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A MODIFIED, STEP-BY-STEP PROCEDURE FOR THE GENTLE BLEACHING OF DELICATE FOSSIL LEAF CUTICLES

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Abstract: Cuticular analysis has long been used by palaeobotanists for the identification of fossil leaves, and a variety of chemical procedures has been developed to extract and prepare fossil cuticles. However, even commonly used solutions may be too harsh for the preparation of extremely delicate cuticles. Here we offer a step-by-step protocol for the preparation of fragile conifer cuticles using sodium hypochlorite, otherwise known as household bleach. Conifer needles from the Miocene lignites of the Adendorf and Hambach open-mine pits in western Germany were prepared using a mild solution of this oxidizing agent. The cuticles had proven to be too fragile for most maceration chemicals, including Schulze's reagent, which even disintegrated the cuticles that were given a protective coating. However, it was discovered that trimming the leaf margins and damaged areas prior to a short exposure to 5–10% sodium hypochlorite solution resulted in the good preparation of the cuticle. Furthermore, this modified method allowed for the preparation of large areas of leaf. While this procedure may not be suitable for all cuticles, it is offered here as an easy and gentle method for preparing extremely delicate conifer cuticles that are destroyed by other chemicals and protocols.

Key words: conifer cuticle, cuticle preparation, cuticular analysis, fossil cuticle, palaeobotanical method, sodium hypochlorite

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Introduction

Cuticular analysis became a useful palaeobotanical technique in 1855 when Philipp Wessel and Otto Weber first identified similarities between fossil cuticles recovered from lignites and the cuticles of living plants (Wessel and Weber 1855). One year after this pioneering work, Johann Georg Bornemann compared Late Triassic cycad cuticles from Thuringia, Germany, to those of extant cycads (Bornemann 1856). Today, cuticular analysis is still one of the most reliable ways to identify fossil leaves, along with the study of leaf architecture and utilizing fluorescence microscopy and SEM (Kerp 1990).

Cuticle plays a key role in the identification of ancient leaves because it is able to persist even after other organic parts of the leaf have been diagenetically altered during fossilization. In leaf compressions, for example, the organic matter making up the mesophyll typically becomes coalified and is no longer viable for anatomical study or taxonomic identification. However, leaf cuticle, as a result of its waxy composition (van Bergen et al. 1995), can preserve diagnostic features evident in the epidermis, such as patterns in stomatal arrangement, guard cells, and subsidiary cells (Dilcher 1974). These features can then be used to identify leaf taxa, even down to the genus or species level (Kerp 1990), or to interpret palaeoenvironmental conditions (for overview, see Barclay et al. 2007).

In most cases, fossil cuticles must first be freed from a rock matrix and have any associated organic matter removed through maceration and bleaching before they are suitable for microscopy. However, maceration and bleaching require the application of caustic chemicals to clear the organic remains which can end up damaging the microscopic structures of the fossil leaf cuticle. The incorrect preparation of leaf cuticle can destroy what was once well preserved. For instance, Kerp and Barthel (1993) observed that abaxial cuticles of the seed fern *Alethopteris zeilleri* are rare in the fossil record in part because they are especially delicate and likely to be destroyed during maceration and preparation.

The most common preparation process for the maceration of fossil cuticle involves Schulze's reagent, which consists of nitric acid and either aqueous or dry sodium chlorate (Schulze 1855, Kerp 1990). This solution can be highly effective for

both extracting and bleaching cuticles, especially for bulk samples of coal. However, depending on the concentration of the nitric acid, it can also be an extremely reactive mixture, and overexposure to the harsh chemicals can result in the disintegration of the cuticles. Choosing the most appropriate preparation procedure depends on the type of fossil and matrix, and there are a number of other oxidizing agents that can be used for bleaching cuticles which include chromium trioxide, hydrogen peroxide, and sodium hypochlorite (see Kerp 1990 for overview). These chemicals are less likely to damage cuticles than Schulze's reagent. However, their effectiveness is variable, and they are only suitable for bleaching cuticles that have already been extracted from the sediment. Furthermore, especially thin and delicate cuticles may still be damaged or destroyed by these milder solutions.

Some preparation protocols have been developed to help preserve fragile specimens, such as the use of polyester overlays (Kouwenberg et al. 2007) or a protective coating of clear nail polish to prevent excessive degradation (Wang and Leng 2011). However, even these methods may still not protect extremely fragile cuticles. This proved to be the case of our conifer cuticles from Miocene lignites near Cologne. In order to prepare our cuticles for future taxonomic and anatomical study, we needed a process that minimized the deleterious effects of the oxidizing agent even more. The procedure that we developed has turned out to be an extremely successful method of preparation for our cuticles and may be helpful to others with other tricky cuticles.

Here we describe a step-by-step, effective, yet gentle method using a weak bleach solution that effectively prepares fossil conifer cuticle for microscopy. This method is modified from previous protocols by the trimming of leaf margins, tip, and any torn edges, before maceration with 5–10% sodium hypochlorite. Multiple attempts using conventional protocols to prepare the cuticles of fossil conifer needles from the Miocene lignites in western Germany either destroyed or rendered the cuticles unusable. In the published literature, sodium hypochlorite has been previously proposed as an alternative to more stringent methods (Rigby 1963, Kerp 1990, Kerp and Krings 1999), but the use of sodium hypochlorite alone did not effectively produce useable results in our specimens. Only a modified protocol involving the preparation of the cuticle before bleaching, limited exposure to chemical solutions, and a reduced number of steps and transfers resulted in consistent results for the delicate cuticles. Thus, while there are a number of different methods for preparing cuticle, we wish to share here the modified protocol that proved to be highly successful in preparing our extremely fragile conifer cuticles for light microscopy.

Materials and methods

Cuticles were extracted from two sets of conifer needles of Miocene age from the Lower Rhine Embayment, Germany. One set was collected from a lignite seam of the Adendorf clay quarry situated southwest of Bonn and the other from a lignite seam in the Hambach open pit mine located west of Cologne. In the lab, the conifer needles were lifted off the rock surface and stored in glycerol.

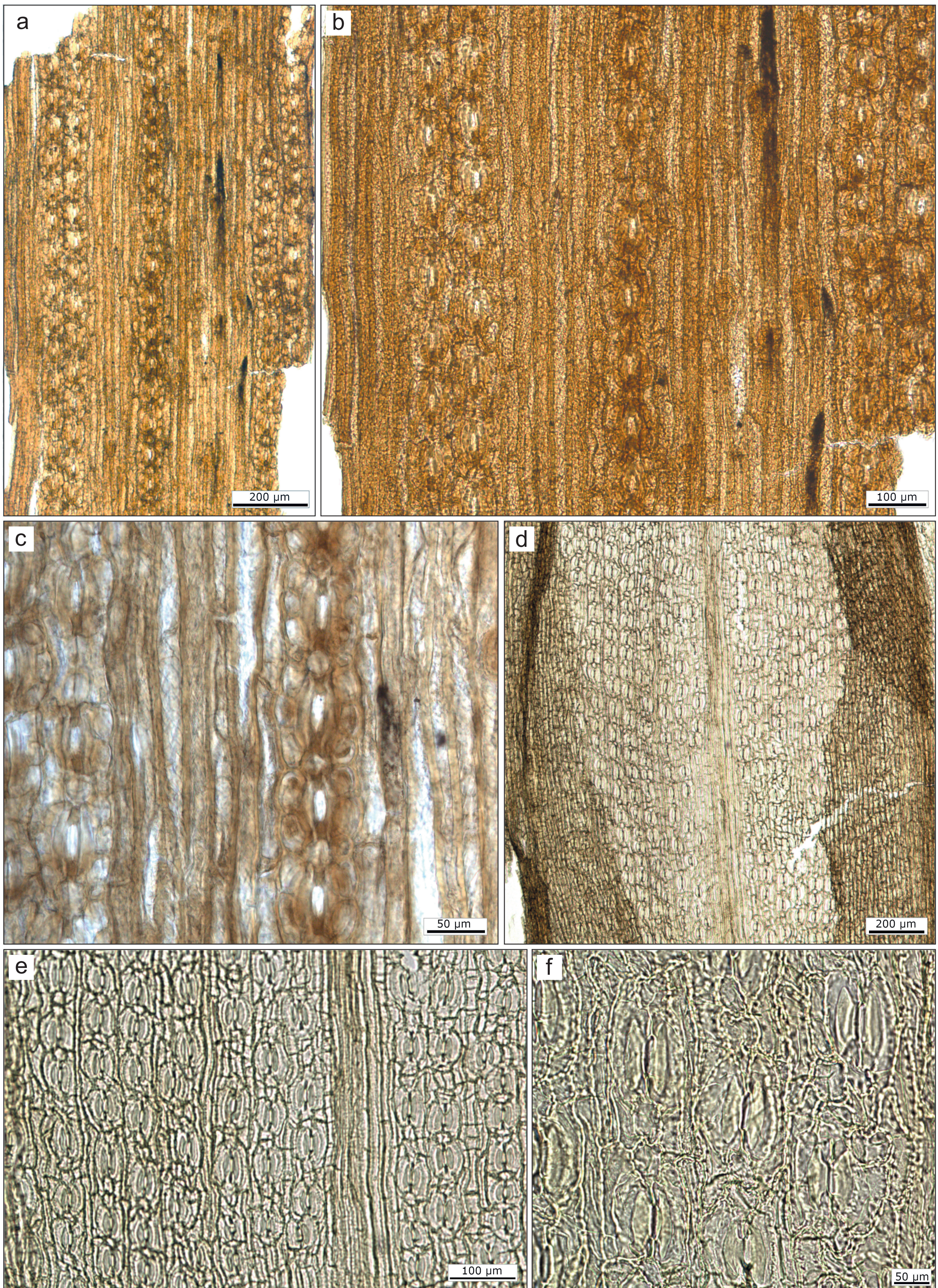
Before maceration with bleach, the needles were treated with 25% hydrochloric acid (HCl) for 10 minutes to remove carbonates, then rinsed with distilled water. This first step was followed by treatment in hydrofluoric acid (HF) for two days to eliminate any remaining silicates (Text-fig. 1, Steps 1–2). The needles were treated with HCl before HF to avoid the formation of calcium fluoride (CaF_2; see also Kerp 1990). After the removal of carbonates and silicates, the conifer needles were dried at ambient temperature overnight (Text-fig. 1, Step 3). Then, a scalpel was used to cut off the tip of the needle, if preserved, and to excise one leaf margin. If there were areas that were torn or otherwise damaged, these were also cut to produce clean edges. Long needles were trimmed to 0.5 to 1.0 cm length (Text-fig. 1, Step 4).

For maceration and bleaching, conifer needles were placed in individual test tubes and covered with 5–10% sodium hypochlorite (NaClO) (Text-fig. 1, Step 5). Needles remained in the NaClO between two minutes and two hours, as necessary; the time required to sufficiently bleach the cuticle depended on the individual leaf specimen. The needles were removed from the NaClO when each individual needle became semitransparent brown or amber, but before it reached the color optimal for microscopy, since some bleaching continued into the rinsing process. Because of the delicate nature of the cuticle, the bleach solution was decanted or siphoned from the test tube instead of physically removing the cuticle out of the solution. The cuticle was then rinsed several times with distilled water until the water ran clear (Text-fig. 1, Step 6).

If the cuticle was removed from the bleach solution too early and remained too dark, more NaClO was added, and

Pretreatment	1. 25% HCl 2. HF or sodium hexametaphosphate
Preparation	3. Dry overnight 4. Remove tips and leaf margins
Bleaching	5. Immerse in 2–10% NaClO • 2 m to 2 hr • Decant solution when amber color is achieved
Rinsing	6. Add distilled water, then siphon or decant it off • Repeat until water is clear • Return to step 5 if needed
Slide Preparation	7. Separate adaxial and abaxial layers 8. Remove mesophyll with tweezers
Mounting	9. Alcohol dehydration series 10. Apply resin, add coverslip 11. Allow to dry (3 days to 6 weeks)

Text-fig. 1. Step-by-step, modified procedure for the gentle preparation of delicate Miocene conifer cuticles for microscopy.



Text-fig. 2. Light micrographs of *Pinus* spp. cuticles prepared with the modified, gentle bleaching procedure. a: Cuticle 1, *Pinus* sp. 1. Nearly the entire width of the leaf has been preserved. Five parallel rows of stomata are visible. b: Cuticle 1, close-up of (a). Two guard cells are visible around each stoma. c: Cuticle 1, close-up of eight stomata. Two guard cells and eight subsidiary cells are visible around each stoma. d: Cuticle 2, *Pinus* sp. 2. Some folding of the cuticle occurred during preparation, but many parallel rows of stomata on both sides of a thin, central midvein are evident. e: Cuticle 2, close-up of (d). Pairs of guard cells surround each stoma. f: Cuticle 2, close-up of (e). Subsidiary and epithelial cells can be observed around the stomata.

the process repeated. Cuticle that was lightened too much was stained with Safranin O, but the staining process increased the potential for damage. In almost all cases, the cuticles were bleached to an optimal color, although a dark, organic strip deriving from the mesophyll sometimes remained intact between the abaxial and adaxial cuticles. In these cases, the leaf was placed on a glass slide and, using a dissection microscope, the abaxial and adaxial cuticles were separated from one another, beginning at the margin that was previously trimmed (Text-fig. 1, Step 7). With needle-nosed forceps and dissection needles, it was normally possible to lift out the remains of the mesophyll in one piece (Text-fig. 1, Step 8).

Because the cuticles were to be mounted later in Euparal resin, which becomes cloudy in contact with water, they were dehydrated by an alcohol series. To this end, solutions of 50%, 75%, 90%, and 99% ethanol were used sequentially (Text-fig. 1, Step 9). Again, to reduce the risk of damage to the cuticles, the needle was covered by each ethanol solution for at least two hours, which was then decanted before the next solution in the series was added to the same test tube. This stepwise dehydration of the cuticle reduced the risk of water being removed too quickly, thus causing shrinkage or adverse changes in the cuticle.

After the cuticle had completed the ethanol dehydration process, it was placed on a glass slide, and one drop of Euparal resin (Carl Roth GmbH + Co, Karlsruhe, Germany) was applied. The resin was spread into a thin layer with a dissection needle, which made the cuticle fragments less likely to drift once the cover slip was placed on the slide. The resin was allowed to sit for at least one minute to allow any bubbles to develop and escape, and for the resin to stabilize the cuticle. Under a dissection microscope, the cuticle's position was adjusted, and any visible bubbles removed. The cover slip was then applied by gently rolling it across the cuticle on the glass slide. Pressure was applied to the coverslip in gentle circles to remove any remaining bubbles, and extra Euparal was added to the edge of the coverslip if the seal was incomplete (Text-fig. 1, Step 10). The glass slide was allowed to dry horizontally for at least three days before excess Euparal was removed with a scalpel or with acetone-soaked cotton swabs, then returned to horizontal storage for another six weeks (Text-fig. 1, Step 11).

Results

Using the modified bleach method presented here, we were able to obtain large, well-preserved areas of conifer cuticle showing important anatomical details. Although the quality of the individual needles varied, in general, the fossil conifer cuticles showed very little degradation in response to this gentle oxidation process. In all cuticles, stomata, associated guard cells, subsidiary cells, and epithelial cells were visible. Because large areas of cuticle remained intact, stomatal arrangement across the entirety of the fossil needles could be observed in all cuticles.

Cuticle 1 (*Pinus* sp. 1, Pinaceae)

Five parallel rows of stomata are visible, with one row located centrally, and two rows to the left and two rows to the right. Two guard cells are positioned around each stoma (Text-fig. 2a–c), while eight subsidiary cells surround the guard cells (Text-fig. 2c).

Cuticle 2 (*Pinus* sp. 2, Pinaceae)

Although some folding of the cuticle at the leaf edges occurred, a well-preserved cuticular surface covered by multiple rows of stomata can be observed in parallel alignment on either side of a thin, central midvein (Text-fig. 2d). A pair of guard cells surrounds each stoma, and subsidiary and epithelial cells are clearly visible around the stomata (Text-fig. 2e, f).

Discussion

The modified mild bleach method given here was highly successful in our preparation of delicate fossil conifer cuticles, whereas attempts with conventional preparation methods with gentler methods in the published literature resulted in the degradation or disintegration of these specimens. Overall, large areas of leaf cuticle macerated with our modified bleach method remained intact in which the arrangement of the stomata across the leaf was clearly evident. The prepared cuticle also exhibited other important diagnostic features, such as the shape, size, quantity, and placement of the guard cells, subsidiary cells, and epidermal cells (Text-fig. 2), in part because any exposure to chemicals that might have degraded the specimens was limited. Cuticles were also found to be consistently lightened across the lamina and the use of stain was unnecessary, which further contributed to the structural integrity of the cuticles.

Although the use of a weak bleach solution for oxidizing fossil cuticle has been previously described (Rigby 1963, Dilcher 1974, Kerp 1990), the preparation of the cuticle beforehand by removing at least one margin, as we suggest here, improves the consistency of the results and reduces overall damage to the cuticle. Removing the leaf apex and margins, as well as trimming torn parts of the needles, increases the rate at which the NaClO bleaches the cuticle, thereby reducing the time that the cuticles are subjected to harsh oxidizing agents. It also leads to more consistent bleaching across the leaf, instead of more rapid bleaching near torn edges and slower bleaching in the interior areas of the leaf. Uneven bleaching often causes the more rapidly bleached areas to begin degrading or to lighten too much from overexposure at the same time the other parts of the cuticle remain too dark. By removing damaged areas and at least one margin, uneven bleaching is nearly eliminated.

Our gentle bleaching method also generally avoids the need for staining, which can lead to damage in delicate cuticles due to the additional handling that is required. For fragile cuticles, it may thus be preferable to bleach for a shorter length of time to avoid the need for staining. While the bleaching process could be completed in as little as two minutes, the observed change is gradual and can be readily stopped or restarted as needed. However, if the prepared cuticle is too transparent for light microscopy, fluorescence microscopy will allow for the clear observation of cellular details without staining the specimen (van Gijzel 1978, Kerp 1990, Kerp and Krings 1999).

Additional modifications can be made to our method, depending on the resources that are available and the requirements of the individual leaf specimen. Although the cuticles in our study required only 10 minutes in 10% HCl solution to fully remove carbonate sediments, it may take up to several hours with other leaf samples. In this case, the cuticle should remain in the HCl until the solution has stopped bubbling. While rinsing, pH test strips or a pH meter can be used to ensure that all the HCl has been removed. Secondly, our preparation involved the use of HF, which is a highly dangerous and corrosive acid that should only be handled by certified technicians. An alternative to HF is sodium hexametaphosphate $Na_6[(PO_3)_6]$, which has been deemed as safer, more accessible, and more environmentally friendly (Riding and Kyffin-Hughes 2010).

Our modified method calls for drying the cuticle overnight, but some cuticles may become excessively fragile when dried. Thus, while our conifer needles were easily trimmed when dry, it might be necessary to trim other cuticles without drying first. Similarly, physical handling with forceps may damage cuticles. An alternative to forceps is the use of 2–5% KOH to remove the organic substances left behind after bleaching. Manual removal of the mesophyll remains is recommended when the mesophyll remains are so compacted that they can be readily lifted out, as was the case in our cuticles.

Furthermore, different concentrations of bleach can be applied, including those in household cleaners. The solution used in our method is stronger than those typically reported in the literature, which range from 1% to 5% dilution (e.g., Kerp 1990, Wagner-Cremer et al. 2010). The higher concentrations (up to 10%) utilized in our many trials did not negatively impact the quality of the cuticles, although a more diluted solution may be equally effective and more affordable. Additionally, as in the HCl treatment, pH test strips or a pH meter can also be used when rinsing to verify that all the sodium hypochlorite has been washed out.

Other methods of preparation were carried out on these same sets of Miocene conifer needles, but none of them achieved satisfactory results. The application of a dry Schulze's reagent was attempted, but after even a short time in the solution, the needles completely disintegrated when neutralized in 5% potassium hydroxide (KOH). Rinsing in water instead of KOH resulted in severe fragmentation of the cuticle, which was a problem also noted by Rigby (1963). In another trial following the method recommended by Wang and Lang (2011), the needle was coated in clear polish to increase its integrity and durability before it was subjected to Schulze's reagent, then rinsed with water. Unfortunately, this procedure did not help preserve the specimens. Finally, cuticles were placed in sodium hypochlorite without trimming the leaf margins first. In these trials, bleaching was uneven; some areas, generally those close to the margins or in damaged areas, lightened more quickly than the leaf blade. By the time the leaf blade had been sufficiently bleached, the more exterior portions had begun deteriorating. This uneven bleaching occurs with any method in which the oxidizing chemicals enter into different areas of the cuticles at different rates.

The one disadvantage of the modified bleach method is that the removal of the cuticle from the bleach solution is imprecise and nonintuitive, because the cuticle must be removed from the bleach before it appears to be ideal for microscopy. In fact, in our many trials, it was often observed that the greatest change in color occurred during the rinsing process, though this was not always the case. Therefore, it is easy to either over- or under-bleach the specimens until familiarity with the process is developed. Moreover, unlike protocols involving Schulze's reagent, this method is not suitable for macerating bulk samples, although it could be used on specimens that have been freed from the matrix but still require additional bleaching (Kerp 1990). It may also not be as effective on all leaf types; the long, thin, nearly complete needles examined here were easily altered with a scalpel to remove damaged areas and to remove margins to allow for more even bleaching. Cuticle from leaves with broader shapes, such as those of dicots, may not benefit from trimming the leaf margins, though the use of sodium hypochlorite itself may still be effective (cf. Dilcher 1974).

Conclusions

Our search for a gentle method of cuticle preparation led us to develop a step-by-step procedure for the gentle bleaching of delicate fossil cuticles. Previously, the fossil conifer needles from Miocene lignites were consistently destroyed by the use of Schulze's reagent and produced unusable results with only 5–10% sodium hypochlorite solution. By using the modified weak bleach method given here, large areas of cuticles could be prepared, remained intact, and yielded good diagnostic information on the leaves. The trimming of the apex, margins, and damaged areas of the needles resulted in even bleaching and less exposure to destructive chemicals. Furthermore, reducing the number of steps and physical transfers of the cuticular material were also crucial to maintaining the integrity of specimens. Although the best results for individual cuticles can only be achieved on a case-by-case basis, the modified method described here provided consistently high-quality results in otherwise unpreparable cuticles and may be useful for other difficult-to-prepare specimens.

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