Research paper

Changes in spore chemistry and appearance with increasing maturity

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ABSTRACT

Sporopollenin is the primary biopolymer found in the walls of pollen and spores; during maturation sporopollenin undergoes a number of discrete chemical changes, despite maintaining identifiable morphological features which can be exploited for palynological study. Here we report the results of heating experiments performed using *Lycopodium clavatum* spores designed to investigate the changes that occur within sporopollenin across a wide range of temperatures (0–350 °C) to simulate different degrees of maturation. Changes in sporopollenin functionality were assessed using Fourier transform infrared (FTIR) microspectroscopy. Our analyses show that the chemical structure of sporopollenin remains relatively stable over a wide range of simulated maturation conditions, until a threshold of 250–300 °C is reached, at which point a reorganisation of chemical structure begins. Comparison of these artificially matured spores with fossil material obtained from a Carboniferous-age section in the United Kingdom shows a strong chemical resemblance, suggesting that our experimental procedure accurately reflects the process of maturation and provides an insight into the chemical stability of sporopollenin in the geosphere.

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1. Introduction

The outer wall of spores and pollen is primarily constructed from the biopolymer sporopollenin, a chemically, physically and biologically inert biomacromolecule. The recalcitrant nature of sporopollenin is likely a key factor in the high preservation potential of palynomorphs, as observed in the geological record. This inert chemical nature provides a wealth of evidence on key palaeobotanical events in, for example, the timing and diversification of the earliest plants (Wellman et al., 2003), vascular plants (Steemans et al., 2009), and the diversification of early angiosperms (Lupia, 1999). Paradoxically it is the incredibly inert character of sporopollenin that has prevented a definitive chemical structure from being elucidated, with the exact structure and monomeric composition still debated. However, it may be that the structure of sporopollenin does yield to some chemical changes given sufficient time and suitable maturation conditions.

Recently a body of work has been produced establishing sporopollenin as an effective biogeochemical proxy for historical UV-B flux (Rozema et al., 1997, 1999, 2001a,b; Blokker et al., 2005; Watson et al., 2007; Lomax et al., 2008, 2012; Willis et al., 2011; Fraser et al., 2011, 2012). To date the longest sporopollenin-based proxy spans c. 5,000 years into the past (Willis et al., 2011). One potential limitation to the applicability of a sporopollenin-based proxy is its longevity in the geological record, with increasing maturity as a confounding factor, potentially altering the chemical functionality used to construct the proxy in the above work. Thus, it is of importance to establish the geological longevity of this geochemical proxy. The discovery of chemically unaltered, Carboniferous (Early Pennsylvanian, ~310 Ma) sporopollenin from the cave infill deposits within Central Quarry, Kendall County, Illinois, USA (Lat. N 41.47669, Long. W 88.43788 Plotnick et al., 2009) has already been achieved (Fraser et al., 2012); however, only in isolated individual specimens. The recognition of relatively unaltered palynomorphs and a fuller understanding of the fate of primary plant biochemistry following death and burial would open the door for reconstruction of deep-time UV-B flux.

The underlying basis of the UV-B proxy described by Rozema et al. (1997), Watson et al. (2007), Lomax et al. (2008, 2012) and Fraser et al. (2011) is the abundance of aromatic-based moieties within the structure of sporopollenin. However, the investigation of fossil spores and pollen has revealed that fossilised sporopollenin appears chemically very different to sporopollenin found in present day plants. Recent

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work by Steemans et al. (2010) has compared naturally occurring fossilised biopolymers from a number of sources using FTIR spectroscopy. The spectra obtained from fossilised sporopollenin differ from that of modern day *Lycopodium* spores reported in the literature (Watson et al., 2007; Lomax et al., 2008; Zimmerman, 2010; Fraser et al., 2011). Overall, fossil sporopollenin FTIR spectra appear to be less complex with fewer prominent peaks than modern counterparts. This simplification reflects a reduction in hydroxyl groups, loss of carbonyl groups, and a relative increase in aliphatic abundance (Watson et al., 2012). The disparity between modern and fossil material has hindered the elucidation of the geological fate of sporopollenin.

Differences between modern and fossil sporopollenin could be explained by a number of hypotheses: 1) Sporopollenin chemical composition is fundamentally different in modern flora compared with that of ancient flora, 2) More than one type of sporopollenin has existed in the past, and multiple forms may continue to exist today, or 3) Post-depositional and burial processes (broadly low-grade diagenesis through to catagenesis) may have altered sporopollenin over geological time.

The first hypothesis, related to the change in sporopollenin chemistry through time, is regarded as unlikely because there are many examples of ancient flora with modern-day analogues. To achieve a fundamental change in chemical composition through time in these plant groups it would be expected that other changes would also be manifest, e.g. in the form and structure of these plants. However, it is evident that within some groups of plants (i.e. 'lower plants') there have been only very limited changes through time. In addition, recent work by Fraser et al. (2012) has indicated that sporopollenin within Carboniferous-age, exquisitely preserved, megaspores from cave deposits in Illinois have a near-identical sporopollenin chemical composition to modern-day analogues.

The second hypothesis, related to multiple forms of sporopollenin is based on the comparison between extant and ancient sporopollenin composition (De Leeuw et al., 2006; Vandenbroucke and Largeau, 2007). However, difficulties in identifying if the chemical composition of the fossil sporopollenin has been altered after the production of the pollen grains mean that the argument eventually collapses to circularity.

The third hypothesis, that of low-grade maturity changes taking place, seems the most plausible as this process has been previously recognised in other types of organic matter (Gupta et al., 2007a,b,c). The testing of this third hypothesis is therefore the focus of the experiments presented in this manuscript.

Insight gained from our work presented here into the stability of sporopollenin chemistry within the geological record could help extend the applicability of the recently developed proxy for UV-B radiation, and could be applied to help answer a number of questions of a similar vein to those of Watson et al. (2007) and Lomax et al. (2008). The use of a geological scale UV-B proxy could act as a gateway to understanding past environments, where UV-B radiation varied in relation to the prevailing environmental conditions, such as shifts in vegetation patterns (Fraser et al., 2011), long-term surface elevation changes (Lomax et al., 2012) or globally significant stratospheric ozone depletion events (perhaps due to catastrophic volcanic eruptions) which have been linked to the end Permian extinction event (Visscher et al., 2004; Beerling et al., 2007; Svensen et al., 2009).

To test the validity of the diagenesis/catagenesis sporopollenin alteration hypothesis (hypothesis three above) we have subjected modern sporopollenin to artificial geological maturation processes using increasing temperature as an analogue for increasing maturity over geological time. Yule et al. (2000) attempted such an approach, but acknowledge that their experiments were conducted under oxygenated conditions, thus oxidation of samples during artificial maturation may have contributed to the observed chemical changes; geological samples will have been subject to elevated temperature and pressure under essentially anoxic conditions. Therefore, to model anoxic geological

conditions, such experiments should be conducted under non-oxidising conditions, i.e. in an inert gas atmosphere or under vacuum.

The work presented here reports on artificial maturation simulations by heating of *Lycopodium clavatum* spores throughout a range of temperatures. This included fresh unheated material through to spores subjected to temperatures of 350 °C under vacuum conditions. The primary objective of this work is to explore the manner in which sporopollenin changes when subjected to elevated temperature conditions commensurate with burial in geological environments. Palynological analysis is used widely in the oil and gas industry for assessing the hydrocarbon source potential of sedimentary rocks, and for evaluating thermal maturity (Hopping, 1967; Staplin, 1977; Lis et al., 2005), thus an understanding of how the chemical composition in visually identifiable palynomorphs changes will help improve maturation studies. Furthermore, this work will provide insight into the potential stability of sporopollenin chemistry for use in palaeoproxy work on reconstructing ultraviolet radiation in the past.

2. Materials and methods

2.1. Fossil samples

Fossil material used in the study was isolated from archive material collected from the Duckmanton Railway Cutting (53°13′44″N, 001°21′58″W), located 6 km East of Chesterfield, Derbyshire, UK. Specimens are late Early Pennsylvanian (~312 Ma Upper Bashkirian, equivalent to Westphalian B). Samples were processed following standard palynological techniques at the University of Sheffield (Traverse, 2007); however processing was halted prior to oxidation of the spore material to preserve the primary biogeochemical signature of the fossil sporopollenin. Macerated residues were then picked and isolated individual megaspores identified and collected for geochemical analysis. Palaeobotanical data presented in this study are from *Tuberculatisporites mamillarius*, which have been previously recovered in situ from sigillarian cones (e.g. Chaloner, 1967), consequently they are a member of the *Lycophyta* most closely related to extant *Isoetales*.

2.2. Experimental sample preparation and artificial maturation

Lycopodium clavatum spores (Sigma-Aldrich, UK) were soxhlet extracted for a period of 24 h in a dichloromethane:methanol mixture (97:3 v/v). Soxhlet extraction results in the removal of 'free' compounds, leaving the spore wall biopolymer for subsequent biogeochemical analysis (Watson et al., 2012). Clean 100 mm borosilicate tubes (6 mm o.d., 4 mm i.d.) were then loaded with ~14 mg of solvent-extracted spores and flame sealed under vacuum (~0.2 Pa) to ensure inert, non-oxidising conditions existed in the tubes. These tubes were then placed in an oven heated to the desired temperature and held at this temperature for a period of 48 h. Temperatures of 0, 100, 150, 200, 250, 300, and 350 °C were used to simulate varying intensities of maturity conditions from immature to thermally mature. After heating, the spores were solvent extracted, as described in Watson et al. (2012).

2.3. Light microscopy

Artificially matured spores were placed into distilled water and mounted on coverslips, and allowed to dry. Images were photographed on a Zeiss axiotope (sic) microscope using a Canon 50D EOS.

2.4. FTIR

All geochemical investigations reported here were performed using a Continuum IR-enabled microscope fitted with a 15× reflechromat objective lens and nitrogen-cooled MCT-A detector operating in transmission mode. This was interfaced with a Thermo Nicolet Nexus (Thermo Fisher Scientific, USA) bench unit to provide microscopic analysis of

spores. Analysis was conducted using a microscope aperture of 100×100 µm at 500 scans per sample; a background spectrum was collected and automatically subtracted from the sample spectrum after each individual analytical run. Analyses were replicated five times per sample. Sample material was dry-mounted on a NaCl IR-transparent disc. Background spectra were collected from an area of the disc that contained no spore sample.

Post-capture interrogation of FTIR spectra follows the method of Steemans et al. (2010). Briefly, the method of Steemans et al. (2010) is as follows. Relative abundances of the various functional groups represented within the spectra are compared across different samples by using ratios; this alleviates any complications arising due to the proportional nature of FTIR analysis, where a longer pathlength of IR beam through a sample increases the absorbance at a particular wavenumber. In particular, the following functional group ratios are used (Table 1): aliphatic (vasCHn, 2925 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}), carboxyl (vC=O, 1710 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}), aliphatic (vasCHn, 2925 cm^{-1})/carboxyl (vC=O, 1710 cm^{-1}), aliphatic (vasCHn, 2925 cm^{-1})/aromatic[b] (vC=C, 1500 cm^{-1}), and carboxyl (vC=O, 1700 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}). Here we exclude one ratio reported by Steemans et al. (2010); that of 2928/2854 cm^{-1}. We exclude this particular ratio from our analyses because it does not provide an estimate of aliphatic chain length, as suggested. By using ratios any relative shifts in abundance of the particular functional groups present within sporopollenin can be assessed.

3. Results and discussion

3.1. Light microscopy

Light microscopy (Plate 1) demonstrates a typical colour shift in artificially matured *Lycopodium*, as would be expected from the well-defined spore colour index (SCI), with increasingly mature samples showing colours through yellow (SCI 3), to orange (SCI 6), and dark brown (SCI 8) to black (SCI 9/10) at greatest maturity (Marshall, 1991; Marshall and Yule, 1999). A quantitative measure of spore colour has recently been developed by Goodhue and Clayton (2010); the Palynomorph Darkness Index (PDI%). Comparison of PDI% analysis of palynomorphs presented in Plate 1 with spore colour index values is shown in Fig. 1.

3.2. FTIR microspectroscopy

A strong absorbance band occurs centred at 3300 cm^{-1}, and decreases in intensity with increasing maturity in both modern and fossil spores (Fig. 2). A pair of peaks is found at 2925 cm^{-1} and 2850 cm^{-1}, which increase in apparent intensity with increasing maturation level. At mid-range maturity conditions a peak at 1710 cm^{-1} is well resolved, but appears only as a shoulder at low and high maturity levels. A peak occurring at 1610 cm^{-1} increases prominence with increasing temperatures. In the region of 1520–1510 cm^{-1} a sharp well defined peak can be seen to become less pronounced with elevated maturity levels. Throughout the maturity range the peak centred at 1450 cm^{-1} remains stable. Finally, in the region 1400–1000 cm^{-1}, detail becomes increasingly poorly resolved with increasing maturity (Fig. 2).

Table 1
Band assignment of observed absorbance bands present in FTIR spectra.

Wavenumber (cm^{-1})	Band assignment
2925	vasCH$_n$
2850	vsCH$_n$
1710	vC=O
1600	vC=C
1500	vC=C
1210	vC–O

3.3. Impact of artificial maturation on spore colour

The results from our light microscopy study confirm only minor changes in appearance, primarily colour, under milder maturation conditions (0–200 °C). The strongest colour changes are seen at higher experimental temperatures of >250 °C (Fig. 1), as is commonly seen in spore colour index analysis (Marshall, 1991). Further reassurance that interpretations are correct is provided by the correlation of Spore Colour Index with the recently developed Palynomorph Darkness Index (Goodhue and Clayton, 2010); both of which correspond well to our geochemical data (Figs. 1, 3). By combining our geochemical data with these colour change observations our data show that the chemical composition of sporopollenin remains stable across a broad range of temperatures, relating to a wide maturity window. Functional group breakdown is initiated at temperatures in excess of 250 °C when a significant colour change is also observed. The broad latitude of chemical stability under simulated maturation conditions suggests the possibility of applying a biochemical proxy to geological material for investigating past variations in UV-B flux.

3.4. Impact of artificial maturation on spore chemistry

The method presented by Steemans et al. (2010) for interrogating FTIR spectra is followed here in order to provide quantification of chemical changes across different samples. The broad absorbance band at 3000 cm^{-1} is due to hydroxyl (OH) groups, most likely hydroxyl groups that are involved in hydrogen bonding given the broad and wide nature of this band (Fig. 3). Absorbance at 2925 cm^{-1} and 2850 cm^{-1} are both due to stretching vibrations of CHn groups (vasCHn, and vsCHn, respectively). The sharp band present at 1740 cm^{-1} is due to C=O bonds, most likely relating to ester linkages within the primary structure of sporopollenin. The final bands of interest here occur in the regions 1605 cm^{-1} and 1520–1510 cm^{-1}; these are attributed to the presence of aromatic ring structures within sporopollenin, specifically as phenolic moieties contributing to the composition of sporopollenin. This interpretation is consistent with data presented previously by Rouxhet et al. (1980), Watson et al. (2007), Lomax et al. (2008), Steemans et al. (2010) and Zimmerman (2010).

Closer inspection of Fig. 3 reveals that the phenolic peak at 1510 cm^{-1} decreases in size, and shifts position to lower wavenumbers with increasing maturation conditions, becoming indistinct from the adjacent peak at lower wavenumbers once an experimental temperature of 300 °C is reached. In fact, a distinct change in peak character is observable from 200 to 250 °C. We interpret this to indicate a change in bonding pattern on the aromatic ring of the phenolic groups. The two identified phenolic components present in sporopollenin, ferulic acid and *para*-coumaric acid share similar bonding patterns, with 1,3,4 and 1,4 substitution around the ring structure, respectively. We suggest that at the highest maturation temperatures, a reorganisation around the ring structure is initiated, resulting in this shift in infrared absorbance band position. Such an interpretation is consistent with the observations of other studies (Lis et al., 2005; Gupta et al., 2007a,b,c; Watson et al., 2012) where clear defunctionalisation and repolymerisation occurs under diagenetic conditions, resulting in an altered chemical structure. However, at lower experimental temperatures (<200 °C), i.e. lower maturity, such changes do not appear to occur, with the aromatic components remaining unaltered.

Both indicators of aliphatic to aromatic balance (i.e. 2925/1600 cm^{-1} and 2925/1500 cm^{-1}) show a similar trend, with a reduction in ratio value to 200 °C, favouring an interpretation of apparent reduced aliphatic and/or increased aromatic contribution with increasing maturity. Comparison of aliphatic and aromatic ratios against carbonyl groups within ester linkages suggests a slight increase in aromatic components above 250 °C, however, aliphatic components remain fairly stable across the maturation range in relation to ester linkages (Fig. 3). The C–O/phenolic (1210/1600 cm^{-1}) ratio is stable across the range of maturation
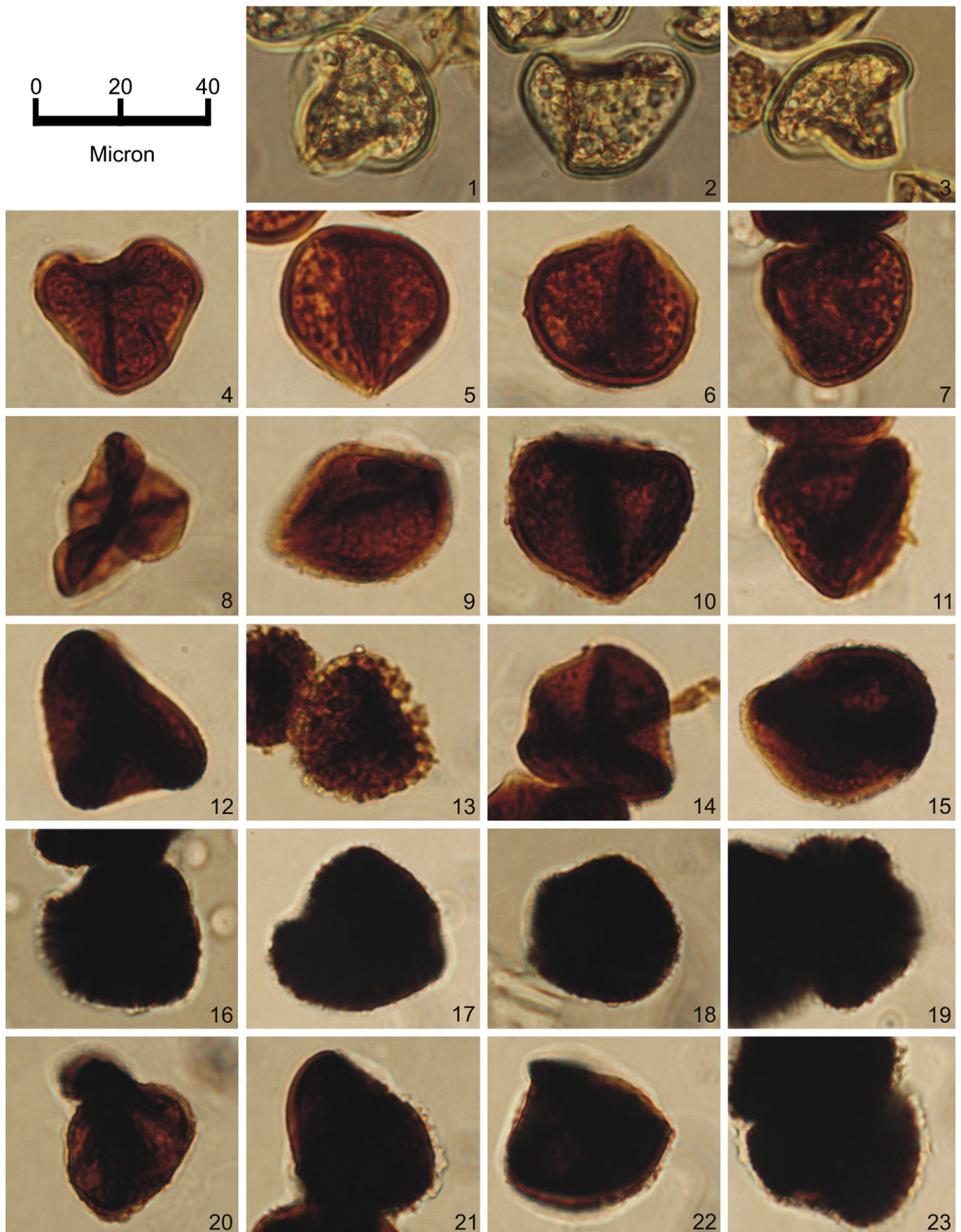
Plate 1. Spores of *Lycopodium* showing differences in colour with increasing artificial thermal maturation taken at ×500 magnification. 1–3) heated to 100 °C, 4–7) treated at 150 °C, 8–11) treated to 200 °C, 12–15) treated at 250 °C, 16–19) treated at 300 °C, and 20–23) treated at 350 °C.
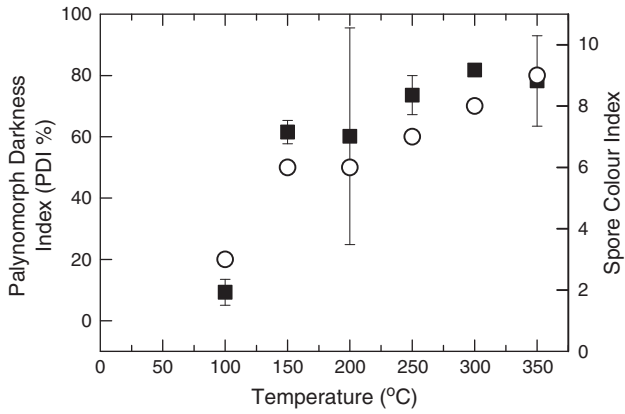
Fig. 1. Assessment of Pollen Darkness Index and correlation with Spore Colour Index of palynomorphs presented in Plate 1. Palynomorph Darkness Index, open circles; Spore Colour Index, solid squares.

temperatures, suggesting that neither phenolic nor aromatic groups vary significantly in relation to each other.

3.5. Comparison with fossilised spore chemistry

Comparison of spectra collected from the higher temperature artificially matured spores under laboratory conditions with naturally fossilised *Lycophyta* megaspores from the Duckmanton (Carboniferous) samples demonstrates strong similarities in their chemical composition using FTIR microspectroscopy (Fig. 2). Such close similarity suggests that the end point in the maturation process is similar, if not identical. This finding is remarkable considering that the naturally fossilised material experienced maturation within an open system, as opposed to the artificially matured spores that underwent heating in a closed system. Thus it can be proposed that, as the phenolic-based signal remains stable across much of the maturity range in our laboratory experiments, it is also parsimonious to suggest that the phenolic signal locked within natural fossil material will also have remained stable under equivalent maturity conditions.
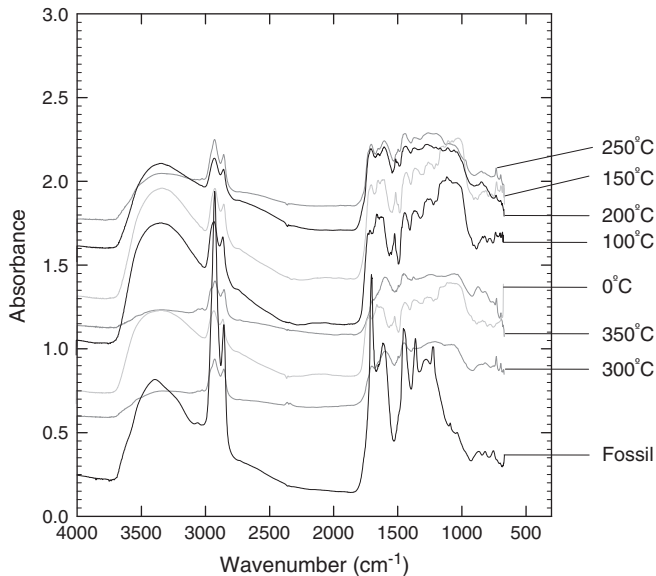

Fig. 2. FTIR spectra of Fossil Lycopsid (*Blogiga bloggsis*) and artificially matured extant *Lycopodium clavatum* spores. Black line, fossil material.
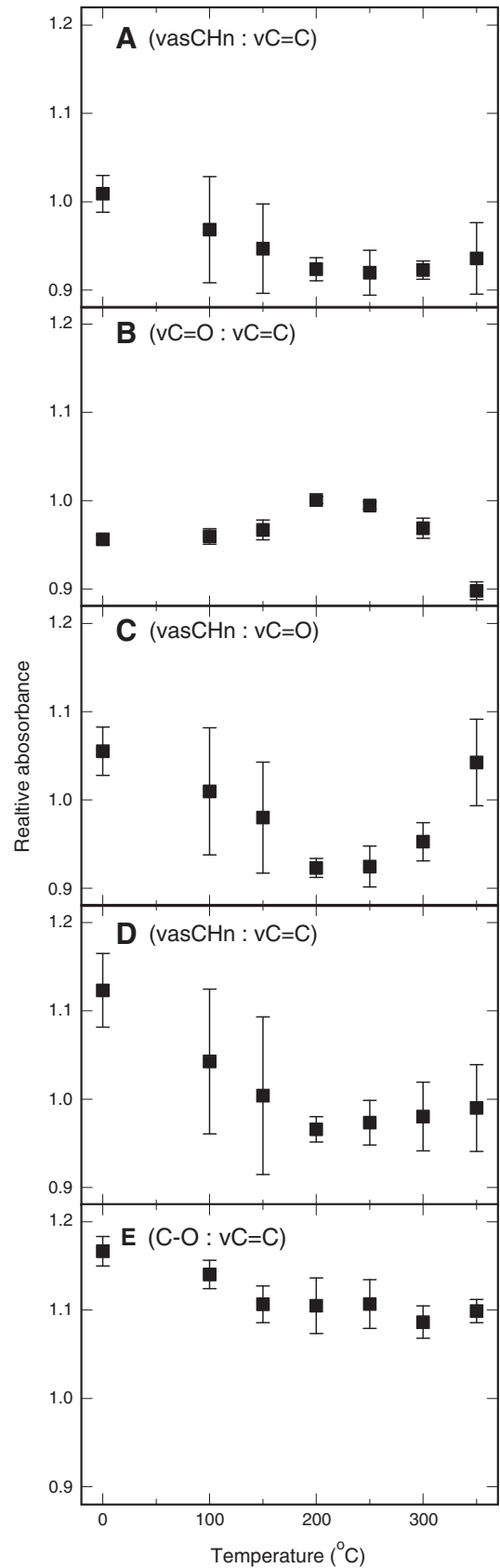

Fig. 3. Functional chemistry ratio plotted against maturation temperature. A) aliphatic (vasCHn, 2925 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}), B) carboxyl (vC=O, 1710 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}), C) aliphatic (vasCHn, 2925 cm^{-1})/carboxyl (vC=O, 1710 cm^{-1}), D) aliphatic (vasCHn, 2925 cm^{-1})/aromatic[b] (vC=C, 1500 cm^{-1}), E) phenolic (vC–O, 1210 cm^{-1})/aromatic[a] (vC=C, 1600 cm^{-1}). Error bars are 1σ.

4. Conclusions

From the evidence presented here it is shown that sporopollenin chemistry remains relatively stable across the lower half of a range of simulated diagenetic conditions, only beginning to alter substantially at higher temperatures, i.e. above 200 °C, approximating to greater maturation. This new chemical information provides evidence of an underlying chemical basis for the preservation of spores and pollen in the geological record. Further to this, such broad apparent chemical stability lends weight to the notion that chemical analyses combined with palynological study may extend the applicability of a new palaeoclimatic proxy (Watson et al., 2007; Lomax et al., 2008, 2012) to deeper geological time.

Acknowledgements

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