Quantifying the impact of chemicals on stable carbon and oxygen isotope values of raw pollen

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ABSTRACT: Purification protocols to extract pollen from lake sediments contain chemicals that alter the carbon and oxygen pollen-isotope values according to pollen characteristics and family affiliation. Modern (raw) pollen of broad-leaved (Alnus glutinosa, Betula pendula, Carpinus betulus, Corylus avellana, Fagus sylvatica and Quercus robur) and coniferous tree species (Picea abies and Pinus sylvestris) were treated with potassium hydroxide (KOH), hydrofluoric acid (HF), sodium hypochlorite (NaClO) and sulphuric acid (H2SO4) to test the impact on δ13Cpollen and δ18Opollen and assess the applicability in purification protocols. Pollen of broad-leaved and coniferous trees reacted differently to chemical exposure, but response patterns are generally alike. Alterations of δ13Cpollen values vary between +1.0‰ (B. pendula, NaClO-treatment) and −5.0‰ (P. sylvestris, H2SO4-treatment). The δ13Cpollen values of raw and chemically treated samples seem to be related after treatments with KOH, NaClO and HF, whereas the application of H2SO4 led to inconsistent changes among species. The impact of chemicals on δ18Opollen are more diverse and offsets range between +1.1‰ (C. avellana, NaClO-treatment) and −17.9‰ (P. sylvestris, H2SO4-treatment). In general, the use of isotope-altering chemicals in purification protocols should be brought to a minimum, but the application of KOH and NaClO seems mostly unproblematic before δ13Cpollen and δ18Opollen analysis.

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KEYWORDS: chemical treatment; pollen; pollen purification protocol; stable carbon and oxygen isotopes

Introduction

Modern (raw) pollen can function as reference material to interpret fossil pollen-isotope values. The application of certain chemicals on raw pollen imitates a fossilization process to enhance comparability, but chemical substances are also used to purify fossil pollen from organic and inorganic remains of a sample. It is already known that chemical application alters the pollen-isotope values (Amundson et al., 1997), but the single effect of certain chemicals on pollen-isotope values of different species with variable pollen characteristics are unknown. Before the approach of pollen-isotope analysis can be applied in palaeoclimate studies, the impact of chemical substances from purification protocols on the δ13Cpollen and δ18Opollen values needs to be thoroughly investigated.

Raw pollen differ from fossilized pollen, because decomposition of structurally weak pollen components and pollen wall coatings leaves only the sporopollenin layer of fossil pollen intact (Loader and Hemming, 2000). Sporopollenin, the main component of a pollen wall, is a highly resistant biopolymer approximately consisting of C90H150O33 (Brooks and Shaw, 1978; Loader and Hemming, 2004; Fraser et al., 2014; Li et al., 2019; Mikhael et al., 2019) and the proportion of sporopollenin within raw pollen of different species ranges between 55 and 85% (Nelson, 2012). Additional to sporopollenin, the outer wall of raw pollen contains lipids, proteins and in some cases pollenkitt, whereas the inner pollen wall is composed of pectin, cellulose and hemicellulose (Fan et al., 2019). The structure and composition of a pollen grain wall varies highly with species (e.g. Moore et al., 1991; Stanley and Linskens, 2012) and therefore also carbon and oxygen isotopes values of raw pollen have species-specific patterns and ranges (e.g. Amundson et al., 1997; Jahren, 2004; Loader and Hemming, 2004; King et al., 2012; Nelson, 2012; Schwarz, 2016; Müller et al., 2020). Chemicals applied on raw pollen change the composition of the pollen wall coating substance-dependently, but the degree of contamination of the sporopollenin layer is unknown. To prevent contamination of the δ13Cpollen during sporopollenin extraction, Loader and Hemming (2000) tested an acid digestion method using sulphuric acid (H2SO4) to remove all organic components from raw pollen except sporopollenin. Stable and species-specific offsets of the δ13Cpollen from Pinus sylvestris (−2.18‰), Zea mays (−1.81‰) and Populus trichocarpa (−3.55‰) were detected and all species yielded strong linear correlations between raw and chemically treated pollen-isotope values, which indicates a consistent impact of the chemicals on the pollen-isotope values and thus implies a replicable outcome (r2 = 0.93; Loader and Hemming, 2000). When Descolas-Gros and Schötz (2007) applied H2SO4 on raw pollen to investigate reflections of the C3 and C4 photosynthetic pathway in δ13Cpollen, they detected offsets of −1.18‰ (Sorghum vulgare) and −0.91‰ (Platanus sp.). Another test of the method using H2SO4 to extract sporopollenin revealed an average offset of −1.5‰ between raw and treated δ13Cpollen of Cedrus atlantica (Bell et al., 2017). In general, carbon pollen-isotope values are depleted after treatments including sulphuric acid, but the impact seems highly species-specific.

Chemicals are traditionally used to extract and purify pollen from lake sediments for palynological studies (Faegri et al., 1989; Nelson et al., 2006; Grieener et al., 2013). These are
mainly: hydrochloric acid (HCl) to remove inorganic carbon particles, potassium hydroxide (KOH) to digest humic acids, hydrofluoric acid (HF) to dissolve siliclastic components and acetylation (glacial acetic acid (CH₃COOH) used together with acetic anhydride (C₄H₄O₆) and H₂SO₄) to remove organic remains and stain the pollen wall. However, traditional protocols were not designed for pollen extraction before stable isotope analysis and can thus lead to carbon-isotopic contamination of the pollen grain wall (Amundson et al., 1997). In particular, a protocol containing acetylation needs to be avoided due to its major impact on the δ¹³C values (Amundson et al., 1997; Descolas-Gros and Schöbel, 2007). Nelson et al. (2006) established a protocol to purify pollen for isotope analysis containing HCl, KOH, HF, NaClO and H₂SO₄. Applied on raw pollen of grass and shrub species, it led to depletions ranging between −1.2 and −3.7‰ in the δ¹³Cpollen (Nelson et al., 2006; Nelson, 2012). Griener et al. (2013) used this protocol on raw and fossil Nothofagus pollen and detected an average depletion of −1.7‰ within the δ¹³Cpollen, and Jahren (2004) suggested a treatment including sodium hydroxide (NaOH), hydrogen peroxide (H₂O₂) and HF to do both: simulate the effect of diagenesis avoiding acetylation and H₂SO₄ and additionally to be used for the isolation of isotopically unaltered pollen. So far, only the identification of four chemical substances (KOH, NaClO, HF and H₂SO₄) and a successive treatment including all chemicals on the δ¹³Cpol and δ¹⁸Opol of raw pollen from eight different tree species. Our aims were to assess species-specific alterations of pollen-isotope values after exposure to the different chemicals and to promote the compilation of a treatment protocol minimizing the impact on fossil pollen isotopes.

Materials and methods
Sample location, collection and preparation
Individual pollen samples contain pollen of several interfossils from individual trees. Modern pollen of three individuals from each species (Alnus glutinosa, Betula pendula, Corylus avellana, Fagus sylvatica, Picea abies, Pinus sylvestris and Quercus robur; Table 1) were collected in March and May 2016 within their respective flowering period in Parc naturel Forêt d’Anlier (Belgium): 49.7899°N, 5.6829°E; average elevation: 385 m a.s.l.). Mean annual temperature at this location is 9.1°C (mean temperature of the coldest month: −0.1°C; mean temperature of the warmest month: 16.1°C). Mean annual precipitation amounts to 1019 mm (based on the high-resolution gridded dataset CRU TS at www.cru.uea.ac.uk/data). Pollen of three individual trees of Carpinus betulus (Table 1) were collected in May 2016 at Nationalpark Steigerwald (Germany): 49.8616°N, 10.5241°E; average elevation: 390 m a.s.l.; Mean annual temperature: 9.4°C; mean temperature of the coldest month: −1.2°C; mean temperature of the warmest month: 17.5°C; Mean annual precipitation: 581 mm).

The samples were kept at 6°C in a refrigerator during fieldwork and dried afterwards in a drying oven with a maximum temperature of 45°C for 7 days. Dry samples were kept frozen at −16°C until further processing. The pollen were separated from the rest of the flower tissue through rinsing with deionized water and sieving with mesh sizes of 10–200 μm. Afterwards, the pollen were transferred to safe lock tubes, frozen again at −16°C and freeze-dried until fully dehydrated. Dry pollen samples were kept frozen for preservation.

Chemical treatment procedure
Pollen samples of 24 individual trees from eight species were treated separately with potassium hydroxide (KOH 10%; 20 min, ca. 70°C water bath), sulphuric acid (H₂SO₄ 10%; 20 min, ca. 70°C water bath) and acetic anhydride (C₄H₆O₃) and H₂SO₄ to remove organic remains and to stain the pollen wall. However, traditional protocols were not designed for pollen extraction before stable isotope analysis and can thus lead to carbon-isotopic contamination of the pollen grain wall (Amundson et al., 1997). In particular, a protocol containing acetylation needs to be avoided due to its major impact on the δ¹³C values (Amundson et al., 1997; Descolas-Gros and Schöbel, 2007). Nelson et al. (2006) established a protocol to purify pollen for isotope analysis containing HCl, KOH, HF, NaClO and H₂SO₄. Applied on raw pollen of grass and shrub species, it led to depletions ranging between −1.2 and −3.7‰ in the δ¹³Cpollen (Nelson et al., 2006; Nelson, 2012). Griener et al. (2013) used this protocol on raw and fossil Nothofagus pollen and detected an average depletion of −1.7‰ within the δ¹³Cpollen, and Jahren (2004) suggested a treatment including sodium hydroxide (NaOH), hydrogen peroxide (H₂O₂) and HF to do both: simulate the effect of diagenesis avoiding acetylation and H₂SO₄ and additionally to be used for the isolation of isotopically unaltered pollen. So far, only the identification of four chemical substances (KOH, NaClO, HF and H₂SO₄) and a successive treatment including all chemicals on the δ¹³Cpol and δ¹⁸Opol of raw pollen from eight different tree species. Our aims were to assess species-specific alterations of pollen-isotope values after exposure to the different chemicals and to promote the compilation of a treatment protocol minimizing the impact on fossil pollen isotopes.

Table 1. Overview of species, taxonomic classification and pollen characteristics (Beug, 2004) of eight tree species examined in this study including their common names and family affiliation.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Common name</th>
<th>Size of pollen (μm)</th>
<th>Shape of pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betulaceae</td>
<td>Alnus glutinosa (L.) Gaertn.</td>
<td>Black alder</td>
<td>24–34</td>
<td>Stephanoportatae, pentagonal</td>
</tr>
<tr>
<td></td>
<td>Betula pendula Roth</td>
<td>Silver birch</td>
<td>26–32</td>
<td>Triporateae, triangular</td>
</tr>
<tr>
<td></td>
<td>Carpinus betulus (L.)</td>
<td>European hornbeam</td>
<td>38–48</td>
<td>Stephanoportatae, roundish</td>
</tr>
<tr>
<td></td>
<td>Corylus avellana (L.)</td>
<td>Common hazel</td>
<td>28–33</td>
<td>Triporateae, triangular</td>
</tr>
<tr>
<td>Fagaceae</td>
<td>Fagus sylvatica (L.)</td>
<td>European beech</td>
<td>28–39</td>
<td>Tricolporateae, roundish</td>
</tr>
<tr>
<td></td>
<td>Quercus robur (L.)</td>
<td>Pedunculate oak</td>
<td>31–40</td>
<td>Tricolporateae, oval</td>
</tr>
<tr>
<td>Pinaceae</td>
<td>Picea abies (L.) H. Karst.</td>
<td>Norway spruce</td>
<td>110–148</td>
<td>Vesicularatae</td>
</tr>
<tr>
<td></td>
<td>Pinus sylvestris (L.)</td>
<td>Scots pine</td>
<td>62–84</td>
<td>Vesicularatae</td>
</tr>
</tbody>
</table>
Table 2. Details of the chemical treatment procedure used to prepare pollen for stable carbon and oxygen isotope analysis including the common names and purity of the chemicals, the specifications of each treatment, the time for each treatment and the effect on the pollen sample.

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>Common name</th>
<th>Purity</th>
<th>Type of treatment</th>
<th>Duration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
<td>10%</td>
<td>Water bath at 70 °C</td>
<td>20 min</td>
<td>Digestion of humic acids</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
<td>96%</td>
<td>Constantly on a shaker</td>
<td>5 h</td>
<td>Extraction of sporopollenin</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
<td>38%</td>
<td>Resting at room temperature</td>
<td>24 h</td>
<td>Degeneration of clastic debris</td>
</tr>
<tr>
<td>NaClO</td>
<td>Sodium hypochlorite</td>
<td>3%</td>
<td>Gentle stirring at room temperature</td>
<td>90 s</td>
<td>Dissolution of small organic matter</td>
</tr>
</tbody>
</table>

96%; 5 h on a shaker) and sodium hypochlorite (NaClO 3%; 90 s; Table 2). The impact of hydrofluoric acid (HF 38%; 24 h) was tested on pollen samples of nine individual trees from three species (C. avellana, P. abies and P. sylvestris; Fig. 1). Repeated and thorough rinsing of the pollen samples with deionized water followed each chemical treatment. To test combined effects of the chemicals, as it occurs following a standard protocol for fossil pollen sample preparation, pollen samples of three individual trees from each of the eight species (in total 24 samples) underwent the chemical treatments with KOH, HF, H₂SO₄ and NaClO successively in this order and with the same length as the single treatments (successive treatment is termed all; Table 2). All samples were visually inspected after each chemical treatment using a Meiji MFT4300L microscope at magnifications of 400× and 600× to ensure intactness of the pollen wall. Additionally, the cellulose content of the pollen wall after H₂SO₄ exposure and the successive treatment (all) was determined with a staining technique using Safranin and Fast Green.

**Stable isotope and statistical analysis**

In total, 220 μg ± 10% of pollen material was weighed directly into silver capsules (0.02 ml; 3.3 x 4 mm) using a high-precision scale (Mettler Toledo AX 26 Delta Range) at the dendrochronological laboratory, section 4.3, GFZ Potsdam. The carbon and oxygen isotope values were determined using a DELTA V isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific, Bremen, Germany). Each sample was weighed and measured with three repetitions and vacuum dried for at least 12 h in a Thermo Scientific Heraeus VT 6060 P at 100 °C before stable isotope measurement. The pollen material was reduced to CO for simultaneous IRMS analysis of carbon and oxygen isotope ratios in a High Temperature Conversion Elemental Analyzer (TC/EA; 1400 °C; Thermo Fisher Scientific) coupled to the IRMS. Isotope ratios are expressed relative to VPDB for δ¹³C and VSMOW for δ¹⁸O. The isotope values were compared against laboratory-internal and international reference material (IAEA-CH₃, IAEA-CH₆, and IAEA 601 and 602). For a single-point normalization, two reference standards with widespread isotopic compositions were used (Paul et al., 2007).

During the chemical treatment procedures, four samples treated with NaClO and one sample of the successive treatment (all) were dissolved and could not be measured isotopically. Each sample was measured three times and in total, and we conducted 296 measurements of chemically treated pollen and 72 measurements of untreated (referred to as raw) pollen of the same individuals (Supporting Information Table S1). Raw and chemically treated pollen have been plotted to visualize the relationship. All calculations and the graphic depiction were made using the programs R (R Core Team, 2017) and RStudio (RStudio Team, 2015). The impact of species and type of chemical applied during treatment of the samples on the mean and between raw and chemically treated pollen was assessed by two-way analysis of variance (ANOVA) in R v4.0.2 (R Core Team, 2017; Table S2).

**Results**

δ¹³C<sub>pollen</sub> after chemical treatment

Mean δ¹³C<sub>pollen</sub> values are mostly depleted after chemical treatment procedures compared to raw pollen-isotope values (Fig. 1; Table 3A). The depletion of mean δ¹³C<sub>pollen</sub> values after KOH exposure ranges from −0.3‰ (F. sylvatica) to −4.0‰ (C. avellana). The HF treatment results in a depletion ranging between −0.4‰ (P. abies) and −1.4‰ (C. avellana) and the impact of sulphuric acid (H₂SO₄) on mean δ¹³C<sub>pollen</sub> values results in a depletion ranging from −1.2‰ (F. sylvatica) to −5.0‰ (P. sylvestris). A successive treatment with all chemicals (all) has the highest impact on δ¹³C<sub>pollen</sub> values compared to the untreated pollen material. The depletions range between −1.6‰ (F. sylvatica) and −6.4‰ (P. sylvestris).

Enriched mean δ¹³C<sub>pollen</sub> values occur only after NaClO treatment for B. pendula (+1.0‰) and Carpinus betulus (+0.8‰). Pollen-isotope values of other species are depleted after exposure to NaClO and range between −0.3‰ (Pinus sylvestris) and −1.4‰ (Corylus avellana). There was no effect on Picea abies pollen measurable for NaClO (Fig. 1; Table 3A).

δ¹⁸O<sub>pollen</sub> after chemical treatment

Mean δ¹⁸O<sub>pollen</sub> values of B. pendula (+0.1‰) and Q. robur (+1.0‰) are enriched after exposure to KOH, whereas values of all other species are depleted in a range of −0.3‰ (Picea abies) to −2.6‰ (A. glutinosa; Fig. 2; Table 3B). The HF treatment leads for C. avellana to enriched pollen-isotope values of +0.5‰, whereas the values of P. abies (−1.8‰) and P. sylvestris (−1.7‰) are depleted.

All broad-leaved species have enriched δ¹⁸O<sub>pollen</sub> values after the treatment with NaClO. The enrichment ranges between +1.1‰ (C. avellana) and +3.1‰ (B. pendula). NaClO had no measurable effect on A. glutinosa δ¹⁸O<sub>pollen</sub> values (Fig. 2; Table 3B). Coniferous δ¹⁸O<sub>pollen</sub> values (P. abies: −1.7‰; P. sylvestris: −0.5‰) are depleted as a result of the NaClO treatment.

The H₂SO₄-treatment has distinct effects on δ¹⁸O<sub>pollen</sub>. Mean pollen-isotope values of broad-leaved species are depleted in a range of −0.7‰ (A. glutinosa) to −5.8‰ (C. betulus), whereas coniferous δ¹⁸O<sub>pollen</sub> values are depleted by −9.7‰ (P. abies) and −17.9‰ (P. sylvestris), respectively. The depletion of mean δ¹⁸O<sub>pollen</sub> values after the successive treatment has a broad range between −3.7‰ (A. glutinosa) and −19.2‰ (P. sylvestris; Table 3B).

**Statistical analysis**

Two-way ANOVA indicated significant impacts of species, type of chemical applied during treatment of the samples and their interaction term on the mean differences in and between raw and chemically treated pollen. However, due to the small number of samples, these results should be treated with caution (Supporting Information Table S2).
Discussion

$δ^{13}C_{\text{pollen}}$ after chemical treatments

Chemical treatment alters the stable carbon isotope values of pollen and the amount of the deviation is species- and substance-dependent (Amundson et al., 1997; Loader and Hemming, 2000; Jahren, 2004; Nelson et al., 2006). However, $δ^{13}C_{\text{pollen}}$ values are mostly depleted compared to untreated pollen material (Fig. 1; Table 3A). Even though the individual deviation between raw and treated pollen is species-specific for $δ^{13}C_{\text{pollen}}$, the overall response patterns to each chemical reveal similarities among the species (Fig. 1), which have also been detected for other species (Loader and Hemming, 2000; Jahren, 2004). We assume that the species-specific pollen wall structure and coating as well as pollen shape and pollen size are factors that may determine the impact.

Figure 1. Scatter plots visualizing the relationship between raw and chemically treated $δ^{13}C_{\text{pollen}}$ values (expressed in ‰, relative to VPDB) of eight tree species (Alnus glutinosa, Betula pendula, Carpinus betulus, Fagus sylvatica, Picea abies, Pinus sylvestris and Quercus robur) after treatment with KOH (24 individuals, eight species), HF (nine individuals, three species), NaClO (20 individuals, seven species), H2SO4 (24 individuals, eight species) and the successive treatment all (23 individuals, eight species). [Color figure can be viewed at wileyonlinelibrary.com].
Table 3. Mean $\delta^{13}C_{\text{pollen}}$ values (3A, expressed in ‰, relative to VPDB) and $\delta^{18}O_{\text{pollen}}$ values (3B, expressed in ‰, relative to VSMOW) of the chemically untreated (raw) pollen of eight species and first standard deviation. $\delta^{13}C_{\text{diff.}}$ and $\delta^{18}O_{\text{diff.}}$ indicate depletion or enrichment of the isotope values of after chemical treatment compared to the untreated (raw) pollen-isotope values and the first standard deviation. Chemicals used for treatment are KOH, HF, NaClO, H$_2$SO$_4$ and all (successive treatment procedure following the protocol of four chemicals applied consecutively; see text). Dev. H$_2$SO$_4$/all refers to the difference between values after treatment solely with H$_2$SO$_4$ and after the successive treatment (all).

#### (A)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>$\delta^{13}C$ Raw</th>
<th>$\delta^{13}C_{\text{diff.}}$ KOH</th>
<th>$\delta^{13}C_{\text{diff.}}$ HF</th>
<th>$\delta^{13}C_{\text{diff.}}$ NaClO</th>
<th>$\delta^{13}C_{\text{diff.}}$ H$_2$SO$_4$</th>
<th>$\delta^{13}C_{\text{diff.}}$ all</th>
<th>Dev. $\delta^{13}C_{\text{all}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnus glutinosa</td>
<td>−30.6 ± 0.3</td>
<td>−3.0 ± 1.4</td>
<td>−0.7 ± 0.2</td>
<td>−3.0 ± 0.7</td>
<td>−4.0 ± 0.4</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Betula pendula</td>
<td>−24.6 ± 0.1</td>
<td>−1.6 ± 0.2</td>
<td>+1.0 ± 0.1</td>
<td>−3.9 ± 0.3</td>
<td>−4.6 ± 0.2</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Carpinus betulus</td>
<td>−26.0 ± 0.1</td>
<td>−1.7 ± 0.3</td>
<td>+0.8 ± 0.2</td>
<td>−2.6 ± 0.3</td>
<td>−3.5 ± 0.3</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Corylus avellana</td>
<td>−28.4 ± 0.3</td>
<td>−4.0 ± 0.2</td>
<td>−1.4 ± 0.3</td>
<td>−1.2 ± 0.1</td>
<td>−3.7 ± 0.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Fagus sylvatica</td>
<td>−28.2 ± 0.2</td>
<td>−0.3 ± 0.2</td>
<td>−</td>
<td>−1.2 ± 0.5</td>
<td>−1.6 ± 0.1</td>
<td>0.4</td>
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</tr>
<tr>
<td>Quercus robur</td>
<td>−26.1 ± 0.3</td>
<td>−1.1 ± 0.6</td>
<td>−0.4 ± 0.2</td>
<td>−2.3 ± 0.3</td>
<td>−3.4 ± 0.2</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Picea abies</td>
<td>−27.4 ± 0.3</td>
<td>−0.1 ± 0.2</td>
<td>−0.4 ± 0.2</td>
<td>0.0 ± 0.2</td>
<td>−2.7 ± 0.3</td>
<td>1.0</td>
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</tr>
<tr>
<td>Pinus sylvestris</td>
<td>−24.8 ± 0.3</td>
<td>−1.0 ± 0.2</td>
<td>−0.7 ± 0.2</td>
<td>−0.3 ± 0.1</td>
<td>−5.0 ± 0.3</td>
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#### (B)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>$\delta^{18}O$ Raw</th>
<th>$\delta^{18}O_{\text{diff.}}$ KOH</th>
<th>$\delta^{18}O_{\text{diff.}}$ HF</th>
<th>$\delta^{18}O_{\text{diff.}}$ NaClO</th>
<th>$\delta^{18}O_{\text{diff.}}$ H$_2$SO$_4$</th>
<th>$\delta^{18}O_{\text{diff.}}$ all</th>
<th>Dev. $\delta^{18}O_{\text{all}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnus glutinosa</td>
<td>21.7 ± 0.5</td>
<td>−2.6 ± 1.8</td>
<td>0.0 ± 0.5</td>
<td>−0.7 ± 0.6</td>
<td>−3.7 ± 0.8</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Betula pendula</td>
<td>24.8 ± 0.2</td>
<td>+0.1 ± 0.2</td>
<td>+3.1 ± 0.1</td>
<td>−4.5 ± 0.4</td>
<td>−7.9 ± 0.2</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Carpinus betulus</td>
<td>26.4 ± 0.2</td>
<td>−1.8 ± 0.3</td>
<td>+1.5 ± 0.1</td>
<td>−5.8 ± 0.2</td>
<td>−9.2 ± 0.2</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Corylus avellana</td>
<td>21.5 ± 0.4</td>
<td>−0.9 ± 0.3</td>
<td>+0.5 ± 0.3</td>
<td>+1.1 ± 0.4</td>
<td>−0.8 ± 0.3</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>Fagus sylvatica</td>
<td>23.3 ± 0.2</td>
<td>−1.1 ± 0.2</td>
<td>−</td>
<td>−1.8 ± 0.1</td>
<td>−6.2 ± 0.4</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Quercus robur</td>
<td>27.4 ± 1.0</td>
<td>+1.0 ± 0.7</td>
<td>+2.3 ± 0.5</td>
<td>−4.7 ± 0.3</td>
<td>−9.1 ± 0.3</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>Picea abies</td>
<td>24.5 ± 0.8</td>
<td>−0.3 ± 0.5</td>
<td>−1.8 ± 0.8</td>
<td>−1.7 ± 0.2</td>
<td>−9.7 ± 0.8</td>
<td>10.6 ± 1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Pinus sylvestris</td>
<td>30.1 ± 0.5</td>
<td>−2.9 ± 0.2</td>
<td>−1.7 ± 0.5</td>
<td>−0.5 ± 0.2</td>
<td>−17.8 ± 0.5</td>
<td>19.1 ± 0.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Impact of KOH, HF and NaClO on $\delta^{13}C_{\text{pollen}}$**

Even though the mean depletion of carbon isotope values after KOH exposure varies between −0.3‰ (F. sylvatica) and −4.0‰ (C. avellana), species react in a similar fashion and a relationship between raw and treated $\delta^{13}C_{\text{pollen}}$ values can neither be confirmed nor denied (Fig. 1). The impact of NaClO on $\delta^{13}C_{\text{pollen}}$ varies between +1.0‰ (B. pendula) and −1.4‰ (C. avellana) but the overall response is similar for most species and the carbon isotope values of raw and treated pollen seem linearly related (Fig. 1; Table 3A).

**Impact of H$_2$SO$_4$ on $\delta^{13}C_{\text{pollen}}$**

Average depletion of $\delta^{13}C_{\text{pollen}}$ values after treatment with H$_2$SO$_4$ is −3.2‰, but the isotopic shift varies highly among species and differ between pollen characteristics (Fig. 1; Tables 1 and 3A). A species-specific level of depletion for the $\delta^{13}C_{\text{pollen}}$ values after H$_2$SO$_4$ treatment has already been shown in other studies (Loader and Hemming, 2000; Nelson et al., 2006; Desclós-Gros and Schölzel, 2007; Nelson, 2012). $\delta^{13}C_{\text{pollen}}$ values of herbaceous plants and broad-leaved trees are on average −2.2‰ depleted, ranging between −0.15‰ (pollen of the family Asteraeae; Nelson, 2012) and −3.7‰ (grass pollen, Nelson et al., 2006). That is in accordance with the depletion of isotope values of broad-leaved trees detected in this study, which averages −2.8‰ after H$_2$SO$_4$ exposure (Table 3A).

In our study the impact of H$_2$SO$_4$ on $\delta^{13}C_{\text{pollen}}$ of the coniferous tree species P. sylvestris is distinct and the carbon isotope values are on average −5.0‰ depleted (Fig. 1; Table 3A). Loader and Hemming (2000) detected an average depletion of −2.18‰ for P. sylvestris $\delta^{13}C_{\text{pollen}}$ after treatment with H$_2$SO$_4$ and the comparison of chemically treated and raw pollen in their study gave a high correlation coefficient of $r^2$ = 0.93. A methodological reason for the different offsets between raw and chemically treated $\delta^{13}C_{\text{pollen}}$ values of P. sylvestris among the studies might be the usage of a Whatman microcentrifuge tube with a mesh to extract the pollen from the sulphuric acid by Loader and Hemming (2000). In our...
study, we slowly diluted the acid with deionized water until the velocity of the sulphuric acid was low enough to extract the pollen via centrifugation. The dilution had a warming effect on the liquid, whose impact on the $\delta^{13}C$ pollen values remain unknown. A similar method of slowly diluting the sulphuric acid was used by Bell et al. (2017), where a constant and low depletion of $-1.5‰$ could be found for coniferous Cedrus atlantica pollen but no impact of a warming effect was reported.

Loader and Hemming (2000) tested the impact of the treatment time of H$_2$SO$_4$ on P. sylvestris pollen and had most stable results after a treatment duration of 8 h. They suggested that the treatment time should not be <30 min, otherwise cellulose residues remained on the pollen. However, a longer treatment resulted in partly degraded pollen grains (Loader and Hemming, 2000). Based on these findings, we settled on a treatment time of 5 h and additional constant shaking (Table 2).

Figure 2. Scatter plots visualizing the relationship between raw and chemically treated $\delta^{18}O_{\text{pollen}}$ values (expressed in ‰, relative to VSMOW) of eight tree species (Alnus glutinosa, Betula pendula, Carpinus betulus, Fagus sylvatica, Picea abies, Pinus sylvestris and Quercus robur) after treatment with KOH (24 individuals, eight species), HF (nine individuals, three species), NaClO (20 individuals, seven species), H$_2$SO$_4$ (24 individuals, eight species) and the successive treatment all (23 individuals, eight species). [Color figure can be viewed at wileyonlinelibrary.com].
Impact of successive chemical treatment (all) on $\delta^{13}$C$_{\text{pollen}}$

The successive treatment protocol imitates the chemical exposure that a fossil pollen sample might need to be fully cleared of other organic and inorganic debris. For all species, a successive application of KOH, HF, NaClO and H$_2$SO$_4$ caused the highest depletion in $\delta^{13}$C$_{\text{pollen}}$ (between $-1.6\%_\circ$ for F. sylvatica and $-6.4\%_\circ$ for P. sylvestris). Even though this protocol avoids carbon-contaminating chemicals, the overall mean depletion of chemically treated $\delta^{13}$C$_{\text{pollen}}$ in comparison to raw $\delta^{13}$C$_{\text{pollen}}$ is $-4.0\%_\circ$ (Fig. 1).

In most cases, the successive treatment with four chemicals alters the samples only slightly more than sole treatment with H$_2$SO$_4$ (Table 3A). The average additional depletion of $\delta^{13}$C$_{\text{pollen}}$ for the successive treatment is $0.9\%_\circ$ (range: 0.4–1.4%) and the deviation between $\delta^{13}$C$_{\text{pollen}}$ values after applying the full protocol and the samples treated solely with sulphuric acid seems to be related to pollen morphology and family affiliation (Tables 1 and 3A). Thus, application of the full protocol may only in some cases be beneficial to purify samples for certain types of pollen designated for stable isotope analysis.

$\delta^{18}$O$_{\text{pollen}}$ values after chemical treatment

Chemical treatment affects the $\delta^{18}$O$_{\text{pollen}}$ of all species markedly (Fig. 2; Table 3B). The chemical treatment protocols to purify pollen from lake sediments were created to avoid chemical contamination of the $\delta^{13}$C$_{\text{pollen}}$ and was not adjusted for the application to analyse stable oxygen pollen isotope values. Hence, the application of chemicals results in some cases in strongly depleted pollen-isotope values compared to the raw pollen isotopes (Fig. 2; Table 3B).

Impact of KOH, HF and NaClO on $\delta^{18}$O$_{\text{pollen}}$

There are no comparable studies on $\delta^{18}$O$_{\text{pollen}}$ after chemical treatment procedures solely with potassium hydroxide (KOH), hydrofluoric acid (HF) and sodium hypochlorite (NaClO) available at present. Mean $\delta^{18}$O$_{\text{pollen}}$ values of B. pendula (+0.1%) and Q. robur (+1.0%) are enriched after exposure to KOH, whereas the isotope values of all other species are depleted in a range of $-0.3\%_\circ$ (P. abies) to $-2.6\%_\circ$ (A. glutinosa; Fig. 2; Table 3B). Even though the impact of KOH on $\delta^{18}$O$_{\text{pollen}}$ of different species is inconsistent, isotope values of raw and treated pollen seem to be related, especially when broad-leaved and coniferous species are looked at separately. Coniferous pollen have relatively thin pollen wall structures in relation to the grain size and thus may be more vulnerable to chemical treatment. In general, species-specific pollen membrane porosity needs to be considered when chemical substances are applied before stable isotope analysis (Loader and Hemming, 2000).

Treatment with hydrofluoric acid for the broad-leaved species C. avellana leads to enriched mean $\delta^{18}$O$_{\text{pollen}}$ values of $+0.5\%_\circ$, whereas mean $\delta^{18}$O$_{\text{pollen}}$ values of coniferous species are depleted, both in a similar fashion (P. abies: $-1.8\%_\circ$; P. sylvestris: $-1.7\%_\circ$; Table 3B). HF is the only non-oxygen-bearing chemical in this protocol, but nonetheless some diagenetic processes affecting the pollen wall occur during chemical exposure (Fig. 2).

Broad-leaved species have enriched $\delta^{18}$O$_{\text{pollen}}$ values ranging between $+1.1\%_\circ$ (C. avellana) and $+3.1\%_\circ$ (B. pendula) following treatment with NaClO (Fig. 2; Table 3B), whereas the values of coniferous species are depleted as a result of NaClO exposure (P. abies: $-1.7\%_\circ$; P. sylvestris: $-0.5\%_\circ$; Table 3B). What exactly causes the different impact on pollen isotope values remains unknown.

Impact of H$_2$SO$_4$ on $\delta^{18}$O$_{\text{pollen}}$

Raw and chemically treated mean $\delta^{18}$O$_{\text{pollen}}$ values of coniferous species deviate strongly after H$_2$SO$_4$ exposure (Fig. 2; Table 3B). In particular, P. sylvestris responses to H$_2$SO$_4$ treatment are depleted ($-17.9\%_\circ$) and also mean $\delta^{18}$O$_{\text{pollen}}$ Values of P. abies are highly depleted ($-9.7\%_\circ$). Broad-leaved species exhibit a distinctly weaker response to H$_2$SO$_4$ exposure. The average depletion of treated $\delta^{18}$O$_{\text{pollen}}$ values compared to raw $\delta^{18}$O$_{\text{pollen}}$ values of broad-leaved species is $-3.1\%_\circ$. The offset varies markedly between all broad-leaved species, and raw and treated $\delta^{18}$O$_{\text{pollen}}$ values do not seem to be related (Fig. 2).

A strong response of coniferous pollen to chemical exposure may be caused by the size of the pollen grains and a relatively thin pollen wall, thus having a larger surface area on which chemicals can corrode the organic material. This applies in milder form also to C. betula and Q. robur pollen (Fig. 2), which have the biggest pollen among the broad-leaved species examined in this study and a relatively thin pollen wall. However, the effect does not seem to be consistent: A. glutinosa, B. pendula and C. avellana are almost similar in size and shape (Beug, 2004), but the treatment affects B. pendula pollen more than A. glutinosa and C. avellana pollen (Fig. 2; Table 3B).

Impact of successive chemical treatment (all) on $\delta^{18}$O$_{\text{pollen}}$

Depletion of mean $\delta^{18}$O$_{\text{pollen}}$ values of broad-leaved species after successive treatment with all chemicals ranges between $-3.7\%_\circ$ (A. glutinosa) and $-9.1\%_\circ$ (Q. robur), whereas coniferous species pollen-isotopes are depleted by $-10.6\%_\circ$ (P. abies) and $-19.2\%_\circ$ (P. sylvestris, Table 3B). The successive treatment alters the $\delta^{18}$O$_{\text{pollen}}$ values of broad-leaved trees on average $3.9\%_\circ$ more than a treatment solely with H$_2$SO$_4$. However, the average additional depletion of coniferous species $\delta^{18}$O$_{\text{pollen}}$ is only $1.1\%_\circ$ (Table 3B). Thus, applying
the full protocol may have an assessable effect on some species, whereas others are severely altered. Applying chemicals before stable isotope analysis should generally be considered carefully, depending on the type of pollen and necessities based on the original fossil sample.

$\delta^{13}C_{\text{pollen}}$ in relation to $\delta^{18}O_{\text{pollen}}$

Species-specific response patterns are generally alike, also when carbon and oxygen pollen-isotope patterns are compared (Figs 1 and 2). Even though the overall offset is higher for chemically treated $\delta^{18}O_{\text{pollen}}$ values, the changes for each species among the different chemicals are almost similar. This may be caused by the species-specific pollen wall composition (proportion of sporopollenin) and shape of the pollen grain as well as the species-specific pollen wall coating (Table 1). The chemicals can alter the pollen isotopes only to a certain degree in relation to a species-specific pollen structure and vulnerability. Nevertheless, A. glutinosa and C. avellana show differing patterns for $\delta^{18}O_{\text{pollen}}$ and $\delta^{13}C_{\text{pollen}}$ after chemical treatment (Figs 1 and 2). This might be related to their plant-specific time of pollination from January to March, which is why these species have generally low baseline values of raw pollen isotopes (Müller et al., 2020).

Conclusions

Potassium hydroxide (KOH), hydrofluoric acid (HF) and sodium hypochlorite (NaClO) alter the $\delta^{13}C_{\text{pollen}}$ and $\delta^{18}O_{\text{pollen}}$ values of raw pollen species-specifically, according to their affiliation and pollen characteristics. The alterations are possibly caused by the removal of non-sporopollenin components of the pollen wall, whose composition and amount differ among species. However, the raw and chemically treated pollen isotope values seem to be related and thus the use of these chemicals to purify pollen samples before carbon and oxygen stable isotope analysis may generally be unproblematic. Care should be taken during the application of KOH on coniferous species before $\delta^{18}O_{\text{pollen}}$ analysis and the application of HF on pollen of broad-leaved species.

The impact of sulphuric acid ($H_2SO_4$) is highly species-specific and the offset between raw and chemically treated pollen-isotope values can be as high as 5.0% for $\delta^{13}C_{\text{pollen}}$ and 17.9% for $\delta^{18}O_{\text{pollen}}$ (both from Pinus sylvestris). $H_2SO_4$-treated and raw $\delta^{13}C_{\text{pollen}}$ values of broad-leaved species seem to be related, whereas pollen of coniferous species react more strongly to $H_2SO_4$ exposure and the impact seems unpredictable. Raw and $H_2SO_4$-treated $\delta^{18}O_{\text{pollen}}$ values are not related for either broad-leaved species or coniferous species. Even though $H_2SO_4$ is applied to extract sporopollenin while leaving it intact, stronger unknown alterations seem to occur.

In many cases, chemicals are needed to prepare fossil pollen samples before palaeoclimate investigations. Purification protocols for pollen should be adjusted due to the requirements of the source material, so the use of pollen-isotope-altering substances can be minimized. In general, protocols to purify fossil pollen samples and protocols to extract sporopollenin from modern pollen should avoid the use of sulphuric acid.

Supporting information

Additional supporting information can be found in the online version of this article.

Table S1. Dataset of $\delta^{13}C_{\text{pollen}}$ and $\delta^{18}O_{\text{pollen}}$ measurements of raw and chemically treated pollen. Three individual trees of the same species were treated with KOH, HF, $H_2SO_4$, NaClO and a treatment protocol (all) including all four chemical substances. The samples were measured with up to three repetitions.

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Data availability statement

Additional supporting information can be found in the online version of this study are available in the Supporting Information.

Abbreviation. IRMS, isotope ratio mass spectrometer.

References


Mikhael A, Jurcic K, Schneider C et al. 2019. Demystifying and Unravelling the Factual Molecular Structure of the Biopolymer Sporopollenin. Chemistry Department, Memorial University, St John’s, Canada.