) Wang, H.; Potroz, M. G.; Jackman, J. A.; Khezri, B.; Marić, T.; Cho, N.-J.; Pumera, M. Bioinspired Spiky Micromotors Based on Sporopollenin Exine Capsules. *Adv. Funct. Mater.* **2017**, *27* (32), No. 1702338.
(10) Potroz, M. G.; Mundargi, R. C.; Gillissen, J. J.; Tan, E.-L.; Meker, S.; Park, J. H.; Jung, H.; Park, S.; Cho, D.; Bang, S.-I.; Cho, N.-J. Plant-Based Hollow Microcapsules for Oral Delivery Applications: Toward Optimized Loading and Controlled Release. *Adv. Funct. Mater.* **2017**, *27* (31), No. 1700270.
(11) Uddin, M. J.; Liyanage, S.; Abidi, N.; Gill, H. S. Physical and Biochemical Characterization of Chemically Treated Pollen Shells for Potential Use in Oral Delivery of Therapeutics. *J. Pharm. Sci.* **2018**, *107* (12), 3047−3059.
(12) Wu, D.; Liang, Y.; Pei, Y.; Li, B.; Liang, H. Plant Exine Capsules Based Encapsulation Strategy: A High Loading and Long-Term Effective Delivery System for Nobiletin. *Food Research International* **2020**, *127*, No. 108691.
(13) Deng, Z.; Li, J.; Song, R.; Zhou, B.; Li, B.; Liang, H. Carboxymethylpachymaran/Alginate Gel Entrapping of Natural Pollen Capsules for the Encapsulation, Protection and Delivery of Probiotics with Enhanced Viability. *Food Hydrocolloids* **2021**, *120*, No. 106855.
(14) Shahbandeh, M. Production Volume of Sunflower Seed in Major Producer Countries in 2019/2020. *Statista*, 2021. https://www.statista.com/statistics/263928/production-of-sunflower-seed-since-2000-by-major-countries/.
(15) Ahlers, F.; Thom, I.; Lambert, J.; Kuckuk, R.; Wiermann, R. 1H NMR Analysis of Sporopollenin from Typha Angustifolia. *Phytochemistry* **1999**, *50* (6), 1095−1098.
(16) Lutzke, A.; Morey, K. J.; Medford, J. I.; Kipper, M. J. Detailed Characterization of Pinus Ponderosa Sporopollenin by Infrared Spectroscopy. *Phytochemistry* **2020**, *170*, No. 112195.
(17) Gonzalez-Cruz, P.; Uddin, M. J.; Atwe, S. U.; Abidi, N.; Gill, H. S. A Chemical Treatment Method for Obtaining Clean and Intact Pollen Shells of Different Species. *ACS Biomaterials Science & Engineering* **2018**, *4* (7), 2319−2329.
(18) Mundargi, R. C.; Potroz, M. G.; Park, J. H.; Seo, J.; Tan, E.-L.; Lee, J. H.; Cho, N.-J. Eco-Friendly Streamlined Process for Sporopollenin Exine Capsule Extraction. *Sci. Rep.* **2016**, *6*, No. 19960.
(19) Chiappe, C.; Demontis, G. C.; Bussolo, V. D.; Douton, M. J. R.; Rossella, F.; Pomelli, C. S.; Sartini, S.; Caporali, S. From Pollen Grains to Functionalized Microcapsules: A Facile Chemical Route Using Ionic Liquids. *Green Chem.* **2017**, *19* (4), 1028−1033.
(20) Li, F. S.; Phyo, P.; Jacobowitz, J.; Hong, M.; Weng, J. K. The Molecular Structure of Plant Sporopollenin. *Nature Plants* **2019**, *5* (1), 41−46.
(21) Mikhael, A.; Jurcic, K.; Schneider, C.; Karr, D.; Fisher, G. L.; Fridgen, T. D.; Diego-Taboada, A.; Georghiou, P. E.; Mackenzie, G.; Banoub, J. Demystifying and Unravelling the Molecular Structure of the Biopolymer Sporopollenin. *Rapid Commun. Mass Spectrom.* **2020**, *34* (10), No. e8740.
(22) Xue, J.-S.; Zhang, B.; Zhan, H.; Lv, Y.-L.; Jia, X.-L.; Wang, T.; Yang, N.-Y.; Lou, Y.-X.; Zhang, Z.-B.; Hu, W.-J.; Gui, J.; Cao, J.; Xu, P.; Zhou, Y.; Hu, J.-F.; Li, L.; Yang, Z.-N. Phenylpropanoid Derivatives Are Essential Components of Sporopollenin in Vascular Plants. *Molecular Plant* **2020**, *13*, 1−10.
(23) Rogers, R. D.; Seddon, K. R. Ionic Liquids - Solvents of the Future? *Science* **2003**, *302* (5646), 792−793.
(24) Clare, B.; Sirwardana, A.; MacFarlane, D. R. Synthesis, Purification and Characterization of Ionic Liquids. In *Ionic Liquids*; Kirchner, B., Ed.; Springer, 2009; Vol. *290*. DOI: 10.1007/128_2008_31.
(25) MacFarlane, D. R.; Kar, M.; Pringle, J. M. *Fundamentals of Ionic Liquids - From Chemistry to Applications*; Wiley-VCH, 2017. DOI: 10.1007/978-94-007-7711-8_1.
(26) Correia, V. G.; Bento, A.; Pais, J.; Rodrigues, R.; Haliński, P.; Frydrych, M.; Greenhalgh, A.; Stepnowski, P.; Vollrath, F.; King, A. W. T.; Silva Pereira, C. The Molecular Structure and Multi-functionality of the Cryptic Plant Polymer Suberin. *Materials Today Bio* **2020**, *5*, No. 100039.
(27) Bento, A.; Moreira, C. J. S.; Correia, V. G.; Escórcio, R.; Rodrigues, R.; Tomé, A. S.; Geneix, N.; Petit, J.; Bakan, B.; Rothan, C.; Mykhaylyk, O. O.; Silva Pereira, C. Quantification of Structure-Property Relationships for Plant Polyesters Reveals Suberin and Cutin Idiosyncrasies. *ACS Sustainable Chem. Eng.* **2021**, *9* (47), 15780−15792.
(28) Moreira, C. J. S.; Bento, A.; Pais, J.; Petit, J.; Escórcio, R.; Correia, V. G.; Pinheiro, Â.; Haliński, Ł. P.; Mykhaylyk, O. O.; Rothan, C.; Silva Pereira, C. An Ionic Liquid Extraction That Preserves the Molecular Structure of Cutin Shown by Nuclear Magnetic Resonance. *Plant physiology* **2020**, *184* (2), 592−606.
(29) Hegedüs, K.; Fehér, C.; Jalsovszky, I.; Kristóf, Z.; Rohonczy, J.; Vass, E.; Farkas, A.; Csizmadia, T.; Friedbacher, G.; Hantz, P. Facile Isolation and Analysis of Sporopollenin Exine from Bee Pollen. *Sci. Rep.* **2021**, *11* (1), 9952.
(30) Palazzo, I.; Mezzetta, A.; Guazzelli, L.; Sartini, S.; Pomelli, C. S.; Parker, W. O.; Chiappe, C. Chiral Ionic Liquids Supported on Natural Sporopollenin Microcapsules. *RSC Adv.* **2018**, *8*, 21174−21183.
(31) Chiappe, C.; Rodriguez-Douton, M. J.; Mozzati, M. C.; Prete, D.; Griesi, A.; Guazzelli, L.; Gemmi, M.; Caporali, S.; Calisi, N.; Pomelli, C. S.; Rossella, F. Fe-Functionalized Paramagnetic Sporopollenin from Pollen Grains: One-Pot Synthesis Using Ionic Liquids. *Sci. Rep.* **2020**, *10*, 12005.
(32) Petkovic, M.; Ferguson, J. L.; Gunaratne, H. Q. N.; Ferreira, R.; Leitão, M. C.; Seddon, K. R.; Rebelo, L. P. N.; Pereira, C. S. Novel Biocompatible Cholinium-Based Ionic Liquids—Toxicity and Biodegradability. *Green Chem.* **2010**, *12* (4), 643.
(33) Meng, X.; Crestini, C.; Ben, H.; Hao, N.; Pu, Y.; Ragauskas, A. J.; Argyropoulos, D. S. Determination of Hydroxyl Groups in Biorefinery Resources via Quantitative 31P NMR Spectroscopy. *Nat. Protoc.* **2019**, *14* (9), 2627−2647.
(34) Ageitos, J. M.; Robla, S.; Valverde-Fraga, L.; Garcia-Fuentes, M.; Csaba, N. Purification of Hollow Sporopollenin Microcapsules from Sunflower and Chamomile Pollen Grains. *Polymers* **2021**, *13* (13), 2094.
(35) Hesse, M.; Waha, M. A New Look at the Acetolysis Method. *Plant Systematics and Evolution* **1989**, *163*, 147−152.
(36) Mundargi, R. C.; Potroz, M. G.; Park, J. H.; Seo, J.; Lee, J. H.; Cho, N.-J. Extraction of Sporopollenin Exine Capsules from Sunflower Pollen Grains. *RSC Adv.* **2016**, *6*, 16533−16539.
(37) Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. Dissolution of Cellose with Ionic Liquids. *J. Am. Chem. Soc.* **2002**, *124* (18), 4974−4975.
(38) Ferreira, R.; Garcia, H.; Sousa, A. F.; Guerreiro, M.; Duarte, F. J. S.; Freire, C. S. R.; Calhorda, M. J.; Silvestre, A. J. D.; Kunz, W.; Rebelo, L. P. N.; Silva Pereira, C. Unveiling the Dual Role of the Cholinium Hexanoate Ionic Liquid as Solvent and Catalyst in Suberin Depolymerisation. *RSC Adv.* **2014**, *4* (6), 2993−3002.
(39) Southworth, D. Solubility of Pollen Exines. *American Journal of Botany* **1974**, *61* (1), 36.
(40) Jungfermann, C.; Ahlers, F.; Grote, M.; Gubatz, S.; Steuernagel, S.; Thom, I.; Wetzels, G.; Wiermann, R. Solution of Sporopollenin and Reaggregation of a Sporopollenin-like Material: A New Approach in the Sporopollenin Research. *Journal of Plant Physiology* **1997**, *151*, 513−519.
(41) Jardine, P. E.; Abernethy, F. A. J.; Lomax, B. H.; Gosling, W. D.; Fraser, W. T. Shedding Light on Sporopollenin Chemistry, with Reference to UV Reconstructions. *Review of Palaeobotany and Palynology* **2017**, *238*, 1−6.
(42) Ahlers, F.; Lambert, J.; Wiermann, R. Structural Elements of Sporopollenin from the Pollen of Torreya Californica Torr. (Gymnospermae): Using the 1H-NMR Technique. *Zeitschrift für Naturforschung C* **1999**, *54* (7−8), 492−495.

https://doi.org/10.1021/acsomega.4c06524
ACS Omega 2025, 10, 411−421