Photo. Above: Fossil leaf of *Ginkgo huttoni* mid Jurassic (age 150 million years). Collection: Dr Simon Troelstra, FALW, Vrije Universiteit, Amsterdam; Below: Present-day *Ginkgo biloba* (Photograph: Jelte Rozema).

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The occurrence of *p*-coumaric acid and ferulic acid in fossil plant materials and their use as UV-proxy

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Abstract

The applicability of *p*-coumaric acid and ferulic acid concentrations or ratios in (sub)fossil plant remnant as UV-B proxies relies on various aspects, which are discussed in this paper and will be illustrated with some experimental data. A newly developed THM-micropyrolysis–gas chromatography–mass spectrometry method was tested on various spores, pollen and other plant remains, which were analysed for the presence of the UV-absorbing compounds *p*-coumaric acid and ferulic acid. This revealed that these supposed building-blocks of sporopollenin appear to be present in pollen of many plant species but also in moss spores. The development of this micropyrolysis method paved the way for the quantitative analysis of UV-absorbing compounds in case only a small amount of analyte is available, for example for fossil pollen and spores but also other small palynomorphs and plant fossils. The use of this technique will provide a better insight in the plant responses to UV-radiation, the chemistry of pollen and spores, their fossil counterparts and furthermore the means for a further development of a proxy for the reconstruction of past UV-B radiation.

Abbreviations: *p*CA – *p*-coumaric acid; DHP – dehydrogenation polymer; FA – ferulic acid; Fame – fatty acid methyl ester; FTIR – Fourier transform infrared; GC/MS – gas chromatography mass spectrometry; LOD – limit of detection; NMR – nuclear magnetic resonance; py – pyrolysis; SIM – selective ion monitoring; THM – thermally assisted hydrolysis and methylation; TIC – total ion current; TMAH – tetramethyl ammonium hydroxide; UV – ultraviolet

Introduction

In contrast to for example the reconstruction of past atmospheric carbon dioxide concentrations, which has been possible due to the presence of ancient air included in ice cores (Petit et al. 1999), a historic perspective on the natural fluctuations of stratospheric ozone and surface UV-B is virtually absent before 1920 when the first instrumental monitoring started at Arosa (Switzerland) (Staehelin et al. 2002). The formation of a hole in the Antarctic ozone layer in 1974 was detected using a Dobson spectrometer at the Halley Research Station at the Antarctic (Farman et al. 1985).

It is commonly known that a decrease in stratospheric ozone will result in an increase in solar UV-B radiation in the lower atmosphere. However, changes in solar intensity also affect the

UV-B radiation reaching the earth's surface. At solar highs UV-B only slightly increases in contrast to UV-C, which leads to an increase in the amount of ozone in the stratosphere resulting in an anticorrelated solar activity/UV-B signal on the earth's surface (Rozema et al. 2002b).

It is well documented that plants have active defenses against UV radiation (e.g. Bornman 1991; Huang et al. 1997; Krauss et al. 1997; Rozema et al. 1997; Gehrke 1998; Laakso and Huttunen 1998; Olsson et al. 1998; Meijkamp et al. 1999; Meijkamp et al. 2001; Rozema et al. 2001; Sinha et al. 2001; Day and Neale 2002; Rozema et al. 2002a; Rozema et al. 2002b; Sasaki and Takahashi 2002; Feng et al. 2003; Sullivan et al. 2003; Chicaro et al. 2004). Compounds biosynthesised via the phenylpropanoid pathway such as flavonoids, *p*CA and FA are able to shield harmful radiation and are shown to increase in some of the experiments where higher doses of UV-B were applied to the plants (for a recent overview; Rozema et al. 2005). However, it was also shown that not all plants respond similarly to increased UV-B (Rozema et al. 2005). As a consequence, the use of flavonoids or phenols as a UV proxy is not feasible unless it is possible to link concentrations of these compounds to a specific plant. It was recently suggested to use fossil pollen and spores for that purpose (Rozema et al. 2001; Rozema et al. 2002b). Given that these palynomorphs are able to survive millions of years of burial, even dating back to the Ordovician (500 mya) (Edwards 2001) without major morphological or chemical changes, identification on a genus or sometimes even species-level is possible.

It seems plausible that as a consequence of their small size and potential high exposure to solar radiation upon transport from the anthers to the stigma, the parental plant would protect pollen and spores against UV radiation. Since the main protective tissue that composes the exine of pollen and spores, the so-called sporopollenin, remains unaltered upon diagenesis, it was hypothesised that the fossil remains of pollen and spores possibly form a record of past UV-radiation (Rozema et al. 2001). Even so it might be possible to use other fossil plant materials such as cuticles and seed or fruit coats and bud or catkin scales for extracting information about past UV regimes, provided that these plant remnants are diagenetically stable.

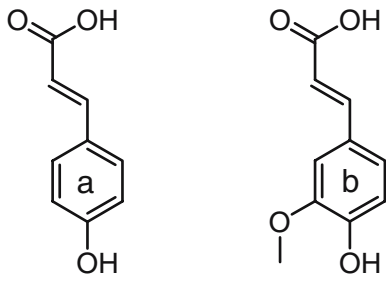

Figure 1. The chemical structure of (a) *p*-coumaric acid; (b) ferulic acid.

The use of chemical information for the reconstruction of past UV-B radiation is hampered by the inconclusive reports on the diagenesis resistant materials composing fossil plant materials, such as sporopollenin. Moreover, the analysis of fossil materials is difficult due to the often low amount of sample material and the lack of analytical instrumentation that can handle these small amounts of material in a reproducible way. This paper describes the potential of using sporopollenin, or more specific, the alleged building-blocks *p*CA and FA as UV-proxies (Figure 1).

Functions of pCA and FA in plants

An important attribute of *p*CA and FA is their function as molecular bridges between lignin and carbohydrates and as cross-linkers between carbohydrates providing rigidity to primary cell walls (Iiyama et al. 1990; Kroon and Williamson 1999; Carnachan and Harris 2000; Lam et al. 2001). Ralph et al. (1992) suggested that these linkages were formed via a catalysed radical reaction by peroxidase comparable to that of lignin formation (Freudenberg and Neish 1968). Although Lam et al. (2001) suggested that such a radical mechanism is possibly unlikely as a consequence of their findings that *p*CA and FA are linked at the benzyl position of a lignin polymer, Boom (2004) shows that a peroxidase/H_2O_2 polymerised DHP of *p*CA can be prepared via a procedure described for artificial lignin (e.g. Hatcher and Minard 1996).

Next to a cross-link function between lignin and carbohydrates, *p*CA and FA are important constituents of unlignified gymnosperm primary cell walls (for an overview of various species Carnachan and Harris (2000)). Comparable to the

lignin–carbohydrate bridges, the carboxylic acid group of *p*CA and FA are ester-linked to the hydroxyl groups of carbohydrates. Cross-linking of cell-wall polysaccharides by diferulate was demonstrated to make the primary cell walls in Poaceae more rigid (Tan et al. 1991) suggesting that similar effects may occur in Gymnosperms (Carnachan and Harris 2000). This rigidity may impede their degradation by fungal carbohydrases, thus providing a defence against pathogenic microorganisms.

Pathogenic defence by bound phenolics may not only have its origin in a physical barrier, but may also impair microbial growth by the release of antibiotic phenolics due to the microbial infestation itself (Liakopoulos et al. 2001). The presence of *p*CA esters in the roots of *Tanacetum longifolium* (Asteraceae) might serve a similar function, though (Mahmood et al. 2003) describes these compounds as being plant growth inhibitors. The occurrence of phenolics in the epicuticular layer covering the guard cells of stomata may be related to a defence against biotic and abiotic factors (Kolattukudy 1981; Holloway 1982). Since stomata provide an entry to the leaf interior for pathogens it seems liable that defence metabolites will be deposited in the surface waxes covering the guard cells. Especially *p*CA and FA are effective phenolic components active in the defence against pathogens (Nicholson and Hammerschmidt 1992; Bennett and Wallsgrove 1994).

Despite some controversy, it is widely accepted that both the epicuticular waxes and the cuticular membrane may form a barrier against the penetration of UV into the mesophyll (Barnes and Cardoso-Vilhena 1996). A number of studies revealed that cuticles isolated from various leaves and fruits exhibited a strong absorption in the UV-range (Krauss et al. 1997). UV absorbance of the cuticle seems to be species specific and may differ between the adaxial and abaxial leaf surfaces of the same plant (Bornman and Vogelmann 1988; Robinson et al. 1993; Liakopoulos et al. 2001). *p*CA and FA have been demonstrated to be important constituent of *Prunus persica* and *Olea europaea* leaf waxes. The actual chemical nature of the intermolecular linkages involved in the binding of *p*CA and FA to the leaf waxes are still unknown, though the fact that both compounds are only released by saponification suggests that either the carboxylic acid or the hydroxyl group is involved. Since it was shown that especially the former group is involved in linkages to carbohydrates (Carnachan and Harris 2000) and fatty alcohols (Mahmood et al. 2003) this suggests that most likely *p*CA and FA are linked to the hydroxyl groups of cutin (the solvent insoluble biopolymer in plant cuticles (Tegelaar et al. 1991))-like monomers (see also; Mösle et al. 1997; Mösle et al. 1998; van Bergen et al. 2004). Other examples of esterified *p*CA can be found in flavonol glycosides such as present in the leaves of *Planchonia grandis* (Crublet et al. 2003).

The former examples of the functions of *p*CA and FA in plants illustrate that a polymer composed of these two phenolic compounds, as suggested for sporopollenin, probably provides a manifold protection to pollen and spores: against UV, microbial and physical damage (cf. Rozema et al. 1997).

Sporopollenin

To discern and isolate spores and pollen from other fossil plant remnants and inorganic material acetolysis is often applied in palynology (Erdtman 1960). Due to the fact that the exine of spores and pollen resist this acetolysis treatment it was assumed that it consisted of a chemically resistant organic polymer, conveniently named sporopollenin (Zetzsche and Kälin 1931; Traverse 1988). The actual chemistry of sporopollenin has been a point of debate for years. Varying hypotheses ranging from a polymerised carotenoid network (Brooks and Shaw 1972, 1978) to an aliphatic biopolymer (for reviews: de Leeuw et al. 1991; de Leeuw and Largeau 1993; Largeau and de Leeuw 1995). More recently, it became evident that much of the chemical information obtained on sporopollenins can be ascribed to incomplete removal of other cell constituents or the incorporation of such materials into a more resistant matrix via chemical linkages, greatly biasing the analytical information. Nowadays there seems to be agreement that the resistant material in spores and pollen may have a phenolic and hydrocarbon component (e.g. de Leeuw et al., this issue; Wierman et al. 2001; Ahlers et al. 2003). Although the presence and chemical nature of a hydrocarbon skeleton is still ambiguous, it is evident that the phenolic part of the sporopollenin is primarily composed of *p*CA

and FA (Figure 1) (Guilford et al. 1988; Ahlers et al. 1999b; Wierman et al. 2001; van Bergen et al. 2004).

Chemistry of sporopollenin

The amount of techniques that can be used for the chemical analysis of sporopollenin is greatly restricted as a consequence of its resilient nature and insolubility in organic solvents. An important exception is the solubility of the exine material from *Typha angustifolia* L. pollen in 2-aminoethanol commonly up to 5 mg/ml (Ahlers et al. 1999b) allowing the application of nuclear magnetic resonance (NMR) for the analysis of the chemical structure. Derivatisation techniques further increased solubility of these exins allowing more advanced 2D-NMR techniques (Ahlers et al. 2003). Generally it is thought that only insoluble highly cross-linked polymeric or macromolecular structures can resist chemical treatments like acetolysis or survive diagenesis over millions of years (de Leeuw and Largeau 1993). Although the dissolvable sporopollenin mentioned above (Ahlers et al. 1999a; Ahlers et al. 1999b; Ahlers et al. 2003) might be of a unique kind, the conclusions drawn from this work agree with the current ideas about the chemical structure of sporopollenin. Interpretation of NMR results of sporopollenin from *T. angustifolia* (Ahlers et al. 1999b; Ahlers et al. 2000; Ahlers et al. 2003) and *Torreya californica* (Ahlers et al. 1999a) clearly illustrates the presence of aliphatic polyhydroxy compounds next to phenolics suchs as *p*CA. The presence of *p*CA as an intricate part of the sporopollenin structure is supported by Wehling et al. (1989) and Mulder et al. (1992) who described the presence of *p*CA in the sacci of *Pinus* pollen by a detailed study using various pyrolysis techniques combined with mass spectrometry. These authors observe an isoprenoid material next to the phenolic material. Although this could be a species specific characteristic, it is possible that these compounds are remnants of other pollen consituents that were not or incompletely removed by the work-up procedure.

The first unambiguous report of sporopollenin based on *p*CA and FA in spores was published by Boom (2004) who described the chemical structure of the walls of *Isoëtes killipii* megaspores as determined by various pyrolysis techniques combined with GC/MS. Though van Bergen et al. (1993) and Bergen et al. (1995) concluded that the compounds vinylphenol and 2-methoxyvinylphenol amongst the pyrolysis products of extant and fresh spores of *Azolla* and *Salvinia* originated from *p*CA and FA, respectively, other biopolymers like lignin might also generated such pyrolysis fragments. Although it seems unlikely that the benthic spores of *I. killipii* as described by Boom (2004) were exposed to high UV-B radiation or will be during their life-cycle, it illustrates that the use of *p*CA and FA as spore and pollen wall building-blocks also provides other modes of defence.

Other reports on the analysis of sporopollenin confirm the presence of aliphatics and phenolics in sporopollenin for example by degradation with potash fusion (Schulze-Osthoff and Wiermann 1987), by using tracer experiments (Gubatz et al. 1993) and by immunocytochemical experiments (NiesterNyveld et al. 1997).

Next to the fact that certain sporopollenins can be dissolved in organic solvents as 2-amino-ethanol, a synthetic *p*CA/FA DHP produced in a similar manner as that described by Boom (2004) was largely soluble in a 0.1 M phosphate buffer of pH 6. Although this will only give a very crude approximation of the polymer size, dialysis over a 2000 Dalton molecular cut-off membrane demonstrated that the polymer contains more than 10 monomers (Blokker et al. 2005). In general it appears that both *p*CA/FA DHP and sporopollenin are more soluble than lignin, which is composed of their alcoholic counterparts (Freudenberg and Neish 1968). This feature could have serious implications for chemical analysis. Differences in sporopollenin work-up procedures will result in a different dissolution of the phenolic part of the polymer. Since literature data suggests that the aliphatic part, possibly due to a better resistance toward oxidation, is less affected by the chemical treatments used to work-up sporopollenin, different protocols will result in discrepancies between analytical data. For example, Dominguez et al. (1999) describe a work-up method that selectively purifies an aliphatic polymer by removing all ether and ester-bound phenols and fatty acids. A material is obtained that chemically resembles that of an oxidatively polymerised fatty acid network. In contrast, prolonged treatment of *T. angustifolia* sporopollenin with relatively mild acidic methanol

(Bubert et al. 2002) was shown to degrade the sporopollenin in a non-selective way. In other words the ratio between the phenolic and aliphatic part was retained upon further degradation (Bubert et al. 2002).

Acetolysis combines the extracting power of acetic acid anhydride with hydrolysis and derivatisation; most hydroxyl groups will be converted into their corresponding acetals. In theory acetolysis will remove all cell contents under relatively mild conditions, however, chemically altering the sporopollenin by acetylating free hydroxyl groups (Hemsley et al. 1995, 1996). Nevertheless, this chemical alteration does not pose analytical problems when using THM-py-techniques, since the strong hydrolysing environment will replace the acetyl group by a methylene moiety analogous to free hydroxyls. Following the former discussion on the effect of sporopollenin work-up procedures it is likely that acetolysis time, the presence of water in the acetolysis mixture, oxygen or maybe even trace metals will probably affect the chemical composition of the final sporopollenin. Future experiments will reveal if the acetolysis time and conditions will affect the relative quantities of sporopollenin components. This knowledge is of paramount importance when the chemical information retained in fossil pollen and spores are used as a proxy.

The preservation potential of pCA and FA

*p*CA and FA are omnipresent in plants performing various functions. Therefore, it is likely that these phenolic compounds can be found in sedimentary or soil organic matter that underwent limited diagenetic stress. However, to use these UV-screening phenols as a proxy for past UV-B radiation it is necessary to link the chemical information to specific plant parts. The most obvious fossil remnants to investigate the past UV-B are leaves. Both the epicuticular waxes and the cuticular membrane may form a barrier against the penetration on UV into the mesophyll (Bornman and Vogelmann 1988; Robinson et al. 1993; Barnes and Cardoso-Vilhena 1996; Krauss et al. 1997; Liakopoulos et al. 2001). This suggests that maybe a UV-signal might be preserved as chemical information in the fossil leaves. In general plant leaves may preserve in sediments or soils over extremely long periods under the right conditions. It was suggested that this might be due to the presence of resistant aliphatic biopolymeric materials like cutan (e.g. de Leeuw et al., this issue; van Bergen et al. 2004), however, depending on the preservation conditions, it seems that less resilient biomolecular structures survive diagenesis very well. (For a recent review on biomacromolecules and their preservation see: de Leeuw et al., this issue; van Bergen et al. 2004).

The presence of the 4-vinyl phenol in the pyrolysate of fresh and fossil cuticles has been reported in fossil leaf material of *Ginkgo* species (Mösle et al. 1997, 1998). 4-vinyl phenol is thought to be the pyrolytic decarboxylation product of *p*CA (Figure 2). Using the presence of *p*CA in fossil cuticles for the reconstruction of past UV-B might pose difficult if diagenesis affects the chemistry of the cuticle. Huang et al. (1998) shows that upon a 23 years degradation field experiment of *Calluna vulgaris* litter, *p*CA is preferentially removed over the syringyl and guaiacyl moieties as deduced from a decrease in 4-vinyl phenol in the pyrolysate in the time series 0, 0.5, 7 and 23 years. Similar observations were done by Kuder and Kruge (1998), van Bergen et al. (1997) and Karunen and Kalviainen (1988). However, despite a significant loss of *p*CA and FA these authors did not observe a complete removal. This might suggest that a labile fraction of these phenolics, probably from other parts of the leaf than the cuticle, is degraded while a more resilient, probably polymeric, fraction remains. Fossil seeds may also contain *p*CA and FA (e.g. Bergen et al. 1995; van Bergen et al. 1997, 2004, and references therein). These authors state that the polyphenolic constituents in seeds are most likely of a lignin origin and *p*CA and FA are incorporated into such polymeric structure via ester and ether-linkages as indicated by 4-vinylphenol and 4-vinyl-2-meth oxyphenol in the pyrolysis products (for the pyrolysis mechanism see Figure 2).

Pollen and spores are amongst the most resistant organic plant remains (e.g. de Leeuw and Largeau 1993; van Bergen et al. 2004), and will thus be a reliable source for information of past UV-B concentrations. Still, like the chemical information on fresh spores and pollen, not all research seems to agree on the presence of

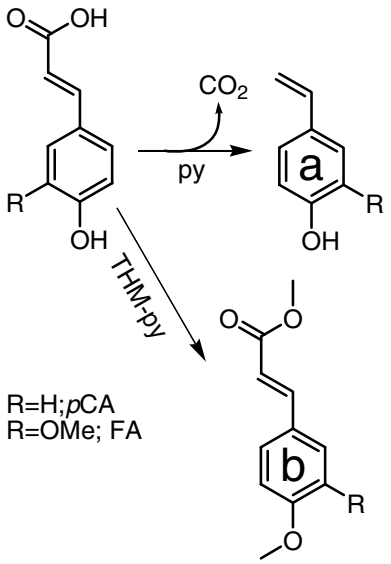

Figure 2. The main pyrolysis pathways of polymeric *p*-coumaric acid (R = H) and ferulic acid (R = OMe) with and without THM. (a) 4-vinyl-phenol R = H and 2-methoxy-4-vinyl-phenol (R = OMe); (b) Methylated *p*-coumaric acid R = H and ferulic acid (R = OMe).

phenolics in fossil spores and pollen. Oxidative conditions will most likely preferentially degrade the polyphenolics in favour of the aliphatic part as demonstrated by Hemsley et al. (1996) using ^{13}C-NMR on *Lycopodium clavatum* spores. Diagenetic modelling by heating *Pinus* sporopollenin furthermore shows that in the early stages the typical polyphenolic signal is replaced by a single aromatic signal, while the aliphatic signal remains. Further heating results in a homogenisation of the signal, resulting in a single aromatic and aliphatic signal without the fine-structure of the original sporopollenin comparable to that of carboniferous megaspores. The most diagenetically altered material consists of only aromatic material as can be seen in fossil samples that are significantly affected by diagenesis (Hemsley et al. 1995, 1996).

The loss of fine-structure and change in the aliphatic vs. aromatic signal was also observed for *Lycopodium clavatum* spores by (Yule et al. 2000) upon simulated diagenesis. They observed a relative intensification of the FTIR aliphatic signal with increasing maturity. The aromatic signal dominated the spores' FTIR spectrum of the most diagenetically altered material.

Development of a micro-scale analytical method for the reconstruction of past UV radiation

The analysis of fossil plant materials from cores is seriously hampered by their low availability. Scarcity of samples requires an analysis method that is non-destructive or uses only limited sample amounts. This is even more crucial for chemical analysis of Herbarium material since destructive sampling of precious collections is unsolicited. Most suitable are these techniques that allow the analysis small samples without laborious pre-treatment, such as pyrolysis-coupled to sensitive GC/MS. It has been demonstrated that such techniques enable the analysis of microscopic entities and would be the analytical method of choice for example fatty acid profiling of flow cytometry isolated microorganisms (Blokker et al. 2002; de Koning et al. 2002). A major drawback of pyrolysis techniques is the often complex fragmentation of macromolecular structures, providing a myriad of compounds that are often unrecognisable by commercial mass spectra-libraries (Haken 2000). In the case of *p*CA and FA conventional pyrolysis will lead to 4-vinyl-phenol and 2-methoxy-4-vinyl-phenol (Figure 2) as primary products (e.g. Boom 2004). Since lignins may also thermally degrade into these compounds amongst others, the presence of 4-vinyl-phenol and 2-methoxy-4-vinyl-phenol does not provide conclusive evidence for the presence of *p*CA and FA in the sample (e.g. Saiz-Jimenez and de Leeuw 1984; van der Hage 1995; Kuroda 2000). However, applying THM-reagents such as TMAH will decrease the degree of fragmentation by simultaneously hydrolysis of macromolecular structures and derivatisation of hydroxyl, carboxylic acid and other functional groups that carry an acidic proton (Challinor 1989). Though the 4-vinyl phenol in the pyrolysate of fresh and fossil *Ginkgo* cuticles (Mösle et al. 1997, 1998) is proposed to originate from *p*CA, only upon using TMAH-pyrolysis this could be unequivocal demonstrated. THM-py on a Paleocene (65–54.8 mya) *Ginkgo adiantoides* leaf (Figure 3) indeed confirmed the presence of pCA next to typical oxidised fatty acid THM-py products. This over 55 million year old fossil cuticle even retained its UV absorbing properties (Figure 4) as compared with absorbance assessed in an extant cuticle of *G. biloba* leaf. These results

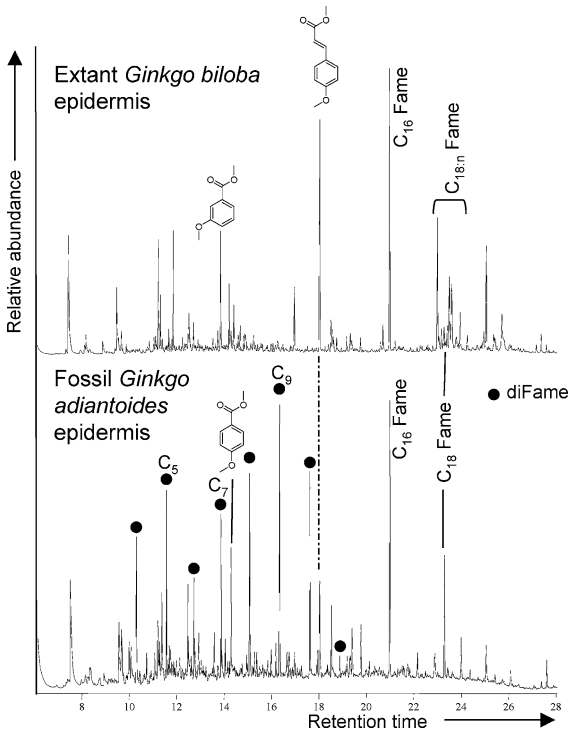

Figure 3. TIC of THM-pyrolysis product mixture of extant and fossil *Ginkgo* cuticles (1×3 mm) (Paleocene, ca. 60 mya). diFame – alkyl dicarboxylic acids methyl ester; Fame – fatty acid methyl esters; C_n = carbon number.

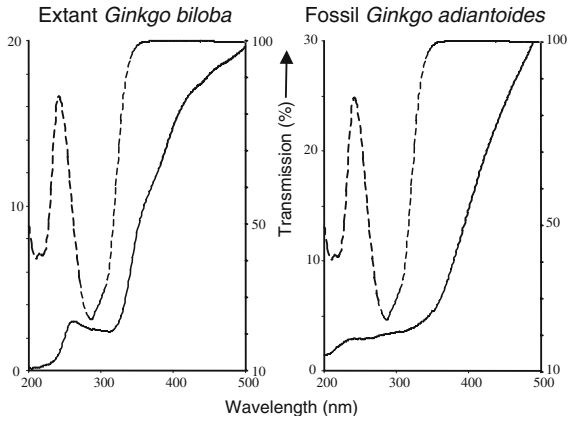

Figure 4. UV-absorption spectra of fresh and fossil *Ginkgo* superimposed on the absorption spectrum of a synthetic *p*-coumaric acid-based sporopollenin (DHP).

indicate that *p*CA is an intricate part of the *Ginkgo* cuticle.

The application of THM reagents and pyrolysis techniques thus supplies the means for the analysis of target compounds in very small sample sizes. To increase the sensitivity of the method, allowing the analysis on a 50–500 pollen grain or moss spore level (depending on the grain size), it was necessary to run the mass spectrometer in selective-ion-monitoring (SIM) mode. This afforded a detection limit of ~60 fresh *Alnus* pollen for pCA and ~10 for FA (S/N = 3) (Blokker et al. 2005).

Though a very low amount of *p*CA and FA can be quantified by using THM-py-GC/MS, absolute quantification of these phenolics as part of a polymeric structure is not possible since a wide variety of different intermolecular linkages will give rise to various pyrolysis products. Therefore, differences observed between samples could be due to differences in linkage types rather than to concentrations. Furthermore, when interpreting results from fossil material it is possible that diagenesis will affect the observed amount of phenolics. Thermal maturation will result in homogenisation of the aromatic signal in NMR and FTIR, possibly due to coalification processes (Hemsley et al. 1995, 1996). Such chemical changes will most likely also be reflected in the pyrograms of such materials and hamper the comparison of fresh and fossil materials.

An alternative to the quantification of pyrolysis products is the use of ratios between different pyrolysis products. Though investigations are ongoing, preliminary results (Figure 5) indicate that next to the concentration of *p*CA and FA, the *p*CA/FA in a THM-pyrolysis mixture of *Vicia faba* pollen is significantly increased by UV-light. THM-py-GC/MS analysis of various fresh spores and pollen (Figure 6) indicate that there is a large variety between the ratio due to interspecies differences of environmental (including radiation regime) conditions. Whether or not UV-B radiation increased the relative amounts of these compounds in the pollen or just affects the degree and/or type of polymerisation remains unknown.

There are still many and large gaps in our knowledge on the effects of UV-B on plants and past UV-radiation. THM-py-GC/MS analysis of *p*CA and FA appears to show perspective for the reconstruction of past UV-B. Especially the analysis of samples from peat deposits and herbarium collections seems to be a fruitful route to gain insight in the impact of fluctuating UV-B on our climate and ecosystems.

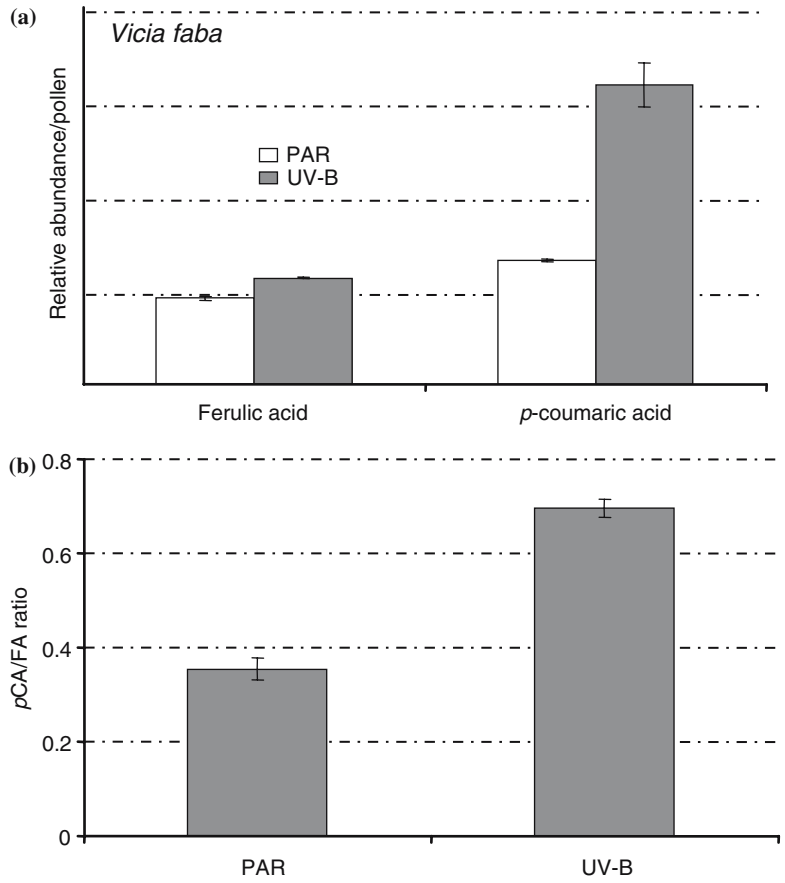

Figure 5. (a) Integrated peak area of *p*-coumaric acid and ferulic acid divided by the number of pollen (between 44 and 68) of unacetolysed *Vicia faba*. Error bars represent the standard deviation. Two-sided *t*-tests (df = 2): ferulic acid, $p = 0.004$; *p*-coumaric acid, $p = 0.007$. (b) Data from (a) plotted as ratio. Error bars represent the standard deviation. Two-sided *t*-tests (df = 2): $p = 0.006$ (Blokker et al. 2005).

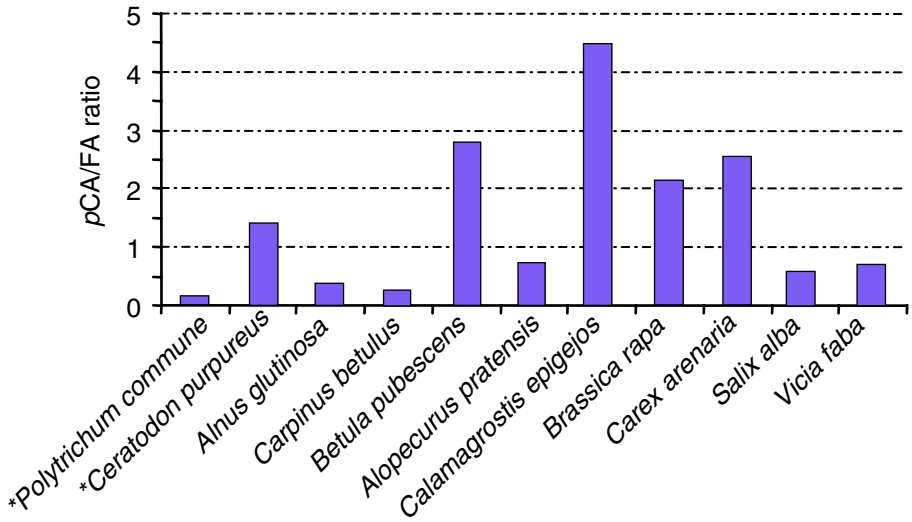

Figure 6. *p*-coumaric acid/ferulic acid ratios of pollen and spores from various higher and lower plants (latter indicated by *).

Experimental

For analysis the samples were placed in a pyrolysis liner (CDS) and 5 μl of a 25% TMAH solution in methanol was added. The sample was allowed to incubate for 2 h at 70 °C and pyrolysed at 550 °C for 5 min. in a CDS AS2500 pyrolysis unit (260 °C interface temperature) coupled to an Agilent 6890 GC equipped with an Agilent 5973 MSD. The GC-oven was programmed from 40 °C (5 min hold time) to 130 °C at 20 °C/min and subsequently to 320 °C at 6 °C/min followed by 10 min isothermal at 320 °C. He was used as a carrier gas at a constant flow of 1.2 ml/min. using a 20:1 splitratio for leaf samples and in splitless mode in case of pollen and spores. The mass spectrometer was operated in full scan mode (m/z 50–800) or in SIM-mode in case of pollen and spore analysis (m/z 192, 222) at 70 eV ionization energy.

The UV spectra were recorded on a Shimatzu UV-1601PC UV–VIS spectrophotometer. The extant leaf was sampled May, 2004 from a 40-year-old *G. biloba* tree on the Vrije Universiteit campus.

Vicia faba was cultured without and with UV-B treatment (biologically active radiation; 10 kJ m^{-2} day^{-1}, representing ~30% ozone depletion) conditions earlier reported by (Meijkamp et al. 2001).

The other pollen and spore materials were collected spring at various places in the north of the Netherlands.

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