Rapid determination of spore chemistry using thermochemolysis gas chromatography-mass spectrometry and micro-Fourier transform infrared spectroscopy

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Spore chemistry is at the centre of investigations aimed at producing a proxy record of harmful ultraviolet radiation (UV-B) through time. A biochemical proxy is essential owing to an absence of long-term (century or more) instrumental records. Spore cell material contains UV-B absorbing compounds that appear to be synthesised in variable amounts dependent on the ambient UV-B flux. To facilitate these investigations we have developed a rapid method for detecting variations in spore chemistry using combined thermochemolysis gas chromatography-mass spectrometry and micro-Fourier transform infrared spectroscopy. Our method was tested using spores obtained from five populations of the tropical lycopsid *Lycopodium cernuum* growing across an altitudinal gradient (650–1981 m a.s.l.) in S.E. Asia with the assumption that they experienced a range of UV-B radiation doses. Thermochemolysis and subsequent pyrolysis liberated UV-B pigments (ferulic and *para*-coumaric acid) from the spores. All of the aromatic compounds liberated from spores by thermochemolysis and pyrolysis were active in UV-B protection. The various functional groups associated with UV-B protecting pigments were rapidly detected by micro-FTIR and included the aromatic C=C absorption band which was exclusive to the pigments. We show increases in micro-FTIR aromatic absorption (1510 cm^{-1}) with altitude that may reflect a chemical response to higher UV-B flux. Our results indicate that rapid chemical analyses of historical spore samples could provide a record ideally suited to investigations of a proxy for stratospheric O_3 layer variability and UV-B flux over historical (century to millennia) timescales.

Introduction

A spore is a reproductive cell produced by plants, the walls of which contain a resistant biopolymer termed sporopollenin. Geochemical analyses of sporopollenin has revealed that it is composed of fatty acid units and simple phenolics, such as ferulic and *para*-coumaric acid (Fig. 1). Current scientific interest in the constitution of sporopollenin has been enhanced by proposals that the phenolic moieties, which absorb harmful ultraviolet radiation in the 280–315 nm wavelength (UV-B) range, are screening compounds synthesized in variable amounts as part of an adaptive biochemical response to the ambient UV environment.[1,2] Sporopollenin chemistry may therefore be used as a proxy for stratospheric ozone levels and their attenuating effect on solar UV-B flux, although some plants do appear well adapted to ambient levels of UV-B and exhibit no chemical or morphological change.[3,4]

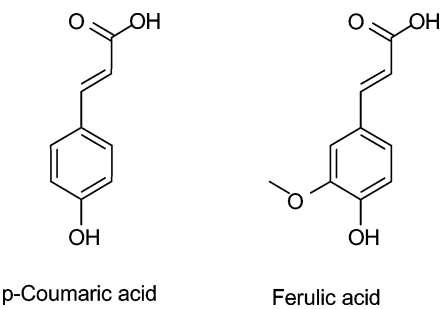

Fig. 1 The UV-B absorbing compounds *para*-coumaric and ferulic acid.

Scientific preoccupation with the recent history of the ozone (O_3) layer is understandable given the recognition in the mid-1980s that man-made halocarbons were causing harmful springtime stratospheric ozone depletion.[5] However, instrumental records of stratospheric ozone only extend back to 1957, whilst those of solar UV-B go back to 1970.[1] Placing modern-day instrumental records in a historical context requires the development of robust proxies for past levels of near-surface UV-B radiation.

It is in this context that the responses of plants to increasing near-surface solar fluxes of UV-B have been investigated.[1,2,6] Plants exposed to increased UV-B radiation typically experience a number of detrimental effects, including damage to proteins, membrane lipids and DNA.[7] To reduce this damage,

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many plants, animals and microbes accumulate UV-B protecting pigments,[1,2,6,8–10] in addition to affecting photorepair and excision repair to damaged DNA.[11]

Of particular interest is the recent observation that a number of herbaceous and woody plants grown in controlled environments under enhanced levels of UV-B radiation, simulating a 10 to 50% ozone depletion, accumulated great quantities of UV-B absorbing pigments in the protective walls of pollen and spores.[1,2] A parallel response has also been seen in the concentration of UV-B protecting compounds of terrestrial mosses from Antarctica between 1957 and 1989.[6]

The first step in providing a method that can access any proxy information in spores is to determine the chemical constitution of sporopollenin. This can be achieved using a combination of thermal and chemical degradation techniques. Such methods reveal both the structural characteristics of UV-B pigments and the nature of how they are bound into the organic network. Thermally assisted hydrolysis and methylation using tetramethylammonium hydroxide (TMAH) results in the breaking of ether and ester bonds concomitant with methylation to ensure the analytical amenability of the products.[12] Temperatures employed (~300 °C) are below those which would break carbon–carbon bonds and therefore only oxygen-bound compounds are released. The individual chemical units which perform UV-absorption retain the majority of their structural integrity facilitating identification. The presence of UV-absorbing compounds not completely liberated from the sporopollenin by thermochemolysis can be subsequently released by pyrolysis at temperatures above 600 °C. Recently published data on pollen, imply that similar methods have a detection limit of approximately 60 fresh pollen grains and standard deviations of around 10% for between to 100 and 600 pollen.[13]

The second step in developing an effective protocol for characterizing spore chemistry is the rapid generation of information on chemical variation. Short analysis times allow large numbers of samples to be processed and effective statistical interrogation of the data to be performed. Fortunately, the UV-B absorbing compounds in sporopollenin contain characteristic chemical groups such as aromatic, olefinic and oxygen-containing functionalities. Once the main structural components are verified by thermochemolysis the rapid *in situ* detection of characteristic functional groups representing UV-absorbing pigments, can be achieved with micro-Fourier transform infrared (micro-FTIR) spectroscopy. Natural biopolymers differ in both the number and intensity of bands, which correspond to certain chemical groups. By internally normalising the various bands a semi-quantitative analysis of functional group content is provided, variations of which may be hypothesized to reflect responses to different UV-B levels.

Here, we test the efficacy of combined thermochemolysis–pyrolysis and micro-FTIR for rapid detection of UV-induced effects on plant protective pigments by taking advantage of natural gradients in terrestrial UV-B environments. UV-B radiation is variable around the globe and is highest where radiation travels the least distance through the atmosphere, such as in the tropics and at high altitudes. For instance, measurements of North American sites at different altitudes and latitudes by the United States Department of Agriculture revealed an approximate 15% increase in UV-B with 1000 m altitude and a 15% decrease with 7° latitude.[14] Therefore we evaluated the potential of spore chemistry to provide a proxy for UV-B flux by analysing *Lycopodium cernuum* spores collected from plants growing across a range of altitudes (650–1981 m a.s.l.) on tropical mountains[15] and which are likely to have experienced different UV-B levels during growth.

Experimental

Spore samples

Spore samples from five populations of the tropical clubmoss *L. cernuum* were obtained from sites in S.E. Asia spanning an altitudinal range of 650 to 1981 m a.s.l (Table 1, ref. 15). All samples were accessed from collections stored at the Natural History Museum, London.

Thermochemolysis and pyrolysis

To liberate UV-B absorbing units from the samples, spores (typically 2–4 for each altitude) were placed in quartz pyrolysis tubes, which were plugged with quartz wool at each end. 10 µl of 25% tetramethylammonium hydroxide in methanol was added to the spores and the methanol allowed to evaporate for 12 h. Samples were heated (300 °C at a rate of 20 °C ms^{-1} then held isothermally for 15 s) in a flow of helium using a CDS Pyroprobe 1000 fitted with a 1500 valve interface (CDS Analytical, Oxford, PA) and coupled to a gas chromatograph-mass spectrometer (GC-MS). After thermochemolysis samples were subsequently pyrolysed at 610 °C (all other conditions as for thermochemolysis).

GC-MS analysis was carried out using an Agilent Technology 6890 gas chromatograph coupled to a 5973 mass spectrometer. Separation was performed on a S.G.E. (U.K.) BPX-5 column (30 m length, 0.25 mm internal diameter and 25 µm film thickness). Helium at column flow rate of 1.1 ml min^{-1} was used as the carrier gas. Injection was splitless and injector temperature was 250 °C. The GC oven temperature was held for 15 min at 30 °C and then programmed at 4 °C min^{-1} to 300 °C, the final temperature was held for 9 min. Identification was based on authentic standards and published data.

Micro-Fourier transform infrared spectroscopy

To determine the functional groups present in the spores, the samples were analysed by transmission micro-FTIR spectroscopy. The spores were mechanically compressed and placed on a sodium chloride disc. FTIR analyses were carried out using a Thermo Nicolet Nexus FTIR spectrometer coupled with a continuum IR microscope. Operation conditions were standard EverGlo mid-infrared source optics, Ge-on-KBr beamsplitter and a MCT-A* detector (11700–750 cm^{-1}). Spectra were obtained in the mid-infrared (4000–400 cm^{-1}) region using an aperture size of 75 × 100 µm. A 15× infrared objective was used. 500 scans were accumulated for each spectrum, with a spectral resolution of 4 cm^{-1}. The instrument was not purged during data acquisition and therefore an absorbance band at 2350 cm^{-1} due to atmospheric carbon dioxide was removed during data analysis. Bands were assigned after the published work of Williams and Fleming[16] and Rouxhet *et al.*[17] Initial assignments were confirmed by direct comparison of sample data with that in the Sigma biological sample library and the Georgia state crime lab sample library databases accessed *via* the Thermo Nicolet Omnic software. For

Table 1 Localities, ratios of UV-active to UV-inactive compounds from GC-MS data and ratios of FTIR bands corresponding to aromatic and unsaturated (aromatic and olefinic) bonds relative to OH band for *Lycopodium cernuum* samples analysed

Sample	Year collected	Altitude/m	Latitude/°	Locality	Ferulic acid/*n*-$C_{18:0}$	*p*-Coumaric acid/*n*-$C_{18:0}$	*p*-Coumaric acid + ferulic acid/*n*-$C_{18:0}$	Aromatic (1510 cm^{-1})	Aromatic or olefinic (829 cm^{-1})
LC1	1965	1981	3	Asia, Malaya	0.05	1.4	1.5	0.233	0.107
LC2	1962	1950	4	Asia, New Guinea	0.20	2.7	3.0	0.348	0.167
LC3	1976	1400	–9	Asia, Mt. Kilkerran	0.18	0.6	0.8	0.327	0.130
LC4	1943	762	4	Asia, Papua New Guinea	0.28	2.2	2.5	0.181	0.099
LC5	1981	650	16	Asia, E. Tongoa	0.08	1.2	1.2	0.152	0.051

tabulated data, band heights were measured relative to a straight baseline using the Omnic software. Aromatic (1510 cm^{-1}) and olefinic (829 cm^{-1}) band heights were normalised to the OH peak (3350 cm^{-1}) which was chosen for normalisation owing to its presence in each sample and stability resulting from its large band area. Heights were measured between three and six times and the mean value quoted. Standard deviations on the measurements were less than 0.09.

Results

Thermochemolysis and pyrolysis

The main products liberated from thermochemolysis of *L. cernuum* pollen spores were *n*-acids (saturated and unsaturated), α, ω-diacids and benzoic acid derivatives (Fig. 2 and 3). The saturated *n*-acids range from C_8 to C_{22}, whereas only C_{16} and C_{18} unsaturated acids are present (including polyunsaturated *n*-C_{18} acids).

Subsequent pyrolysis of the spores after thermochemolysis demonstrated that not all UV-B sensitive material was liberated during thermochemolysis. Both *p*-coumaric and ferulic acid were detectable in addition to the associated saturated acids (Fig. 2 and 3). Other compounds liberated by pyrolysis include *n*-alkanoic acids and minor amounts of *n*-alkenes/alkanes.

Micro-Fourier transform infrared spectroscopy

Micro-FTIR spectra of the spores (Fig. 4) showed significant –OH (3350 cm^{-1}) and aliphatic (2800–3000 cm^{-1}) bands. Between 1650 and 1750 cm^{-1}, there are bands due to esters (1740 cm^{-1}), carboxylic acids (1700 cm^{-1}) and ketones (1680 cm^{-1}). In this region, there may also be contributions from olefinic and aromatic functional groups and molecular water. The band at 1510 cm^{-1} most likely reflects single aromatic rings which is a characteristic feature of sporopollenin, peat and lignite.[17] Amides are a less likely source for this band as they occur at higher wavenumbers (1540 cm^{-1}). The presence of benzoic acid derivatives and the lack of dominant N-bearing compounds released by thermochemolysis (Fig. 2 and 3) supports the assignment of the 1510 cm^{-1} micro-FTIR band to single aromatic rings. *n*-$C_{16:0}$, an *n*-$C_{18:1}$ and 4-hydroxyhydrocinnamic acid are the most abundant compounds. C–H deformations occur at 1440 cm^{-1} and C–O stretches dominate the region 1050–1300 cm^{-1}. The distinct band at 829 cm^{-1} is due to either aromatic or olefinic groups. Comparisons between unextracted and acetone extracted spores showed no difference in micro-FTIR response.

Discussion

Chemical structure of spore biopolymers

Compounds released upon thermochemolysis (Fig. 2 and 3) are only partially consistent with the literature on sporopollenin structure which suggests a lignin-like (phenylpropanoid) biopolymer (*e.g.* ref. 18). From the data presented here the biopolymer is an alkanoic polyester with benzoic and alkanoic acid monomers which would appear more related to the structure of suberin than that of lignin. The FTIR ester absorption band at 1740 cm^{-1} supports this interpretation (Fig. 4). The UV-B absorbing ferulic

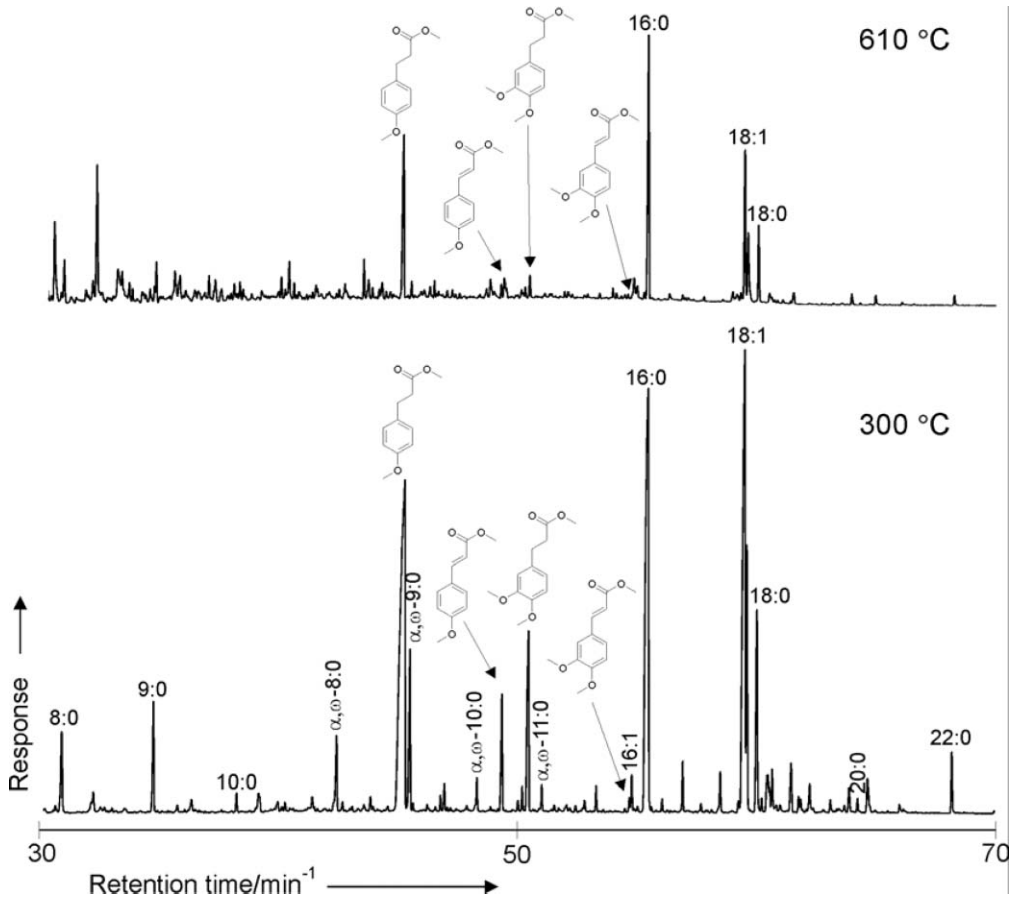

Fig. 2 Total ion chromatograms of products from a typical *Lycopodium cernuum* sample liberated at (a) 300 °C in the presence of TMAH and (b) subsequent pyrolysis at 610 °C.

and *p*-coumaric acids are abundant in the tropical *L. cernuum* spores and are the main aromatic components liberated.

FTIR spectra of the spores reveal bands are generally consistent with previously published data,[19] *i.e.* strong responses from –OH, aliphatic, carbonyl and ether containing units. Responses due to carbon–carbon double bonds are also commonly observed in sporopollenin, yet the intensity of the single aromatic ring absorption in our tropical spore samples is remarkable. Distinct bands attributed to single aromatic rings (1540 cm^{-1}) and esters (1740 cm^{-1}) are absent in data for thermally-mature spores (*e.g.* ref. 19).

When considered together, the FTIR and thermochemolysis–pyrolysis data can be interpreted in the context of high UV-B fluxes associated with tropical mountains from which the samples were collected. Because thermochemolysis–pyrolysis of spores revealed that *p*-coumaric acid and ferulic acid were the main aromatic compounds present, the increased FTIR aromatic absorption must reflect an increase in UV-B pigment content of the spores. This suggestion is consistent with the idea that *L. cernuum* experienced increased exposure UV-B radiation with altitude.

The correlation between thermochemolysis–pyrolysis data and FTIR data has operational benefits for UV-B pigment studies because the latter is acquired much more rapidly (*ca.* 15 min run^{-1}) than the former (*ca.* 2 h run^{-1}). Although, there are obvious limits to the ability of FTIR to track variations in UV-B pigments. For instance, Blokker *et al.*[20] recognised that relative abundance of *p*-coumaric acid and ferrulic acid changes in response to UV light, such variations may be difficult to track by FTIR.

Chemical changes due to altitude and UV-B flux

Thermochemolysis analysis of the *L. cernuum* spores (Fig. 3) showed no clear trend of increased concentrations of UV-B active compounds (ferulic and *p*-coumaric acids) relative to a UV-B inactive compound (*n*-$C_{18:0}$ fatty acid). One possible explanation for this observation is that the two step thermochemolysis–pyrolysis method has disturbed the natural ratio of UV active compounds relative to UV inactive compounds present in the whole spore. UV-B sensitive compounds are present in both thermochemolysis and pyrolysis products and incomplete extraction during the lower temperature thermochemolysis step may be masking any overall trends.

Micro-FTIR is not susceptible to variable extraction efficiencies and simply detects functional groups characteristic of UV-B sensitive compounds throughout the spore organic matter. When normalised to OH there is a general increase in the aromatic band (1510 cm^{-1}) with altitude (Table 1, Fig. 4). This increase is accompanied and confirmed by a corresponding increase in the olefinic or aromatic band (829 cm^{-1}). The relatively closely spaced higher samples deviate from a perfect correlation but taken

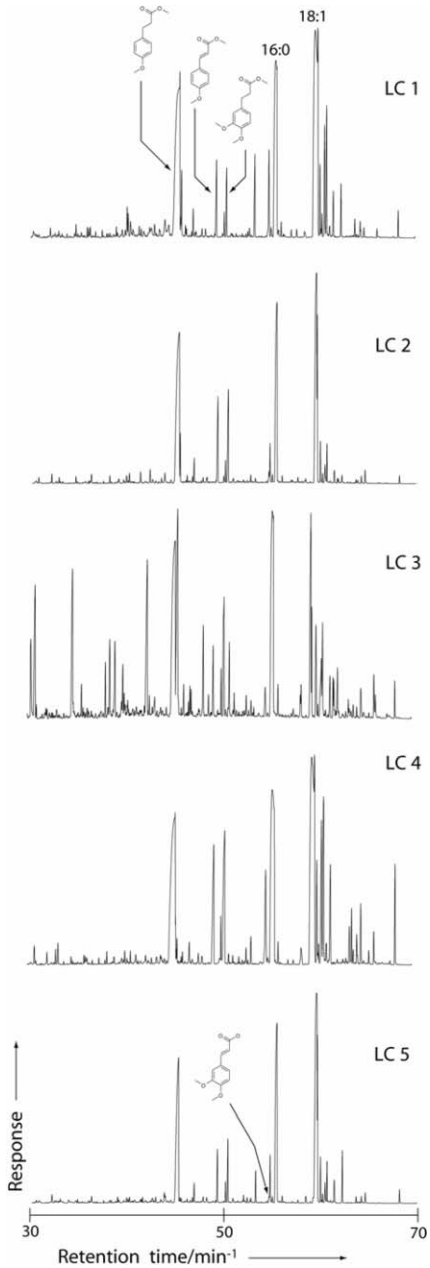

Fig. 3 Total ion chromatograms of products from *Lycopodium cernuum* at 300 °C in the presence of TMAH, sample codes given in Table 1.

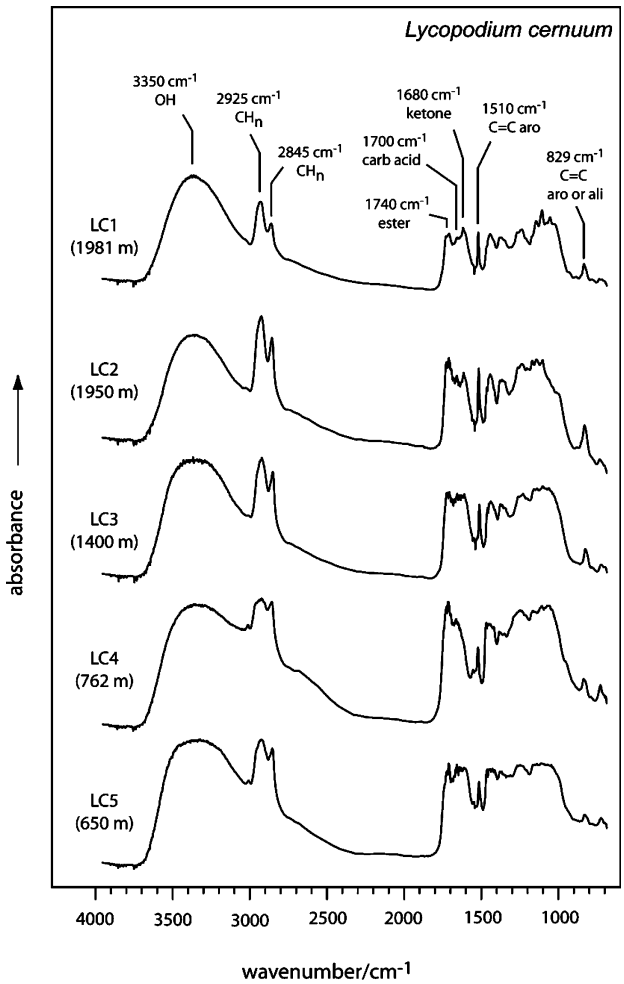

Fig. 4 Micro-FTIR spectra of *Lycopodium cernuum* spores from different altitudes on tropical mountains. The increase in aromaticity with altitude, reflected by the band at 1510 cm^{-1} and in part by the band at 829 cm^{-1}, appears to reflect greater amounts of *para*-coumaric acid and ferulic acid pigments in response to greater UV-B flux.

together are still significantly enriched in pigment relative to the lowest samples. The nature of the increase in aromatic response with altitude makes it unlikely that the data are reflecting random factors such as variations in cloud cover (*e.g.* ref. 21). The micro-FTIR data therefore appear to reflect a genuine increase in pigment concentration with altitude and UV-B flux.

Conclusion

Overall, our results demonstrate the clear potential of combined thermochemolysis GC-MS and micro-FTIR analyses of spores to provide a means for rapidly discerning changes in chemistry that may be related to variations in the stratospheric ozone layer and associated terrestrial UV-B fluxes. In particular, the highly recalcitrant nature of the spore biopolymer sporopollenin[22] means that proxy information may well be preserved intact for thousands of years which suggests the enticing possibility of utilizing our method to develop a proxy record of near-surface UV-B fluxes back into the Quaternary fossil record and beyond.

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