Preserving colour of botanical wet specimens: bibliographic review and tests of historical recipes

Elodie Granget, Marion Dangeon, Joane Latty, Laura Brambilla

Haute École Arc Conservation-restauration, HES-SO University of Applied Sciences and Arts Western Switzerland, 2000 Neuchâtel, Switzerland

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Abstract

This paper presents the results of a study on colour preservation of botanical specimens in fluid. The article includes: 1 - An introduction on plants pigments and discolouration issues specific to botanical specimens, focusing on the leaching of pigments from the specimen into the fluid, and on the denaturation of the pigments (through photodegradation, oxidation or polymerization) either in the specimen or in the fluid; 2 – An extensive bibliographic review of historical recipes invented specifically to preserve the colouration of plants as a preparation step; 3 – Results of testing some recipes on freshly prepared specimens, and their discolouration rate compared to reference specimens that were kept in 70% ethanol solution. None of the tested recipes gave results that are optimal to preserve all aspects of a specimen. In fact, colour preservation or prevention of fluid opacification came at the cost of either loss of structural stability, changes in chemical composition of pigments preserved, or loss of other colours. Ultimately, the choice of preserving the colour of wet botanical specimens should be made on the intended use of the specimen.

Keywords: Botanical specimens, discolouration, pigments, wet collections, colour preservation

Introduction

This is a follow-up paper to the article "Losing colour: the discolouration of plants in spirit preserved collections" published in 2022 (Granget *et al*., 2022). In our long-term experiment, we collected 3 (2021-2024) to 5 (2019-2024) years of experimental data on fluid preserved botanical specimens with the aim of understanding how to better preserve their colour. In addition, a literature search for historical and modern recipes targeting colour preservation and testing on fresh specimens are included.

Fluid preservation techniques, initially developed in the 17th century for animal and human specimens, have evolved significantly over time (Simmons, 2014; Neumann et al., 2022). The process of preservation generally involves several key steps: the collection of the specimen, followed by fixation through injection or immersion in a solution, rinsing, mounting in a jar, filling the jar with a preservative fluid, and finally, sealing the container. The application of fluid preservation methods gradually became more common in botanical collections in the 19th century (Moore, 2010). Although fluid preservation is not the predominant method for conserving botanical specimens, it is still used as a valuable alternative to drying, freeze-drying, or pressing, especially for specimens with significant volume and complex or fragile three-dimensional structures that are

challenging to preserve by other means (Bridson and Foreman, 1998). Botanical collections encompass a wide range of non-zoological specimens, including vascular plants, fungi, bacterial pathogens, algae, and corals. Despite belonging to different biological kingdoms, these specimens have traditionally been grouped and studied within the field of botany (Morton, 1981).

Historically, alcohol-based solutions were employed as the primary preservative fluids (Moore, 1999; Neumann et al., 2022), until aqueous formaldehyde solutions replaced alcohol as a more effective and cheaper fixative in the late 19th century. Formalin solution remained widely used in collections also for long-term preservation, until concerns over its toxicity prompted a re-evaluation of its use. The primary objectives of fixation include enhancing the mechanical strength and chemical stability of tissues while inhibiting autolytic processes such as enzymatic degradation (Venteo & Velot, 2010). Due to the health risks associated with formaldehyde and its limitations in DNA preservation compared to alcohol (Carter, 2003), there has been a shift back to alcohol-based preservatives in the northern hemisphere, particularly ethanol or denatured alcohol such as Industrial Methylated Spirit (IMS), and a growing interest in using glycerol, a non-toxic but denser alternative to formaldehyde solutions (Neumann et al., 2022).

In botanical collections, most specimens are preserved in 70% alcohol, typically IMS or full-strength ethanol. In case of an initial fixation step, 4% formaldehyde solution or a commercial formaldehyde-acetic-acid (FAA) solution is used (Prakash, 2019).

Due to the fundamental differences between the cell structure of vascular plants compared to those of algae and fungi, fluid preservation methods have been adapted to meet specific requirements. This paper focuses on vascular plants only.

Discolouration phenomena

Colour is a crucial feature of botanical specimens, providing insights into the function of coloured organs, such as attracting pollinators, defence mechanisms, and photosynthesis. Unfortunately, most current preservation methods, whether dry or wet, fail to maintain the in-vivo colour, leading to an inevitable loss of valuable scientific information. This issue highlights the importance of accurately recording by other means (e.g., photography or illustration) the in-vivo colours of specimens (Bedford, 1999). Botanical wet specimens are particularly prone to colour loss, resulting from pigment alteration within the tissues and the migration of pigments into the preservation fluid (Butler, 1918; Granget et al., 2022). The degradation of these pigments also impedes exhibition, as discolouration can obscure public understanding and, in extreme cases, render the specimen unrecognizable. In this paper, we consider three main mechanisms for the degradation of pigments, chemically affecting the colouration of a specimen:

- Leaching is the extraction of the pigments into the preservative fluid. It is accelerated by frequent handling of the specimen (Latty 2021) and causes fading or changes in the colouration of the specimen itself. In most cases, the leached pigments colour the fluid (Butler, 1918; Granget et al., 2022).
- Photodeterioration is the fading of colour of the specimen or the leached pigments in the fluid by exposure to ultraviolet radiation (Groeneveld et al, 2023).
- Oxidation or polymerization of some pigments may cause the yellowing or darkening of the specimen and the fluid, and is accelerated by the presence of oxygen or inappropriate pH-shifts of the solution.

Plant pigments

Plant pigments are diverse, making their conservation during fluid preservation a complex task. Understanding pigments is essential for developing preservation techniques that prevent their discolouration and thus support the visual and structural integrity of botanical specimens. Besides the impracticality of analysing each specimen's individual pigments before preservation, some generalizations on plant pigments are possible. This paper groups the main pigments into two categories: i) Colourful pigments, mostly prone to leaching and photodegradation, and ii) brown or transparent pigments that darken in the specimen or opacify the fluid through polymerization or oxidation. A more comprehensive list of pigments and their solubility is presented in Appendix I.

- Green pigments (i): the term chlorophyll indicates a group of pigments from the tetrapyrrole family, which vary in form and structure. Chlorophyll a and b are the most common forms in vascular plants. It is a primary pigment of chloroplasts, the plastid responsible for photosynthesis of all green plants, and has a pivotal role in this process (Davies, 2004; Ralph et al., 1970). Chlorophyll

has two important parts: a ring-shaped structure (called the chlorin ring) that captures sunlight, and a magnesium ion at the centre that keeps the structure stable. Together, they turn sunlight into energy for the plant.

- Blue-red pigments (i): are hydrosoluble pigments occurring mostly in the vacuoles, their hues may be influenced by shifting pH levels. For example, red cabbage turns blue when put in contact with an acid such as lemon juice. They are either betalains, anthocyanins or other colourful flavonoids (Davies, 2004; Delgado-Vargas et al., 2000). Depending on the plant, the same type of pigment will express a different hue on the blue-red spectrum.

- Yellow, (and orange-red) pigments (i): These colours are more challenging to assess. In photosynthetic organs (leaves) or some fruits, the pigments are likely carotenoids and therefore liposoluble (Delgado-Vargas et al., 2000). However, in flowers, these colours could originate from either carotenoids in plastids or other hydrosoluble pigments in vacuoles (betalains or flavonoids), requiring more research before preservation. If carotenoids are known to be very stable, betalains and flavonoids (such as anthocyanins) are way less stable and very sensitive to pH shifts (Davies, 2004; Delgado-Vargas et al., 2000).

- "Tannins"(ii) often refers to a variety of molecules that may cause browning of the preservation fluid and specimen, such as phlobaphenes, heteropolymers with bound anthocyanins, quinones, and mostly phenolic compounds. Polyphenolic compounds are present in all plant parts and characterized by their ability to bind and precipitate proteins, a property that is exploited in tanning industry. In plants, they play crucial roles as defence mechanisms against herbivores, pathogens, and UV radiation, as well as in regulating growth and development (Arbenz & Avérous, 2015). Tannins are not all colourful while in the plant, but they can darken through oxidation or polymerization (Khanbabaee & Van Ree, 2001). It is worth noting that there are exogeneous sources of tannins in fluid preserved specimens, such as camphor introduced as antiseptic, or resin-based denaturant in ethanol.

The rate at which plant pigments leach into common conservation fluids has been assessed in systematic studies (Dangeon et al., 2020; Granget et al., 2022). They show that none of the tested fluids were effectively preventing discolouration and highlight the various rates at which the fluid can opacify. Moreover, the tests imply that chlorophyll and carotenoid-rich specimens stored in alcohol-based preservatives (full-strength ethanol and rum in these studies) leach most of their pigments within the first few weeks, with no significant difference between those with and without prior fixation (formaldehyde 4% or FAA) (Figure 1A and C). The fluid usually turns yellowish, indicating pigment breakdown. Glycerol and aldehyde-based preservatives preserve colour better, though some yellowing and browning still occur. Betalain specimens leach pigment almost immediately, with colour shifting to yellow-orange in alcohol-based preservatives and brownish-red in formaldehyde-based solutions (Figure 1B). Tannin-rich specimens, such as walnuts, leach dark pigments, with glycerol slowing the process but eventually matching the colour of other fluids after two years (Figure 1D) (Dangeon 2020, Granget 2022).

The experimental set-up for assessing the discolouration rate in common fluids is the same as for the study of colour-preserving recipes (see materials and methods).

Bibliographic review

The recipes tested in this study were selected after an extensive literature review (Figure 2; complete list of references in appendix II). This paper will only give an overview of the chronological appearance of recipes aimed at maintaining the colour information of botanical specimens. The aim of this section is to give context regarding the major principles behind various recipes. Several variations of these recipes exist, only the ones tested are detailed in the material and methods section of this paper.

Attempts to preserve the colours of plants in fluid began in the late 19th century, inspired by a common practice in the canned food industry, particularly in France (Wiley et al., 1887). This practice involved maintaining the green colour of vegetables by boiling them in copper-alloy vessels, which is still applied today on a larger scale with additives such as copper sulphate. Before the various structures of the chlorophyll were discovered and explained by Fischer and colleagues in 1930 (Seely, 1966), scientists including Tschirch and Schunck (Wiley et al., 1887) rightly suggested that copper might be attaching to this molecule rather than just acting as a dye. It is indeed a complexing action.

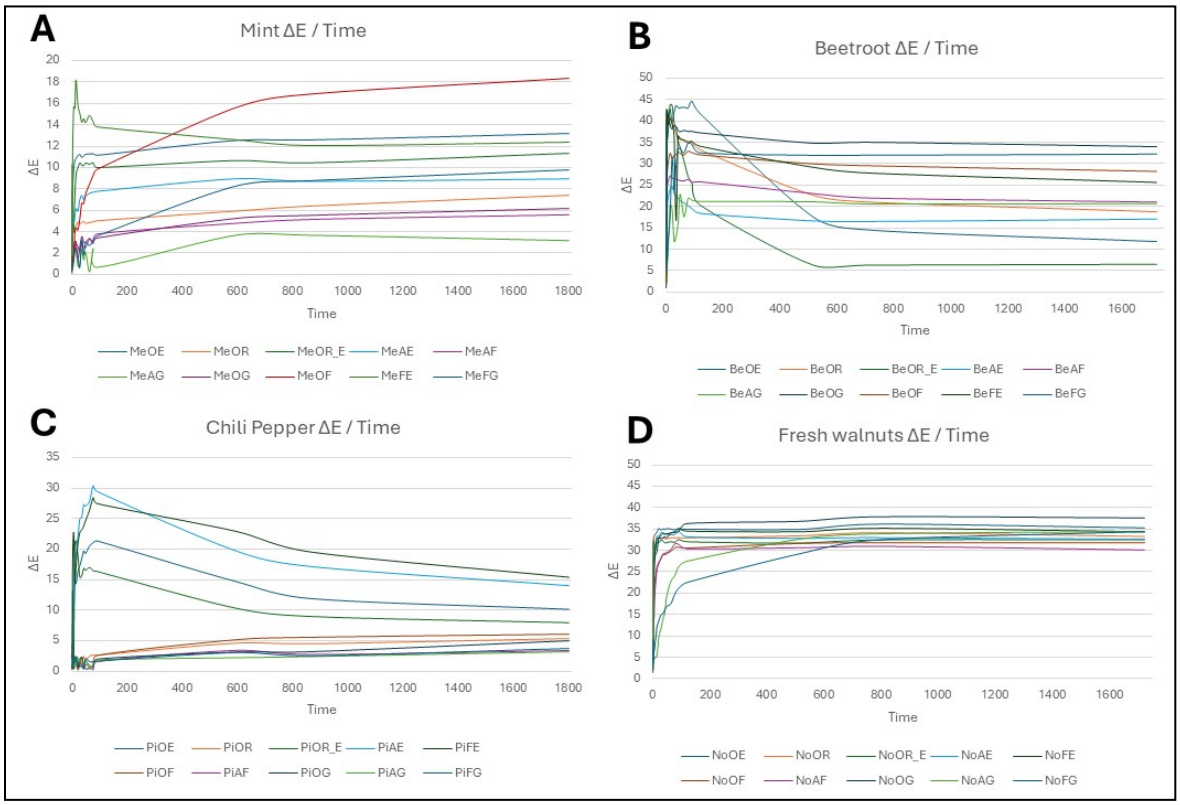


Figure 1: Evolution of Delta E for tested specimens. A. Mint (Me), B. Beetroot (Be), C. Chili pepper (Pi), D. Walnuts (No). Abbreviations are specimen + preparations. OE: no fixation, stored in undenatured EtOH 70%, OR: no fixation, stored in commercial rum, OR_E: temporary storage in rum, stored in EtOH 70%, AE: fixed in FAA, stored in EtOH 70%, AF: fixed in FAA, stored in formaldehyde 4%, AG: fixed in FAA, stored in 70% glycerol, OG: no fix. stored in glycerol, OF: fixed and stored in formaldehyde, FE: fixed in formaldehyde, stored in EtOH 70%, FG: fixed in formaldehyde, stored in glycerol 70%. Summarizing results from Dangeon 2020 and Granget 2022. ©HE-Arc 2024

Chlorophylls are chemical complexes with a cyclical tetrapyrrole as ligand and magnesium (Mg^{2+}), as complexed ion. The ligand acts as a claw, holding the ion in its centre, but this Mg^{2+} ion is easily substituted by two hydrogen ions (H^{+}), through protonation, under the influence of heat or acid, resulting in duller and darker pheophytin. This degradation process eventually ends with cleavage of the porphyrin ring (tetrapyrrole), leading to colourless derivatives. Mg^{2+} can also be substituted by other ions such as zinc (Zn^{2+}) and copper (Cu^{2+}), because they have higher electrophilic affinity with the chlorophyll-complex and replace the Mg^{2+}-ion easily. Thus, the recipes introduced above employ different copper salt solutions to preserve green colour in plants. It seems that the first reports of preserving colour in botanical collections using copper salts date back to around the mid-1890s. The ability of maintaining colours in the original plant material through transformation of their colourising components rather than artificially dyeing the specimens likely influenced the decision to use these methods.

After the fundamental studies by Woods (1897) and Trail (1908), numerous botanists and others have revised, adjusted, or developed new methods to preserve colours of plants in fluid throughout the 20th century (see review in Hangay & Dingley, 1985). The primary focus was on keeping the green shade with copper salts, while only few others investigated the potential of conserving other pigments in plants. Though these copper salts recipes were designed to maintain the green colour of plants, they were also applied to specimens with other colours (Woods, 1897).

Most other attempts to specifically preserve colours were directed towards preventing oxidation. Sulphites were mainly suggested as antioxidants for prevent the browning of clear specimens , e.g., in fruits and parasitical plants (Strasburger, 1911; Butler, 1918; Nieuwland & Slavin, 1928; van Steenis, 1935). Additionally, the use of antioxidant was also proposed to preserve colours, mostly yellow, orange and red (Cruess and Christie, 1922; Adriano and Yonzon, 1933; van Steenis, 1935; Scully 1937). Scully noted that these recipes were not effective for red or blue

colours (Scully 1937). The effectiveness of antioxidants such as sulphites in keeping red colour information in plants likely varies depending on the pigments involved, such as anthocyanins, betalains, or carotenes, which have different structure, solubility, and stability.

The most common red and blue pigments are anthocyanins or betalains, they are hydrosoluble and their colour-information is easily affected by pH shifts. Wagstaffe and Fidler (1968) suggested using a zinc chloride solution ($ZnCl_2$) dissolved in a mixture of formaldehyde and glycerol to preserve these colours (Wagstaffe and Fidler, 1968). Interestingly, they also proposed another solution including a tert-butyl-alcohol, with the addition of a reducing agent, and a complexing agent (thiourea and sodium citrate/citric acid), to preserve red and blue flowers, which contain delicate anthocyanins.

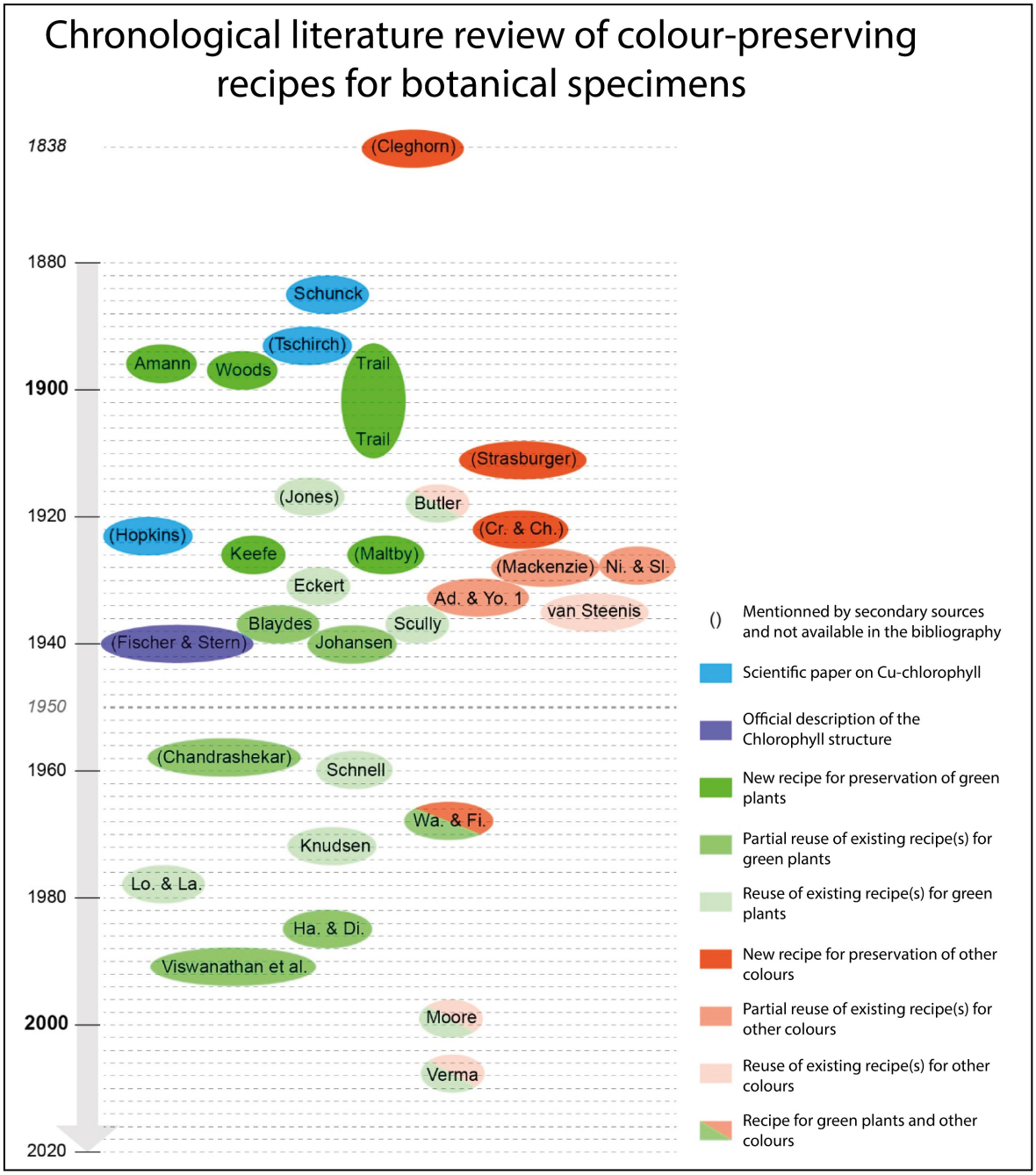


Figure 2: Chronology of colour-maintaining recipes, by author. Translated from Latty 2021. ©HE-Arc 2024

Materials and Methods

To assess how effective the colour-preserving recipes found in literature are, the methodology introduced in Granget et al. (2022), presented below, was followed.

Monitoring

Test-specimens were freshly prepared, following a selection of recipes presented in the next section. They were then kept in a dark environment in a solvent cabinet and underwent both qualitative and quantitative assessments periodically. Test-specimens were monitored daily over the first week, then weekly for 2-3 months and then monthly for 12-18 months. All samples were measured for long-term assessment in 2024 (3-5 years after first measurements). The qualitative and comparative approach consisted of photographic documentation, while the quantitative analysis was conducted on the fluid using a portable spectrophotometer measuring in the visible light range (X-rite® Ci62). For this quantification, 6 mL of the preservative fluid sampled from the individual jars containing the specimens, was put in a special vial for the colourimetric measurements and returned into the jars after measuring.

Fluid colourimetric values in the CIELAB colour space (ISO/CIE 11664-4:2019(E)) were extracted from the spectra acquired.

The CIELAB colour space characterizes colour using three parameters: L* for lightness ranging from 0 (black) to 100 (white), a* for the green-red axis ranging from -a* (green) to +a* (red), and b* for the blue-yellow axis ranging from -b* (blue) to +b* (yellow). These values are relative to a specific illuminant, with D65 (standard daylight) utilized for this protocol.

The extent of colour change was quantified using Delta E (ΔE), representing the Euclidean distance between two points in the colour space. In this case, the comparison was made between the L*a*b* values for the fresh preservative solution (T0) and those on the monitoring day (Tx). A Delta E value approaching zero indicates minimal perceptible colour difference, while a Delta E >2 is perceivable by the human eye. In this study, values exceeding 10 were considered as notable colour deviations (Granget et al, 2022).

Selected recipes

Some of the recipes (Table 1) presented in the bibliographic review have been subjected to close monitoring for at least 3 months, and their usefulness for longer term conservation for 3 to 5 years. For control, fresh specimens were prepared and directly preserved in 70% full-strength ethanol (EtOH) without initial fixation step or other specific colour-preserving treatment. Both the colour retention and the specimen's integrity were evaluated during monitoring.

In the following sections, unless specified differently, "EtOH" or "ethanol" indicates the use of full-strength, non-denatured ethanol, and "water" implies demineralised water.

Table 1. Overview of all tested recipes

Colour	Recipes tested
Green	Copper based recipes: $CuSO_4$ Cu Acetate (II) $Cu(CH_3COO)_2$ $CuCl_2$
Yellow	Scully B: copper salt initial treatment, preservation in sulfuric acid and sodium sulfite solution. Verma 2: propionic acid, formaldehyde, and water for fixation, with glycerol added for preservation
Blue-red	Wagstaffe & Fidler 4: tertiary butyl alcohol, thiourea, and a pH modifier Kew mixture: IMS, water, formaldehyde, and glycerol Copenhagen: IMS, water, and glycerol
Tannins	Antioxidants recipes: aqueous SO_2 Polymerisation recipes: Aldehyde fixatives

Treatments to retain Green colour

Recipes aimed at retaining green colour of pigments in plants are numerous (see Figure 2). Most of these seem to rely on the substitution of metal ions in the chlorophyll complex, thus creating a new and more stable pigment. Variants of these recipes were tested (Latty, 2021) on

young pea leaves (*Pisum sativum* L.). Because they are globally the most effective, only recipes based on copper salts will be presented in this paper. Different copper salt and solvent combinations were tested by using them in an initial step before storing the specimens either in 70% ethanol or leaving them in their initial solution (Table 2).

Table 2. Copper salts recipes used for the preliminary treatment of the samples

Copper salt	Solvent	parts
$CuSO_4$	Water	1:20
	FAA	0.2:100
Cu Acetate (II) $Cu(CH_3COO)_2$	Acetic acid 50% (in water)	19:150
	FAA	11:200
$CuCl_2$	Water	1:100
	FAA	1:100
Control: No salt	Full-strength EtOH 70% in water	

More recipes aimed at maintaining the green colour proposed in the literature were tested rapidly, followed by visual observation and documentation. They included boiling the specimen in Cu acetate (II) before preserving it in 70% EtOH (supposed to enhance and prevent the leaching of pigments) or, in some variants of $CuSO_4$ preparations, adding 5% aqueous SO_2 solution ($SO_{2(aq)}$) (Latty, 2021). These results will not be presented in this paper.

Yellow pigment

Yellow Yarrow (*Achillea millefolium* L.) were used to test two recipes to maintain the yellow colour (Table 3). The yellow colour of yellow yarrow is primarily attributed to carotenoids, though some flavones are co-pigments contributing to their colour (Raudone et al, 2024).

Blue-red pigments

For chemically delicate colours such as blue, red, and purple, tests were carried out on *Glandularia peruviana* L. Small. (red and purple colour) and *Lobelia erinus* L. (blue colour). The vibrant colours of these flowers are mostly due to the presence of anthocyanins and other flavonoids (Chai et al, 2024). Three recipes were tested (Table 4).

Table 3. Recipes tested for the preservation of yellow colour

Scully B	Fixation in 5% copper sulphate; subsequent transfer and preservation in a mixture of 1000 mL water with 16 mL commercial sulphuric acid, and 21 g sodium sulphite.
Verma 2	Fixation in 1 mL propionic acid ($CH_3CH_2CO_2H$), 1 mL formaldehyde, 100 mL water; preservation in same mixture + 2 mL glycerol.
Control	70% EtOH in water

Table 4. Recipes tested for the preservation of blue and red colours

Wagstaffe & Fidler 4	100 g tertiary butyl alcohol, 1 g thiourea, 2 g sodium citrate (for blue flowers) or 2 g citric acid (for red flowers).
Kew mixture	53% IMS, 37% water, 5% formaldehyde and 5% glycerol
Copenhagen	70% IMS, 28% water, 2% glycerol
Control	70% EtOH in water

Tannins

The preservative fluid especially of tannin-rich plant specimens often darken significantly due to extreme leaching of tannins. These include compounds that are either dark in vivo or subsequently darken through polymerization and oxidation as the specimen degrades (this is the case of parasitic plants). To prevent oxidation, a SO_2(aq) solution can be used, prepared by adding $NaHSO_3$ to 70% ethanol, buffering the solution to pH 1.8 with hydrochloric acid, and filtering. Two other methods to prevent pigment migration involve using a non-polar fluid, like liquid paraffin, or polymerizing phenolic compounds with 3.5% formaldehyde and/or phenol. These recipes are only suitable for specimens that are naturally dark, as the fixation process further darkens the tissues (Latty, 2021; Latty et al., 2021).

Table 5. Recipes tested for the prevention of fluid opacification through oxidation of the tannins in parasitical plants.

Fixation step	Preservative fluid
No pre-treatment	$SO_{2(aq)}$ in EtOH 70%
Paraffin impregnation	$SO_{2(aq)}$ in EtOH 70%
Paraffin impregnation	EtOH 70%
Paraffin impregnation	Paraffin
Formaldehyde 3.5%	Glycerol 70% (polar fluid causing less leaching than EtOH)
Control 1: No pre-treatment	Glycerol 70%
Control 2: No pre-treatment	70% EtOH in water

Table 6. Recipes tested to prevent opacification of the fluid through prior fixation of tannins in Pinus *sp. bark.*

Fixation	Recipe	Time
Formaldehyde	35% in water	1 day
	17.5% in water	1 day
	3.5% in water	1 day
	3.5% in water	1 day (kept in the dark)
	3.5% in water	3 days
Formaldehyde / phenol	17.5 + 5%	1 day
	17.5 + 0.5%	1 day
	3.5 + 0.5%	1 day
	3.5 + 5%	1 day
Control	70% EtOH in water	No fixation

Orobanche hederae (Duby) was chosen to test the effects of antioxidants and a non-polar solution, based on studies of browning in parasitic plants. The recipes are summarized in Table 5.

To test the polymerization of phenolic compounds, the bark of *Pinus* sp., known for its high content of condensed tannins (Pizzi, 2008), was used. Various concentration and fixation times, as listed in Table 6, were tested, and all specimens were then stored in ethanol 70%.

Results and discussion

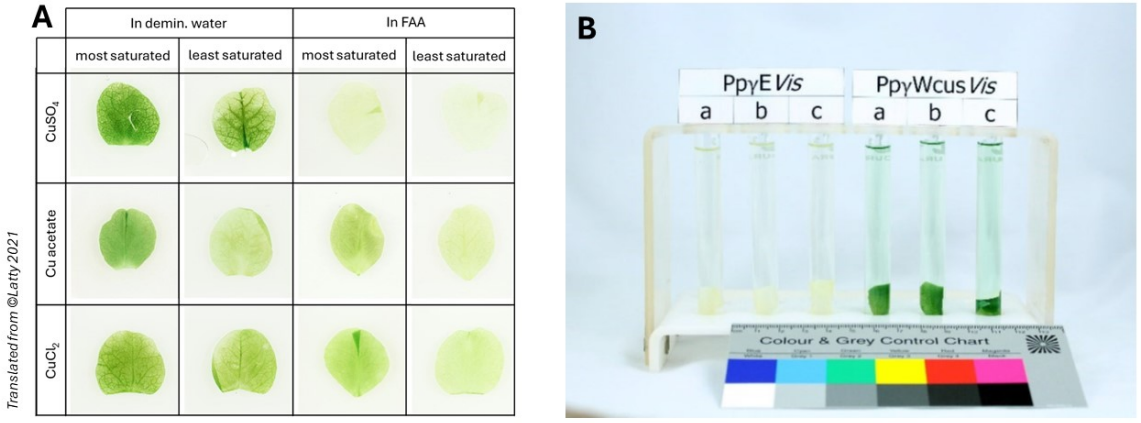
The main observations after several months of monitoring and long-term assessment of all the samples for 3 years (with some results after 5 years) are presented grouped by colour. More detailed results for the green pigment and browning of specimens have been published separately in French (Latty, 2021; Latty et al., 2021). Only main conclusions of these tests are summarised in English in the next section.

Green pigments

Overall, copper salts are efficient at enhancing the colour stability of green leaves, as the specimens subjected to photo-aging demonstrate (Figure 3). However, the hue of the fluid diverges from the untreated specimens kept in 70% ethanol, shifting from the usually observed yellow-green to a bluish -green in the $CuSO_4$-treated samples. This is due to the substitution of the magnesium ion with copper, as confirmed analytically with LC-MS (Latty, 2021), showing the stabilization of tetrapyrrole by Cu^{2+} ions.

The choice of the solvent for the copper salt treatment also affected the results, with FAA solutions permeating faster than the samples in water, but also extracting chlorophylls more rapidly. Thus, FAA recipes yield paler results than water due to this rapid extraction. Regarding the choice of salt, while $CuCl_2$ initially saturates the leaf colour, subsequent pigment loss occurs once the specimen is placed in the preservation fluid. Keeping the specimen in the copper salt solution did preserve the colour better, however, it is important to note that the fluid is noticeably blue and that neither the copper-salts nor the water-based solution provide any fixing or preserving properties of tissues, and does not act as a biocide and thus do not support long term preservation of the tissues.

Additional treatments aimed at enhancing the diffusion of the $CuSO_4$ into the tissues were tested such as prior boiling and submersion in full-strength, concentrated ethanol. Finally, the use of aqueous SO_2 to prevent oxidation was also tested. Boiling the specimen preserved and enhanced its green colour, but caused structural damage, compromising the specimen's integrity. Prior immersion in 95% ethanol removed the



A. Most saturated and least saturated results for Pea leaves after 49 days in 70% ethanol. Prior fixation with various copper salts in water or FAA.

B. Triplicats of pea leaves in ethanol 70%, no colour fixation (left) and $CuSO_4$ fixation (right) after 3 days of light exposure.

Figure 3: Results for Pisum sativum*: A. specimen colour for all tested recipes, B. comparison of specimens stored in ethanol, without and with Cu-salts treatment (dissolved in water). Figure adapted from Latty (2021). ©HE-Arc 2024*

hydrophobic barrier from the leaves, allowing for a more uniform diffusion of the $CuSO_4$ into the specimens. The addition of 5% aqueous SO_2 solution ($SO_{2(aq)}$) as an antioxidant in the 5% $CuSO_4$-water solution enhanced the colour stability, however it was again at the cost of the structural integrity of the specimen. The use of a vacuum pump should also help the penetration of the $CuSO_4$ but could not be explored in this work.

Yellow pigment

The results obtained with the Scully B solutions (Scully 1937) are rather good, and despite a small dulling of the colours, the specimens are still stable and recognizable after 90 days (and after 3 years).
The original Verma 2-recipe (Verma, 2008) requires boiling the specimen in the solution for better colour retention. However, since prior tests demonstrated that boiling compromised the physical integrity of the specimens, making their manipulation outside the fluid perilous for the tissues, the Verma 2 recipe was tested without the boiling step. Even with this modification this method still performed better than the Scully B procedure. The plants keep a vibrant yellow colour during 90 days after the preparation, and are still showing recognisably yellow colour three years after. The specimens floated in the fixation and in the preservation fluids for about 10 days before sinking to the bottom. Using a vacuum pump could help accelerate the diffusion of the fluid and thus further improve the preservation of the colours while probably also avoiding the floating of the specimens.

Yellow yarrows preserved in the ethanol 70% for control showed that both the chlorophyll and the yellow pigment leached, and the specimen stiffened. The flowers were completely bleached after the 90 days of monitoring and the plant stem had browned (Figure 4).

Blue-red pigments

The Wagstaffe & Fidler 4 recipe (Wagstaffe & Fidler 1968) preserved the colour perfectly (Figure 5A), despite showing similar specimen's flotation issues as the Verma 2-recipe. However it embrittles the specimens making them very vulnerable for breakage during manipulation (Figure 6A). It is therefore one of the most suited options to prepare fresh specimens for exhibition but is not recommended for frequently handled specimens.

The Kew mixture (Bridson & Forman 1998) preserves the red verbena's colour, even if a pale pink colouration of the fluid can be observed. However, it failed in preserving the blue and purple pigments. This is probably because these pigments are less stable and highly soluble in alcohol (Figure 5B). This recipe would therefore be a good choice for long term preservation of plant tissues, keeping in mind that some degree of colour loss is inevitable.

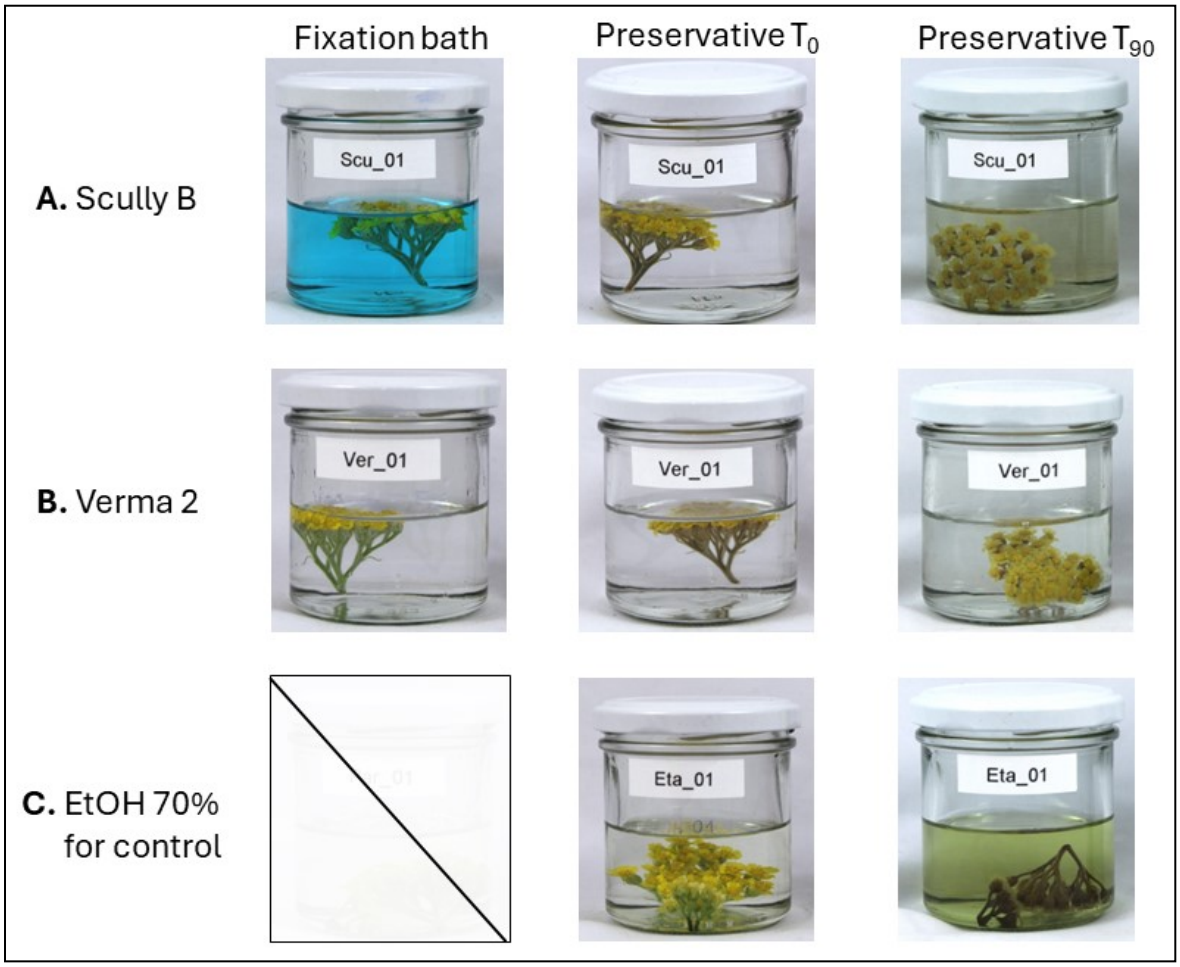


Figure 4. Overview of the tests for yellow colour preservation. ©HE-Arc 2024

The Copenhagen solution (Tredwell 2006) is a widely used recipe in botanical collections supposed to be effective at preserving colours. However, the tests carried out on fresh specimens did not yield satisfying results (Figure 5C). All flowers lost their colours within days and almost as fast as the control specimens in 70% ethanol (Figure 5D).

Tannins

The solution of 70% ethanol saturated with $SO_{2(aq)}$ as an antioxidant avoided the subsequent browning of the specimens but developed a noticeable pink hue just one day after preparation (Figure 7 A1), indicating that anthocyanin pigments of the flower petals leached into the fluid. The colouration of anthocyanins can be modified by adjusting the pH of the solution (Figure 7 A2). The pH of the antioxidant solution as prepared is aimed to be 1.8 to obtain enough $SO_{2(aq)}$. Another solution was tested, saturated with sodium bisulphite in 70% ethanol but without pH adjustment, giving as a result a pH of 4.7 (Figure 7 A2). With increasing pH the anthocyanins normally turn more purple and less red. However, the solution turned out to be colourless, probably due to the reduction of the flavylium cation by the bisulphite anion (Morata et al., 2019), or the formation of a hemiacetal form that usually develops in hydroalcoholic conditions (He et al, 2012). Both are colourless compounds. It should be noted, however, that the fluid will turn back to pink through the continuous drop of the pH to below 3, which also indicates that alcoholic sodium bisulphite solutions are highly susceptible for pH-shifts and should be buffered to a pH>3 to stabilize them. As the pH fluctuates, the $NaHSO_3$ salt can partially precipitate, causing a slight lowering of the transparency of the fluid.

Impregnating the specimens with paraffin was not satisfactory, because the paraffin slowly leached with the darkened pigments into the preservation fluid (Figure 7 B). While preserving the specimens in paraffin prevents opacification of the fluid (Figure 7 D1), the specimens subsequently darken and make them susceptible for mould growth

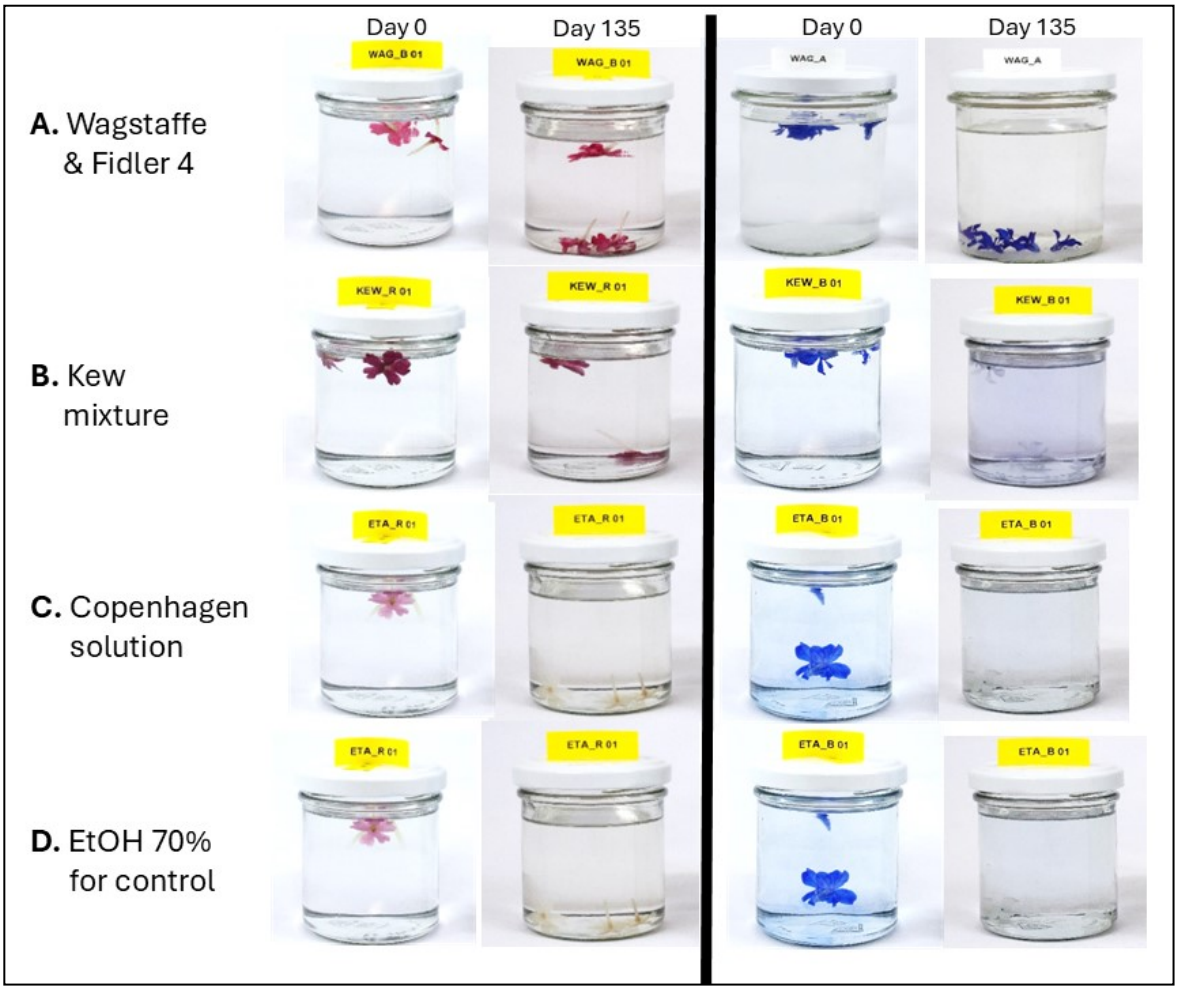


Figure 5. (Above) Overview of the tests for red and blue colour preservation. ©HE-Arc 2024

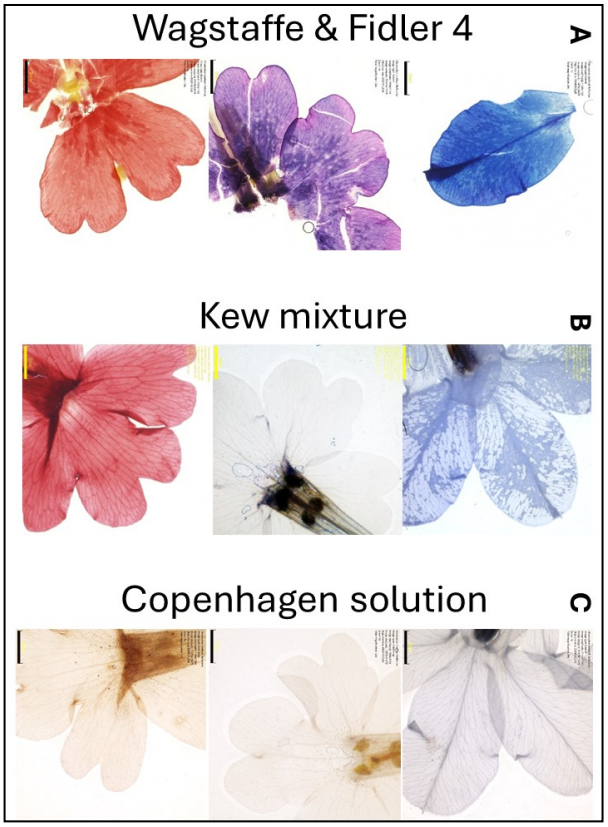


Figure 6. (Right) Optical Microscopy image, Olympus DSX100, x24 magnification. Blue Lobelia erinus *and red and purple* Glandularia peruviana *after 3 months in A. Wagstaffe & Fidler 4, B. Kew mixture and C. Copenhagen solution. ©HE-Arc 2024*

(Figure 7 D2). The high viscosity of paraffin prevents a thorough and close contact with specimens with a complex surface texture. The resulting moist air space between the specimen and the paraffin can accelerate the development of mould. A slow impregnation and surface wetting with paraffin improves the efficacy of this preservation method.

Prior fixation of the *Pinus* bark with formaldehyde and subsequent transfer into ethanol 70% keeps the preservative fluid clear, unlike the non-fixed control samples (Figure 8 A-B). The different concentration or fixation times revealed no noticeable visual differences, probably because the samples were small in size. The recipes mixing

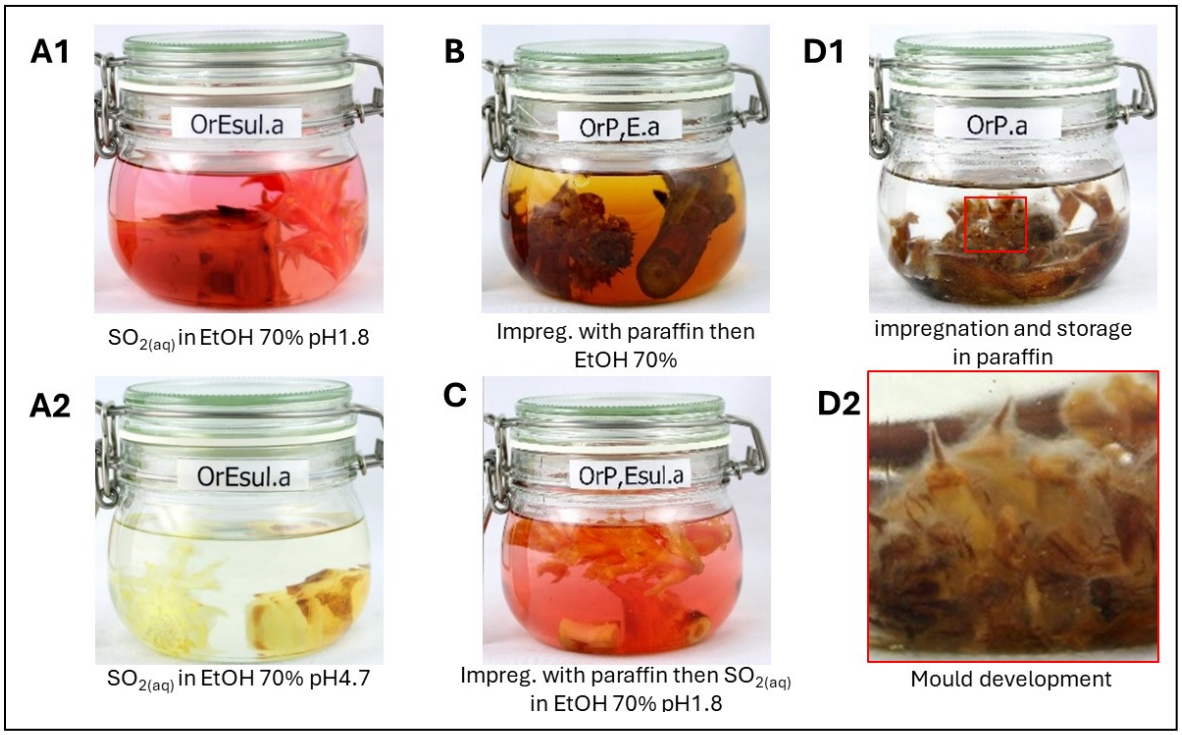


Figure 7. Orobanche hederae *Duby, A1. In $SO_{2(aq)}$ in EtOH 70% at pH 1.8 and A2. pH 4.7, B. Impregnation with paraffin then EtOH 70%, C. Impregnation with paraffin then $SO_{2(aq)}$ in EtOH 70% pH1.8, D1. Impregnation and storage in paraffin, with D2. Mould developing. Figure adapted from Latty 2021. ©HE-Arc 2024*

formaldehyde with phenol cause a slight yellow tint (Figure 8 C) that intensifies with increased concentrations. In addition to worse performances in preventing colouration of the fluid, phenol is highly toxic. Thus, its use should be avoided and we do not advocate for it. Instead, the use of formaldehyde on its own seems more appropriate.

The methods tested are effective to prevent the browning of the preservation fluid, but none preserved the original colours of the specimens. Preparation of fresh specimens therefore requires a choice between accepting the subsequent browning of the tissues because of autolysis or artificial polymerization, and discolouration of less stable pigments in case $SO_{2(aq)}$is added. Like many other light-coloured flowers, the *Orobanche hederae* we used for our tests contains anthocyanins that are prone to pH-induced colour -changes. Highly acidic pH-values turn specimens pink, while less acidic conditions sustain the colourless form(s) of the anthocyanin pigments.

Liquid paraffin, though an interesting alternative for non-toxic preservation fluids was found to be ineffective. It preserved the original colours longer without preventing eventual browning, possibly because it does not stop autolysis or does not diffuse quickly enough into the tissue of specimens. Testing lighter non-polar solvents such as xylene might help to sustain colour information, however, the high toxicity and flammability are major concerns, especially in museums. Using solvents at the beginning of the preservation process and then transferring the specimens to heavier hydrocarbons for long-term preservation could be beneficial, with addition of appropriate antiseptics to prevent microbial growth. This option remains to be tested.

Conclusion

Preserving or maintaining the colour information in botanical specimens in museum collections is to a certain extent achievable when starting with fresh specimens. In the case of historical preparations, the colour loss through leaching and degradation of the pigments is however fully irreversible. Simple application of published recipes does not necessarily return the desired and satisfactory results. Some cases such as the Copenhagen solution or the Kew mixture, though reportedly effective, do not seem to preserve all colours consistently. Some recipes, such as pre-treatment of green specimens with copper salts, lead to chemical transformation and altering of the original chlorophyll pigments, thus changing the natural colour of the specimen even though a colour information close to the original hue can

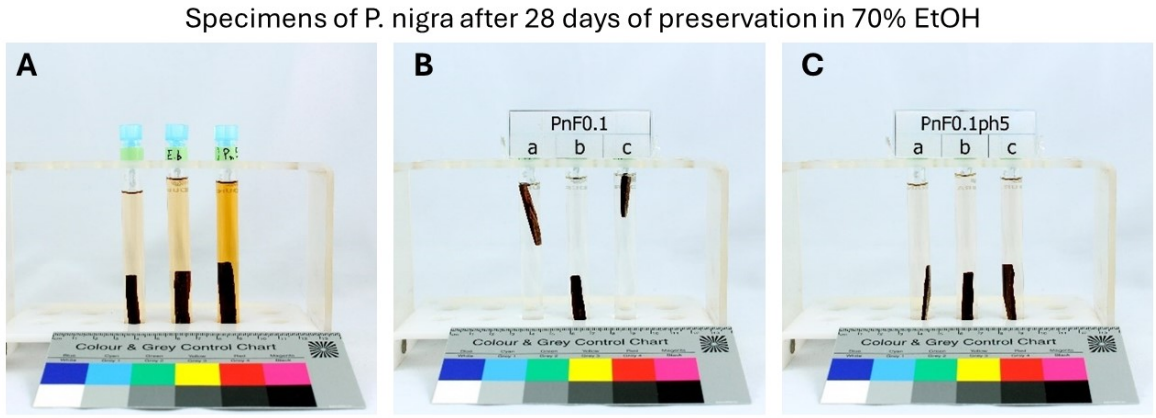


Figure 8. Specimens of P. nigra *after 28 days of preservation in 70% EtOH A No fixation, B. Fixation with formaldehyde, C. Formaldehyde + phenol. Figure adapted from Latty 2021. ©HE-Arc 2024*

be maintained. Wagstaffe & Fidler 4 mixture for blue-red flowers perfectly preserved colour, but may compromise the structural integrity of the specimen, making it fragile and difficult to handle, which causes concern for the long-term preservation and scientific usefulness of the specimens.

The addition of an aqueous solution of $SO_{2\,(aq)}$ to the preservative fluid can prevent the opacification of the preservation fluid in fresh and in historic specimens. However, also this treatment requires a lot of caution. Even if it is essential for maintaining the clarity of the fluid for tannin rich specimens, the use of antioxidants often results in the loss of more delicate pigments such as anthocyanins. It requires a close monitoring of the pH fluid level in specimen jars to avoid precipitation of the salt. It is worth noting that we have no detailed information on how the antioxidant affects future analysis on the preserved tissues.

It is essential to recognize that any intervention during the preparation process inevitably alters the specimen. Each compromise made during preservation comes at the cost of losing certain characteristics, albeit hopefully outweighed by the preservation of more valuable ones. Therefore, a deep understanding of the intrinsic values associated with botanical specimens, their intended use, and their characteristics is paramount when making decisions regarding their preservation. Thus, maintaining the colour of a specimen might not always be worth compromising on other aspects. Nevertheless, some recipes such as the Wagstaffe & Fidler 4 mixture for blue and red flowers, or the addition of antioxidants to tannin-rich specimens, would be a great option for preparations dedicated for display.

Finally, the design and use of individual preservation recipes tailored for specific specimens within an institution may not always be financially or practically viable within the constraints of their budget and resources. This underscores the necessity of employing sound judgment when selecting preservation methods, ensuring that the chosen approach is in line with the goals of the institution as well as with the maintaining and management of its collections. It is also crucial to document and file any interventions made during the preservation process. Transparency regarding these interventions is vital, as it enables researchers to accurately interpret and contextualize their findings when studying the specimens in the future.

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Appendix 1: Summary of pigments and characteristics (translated from Latty 2021 [FR])

Family	Hue	Main location	Stability (qualitative categorization)	Solubility
Chlorophyll		Leaves	-	Liposoluble
Carotenoid		Leaves	+	Liposoluble
Flavonol, aurone, chalcone, flavone	UV	Flowers	+/-	Hydrosoluble
Anthocyanin		Flowers	--	Hydrosoluble
Proanthocyanidin		Young growth	+	Hydrosoluble
Phlobaphene		Bark	++	Liposoluble
Dihydropyran		Ligneous tissues	-	Hydrosoluble
Betalain		Roots, flowers	-	Hydrosoluble
Anthraquinone		Roots	+	Hydrosoluble
Naphtoquinone		Bark	+	Hydrosoluble
Indigo		Leaves	+	Liposoluble (oxidized)
Hydrolysable tannins		Bark and ligneous tissues	+	Hydrosoluble

Appendix II: Colour preservation literature (adapted from Latty 2021 [translated from FR])

The following table presents recipes in chronological order. It is not an exhaustive list regarding vascular plants preserved in fluid. Moreover, this table omits most recipes that are specific for algae and mushrooms. It also omits the recipes intended for green plants but not specifically designed for colour fixation or retention. Reading guide:
Code: This is how the recipe is referred to in the paper. It is often the primary source author's name, and if multiple recipes are listed, numbering is used. This cell is coloured following the main specimen's colour target. A brown colour indicates both brown specimen and white ones prone to browning.
Date: Date of publication of the primary source.
Author: Name of the author of the primary source.
Source: When the primary source could not be consulted, a secondary source in which the detailed recipe can be found is briefly mentioned in this column. An extensive list of references is provided in the bibliography.
Type of specimens: The information provided based on the point of view of the predominantly affected pigments rather than specific species (sometimes specified).
Colour retention: Indicates whether the colour preservation method is based more on "fixation" in a first bath prior to the transfer of the specimen in the preservation fluid (= Fix), or if colour retention is achieved by the same fluid used for

long-term preservation (= Cons). In the case where the principle is unclear (usually in the case of non-detailed recipes), a "?" is used.
Antioxidant: If mentioned, the means of removing oxygen, or generally preventing oxidation, is indicated in this column. The methods can be physical (vacuum), or chemical (addition of sulfites in the solution).
Preservative: Clarification on the preservation fluid, if different from the fixation one, otherwise "Same".

Code	Date	Author	Source	Type of specimens	Colour retention	Antioxidant	Preservative
Cleghorn	1838	Cleghorn	Verma	Coloured plants	?	-	Same
Amann 2	1896	Amann	-	Green plants, mainly algae and bryophytes	Cons	?	Same
Woods	1897	Woods	-	Green plants (also preserves yellows, browns, etc.)	Fix	Physical	Same without Cu, or other than full-strength EtOH
Trail	1908	Trail	-	Green plants (not too fragile, as boiling)	Fix	Physical	Formaldehyde or EtOH base
Strasburger	1911	Strasburger	Butler	Monotropes	?	Sulfites	?
Jones	1917	Jones	Verma	Green plants	Fix	Physical	?
Butler 1	1918	Butler	Trail	Green plants with dark pathologies	Fix	Physical	Formaldehyde
Butler 2	1918	Butler	Strasburger	Colourless or pathological specimens with liposoluble pigments	Fix	Sulfites in fixation	Formaldehyde
Cr. & Ch. 1	1922	Cruess & Christie	Adriano & Yonzon	Yellow fruits and vegetables	Cons	Sulfites in preservation	Same
Cr. & Ch. 2	1922	Cruess & Christie	Adriano & Yonzon	Red and multicoloured fruits and vegetables	Cons	Sulfites in preservation	Same
Maltby 1	1926	Maltby	Verma	Green parts	Cons	Sulfites in preservation	Same
Maltby 2	1926	Maltby	Verma	Green parts	Fix	Physical	?
Maltby 3	1926	Maltby	Verma	Other colours?	?	?	?
Keefee	1926	Keefe	-	Green plants	Fix	-	Same, drying, other?
Mackenzie 1	1928	Mackenzie	Verma	Green leaves	Fix	Sulfites in fixation and preservation	?

Code	Date	Author	Source	Type of specimens	Colour retention	Antioxidant	Preservative
Mackenzie 2	1928	Mackenzie	Verma	Pears, quinces, and soft fruits	?	Sulfites	?
Mackenzie 3	1928	Mackenzie	Verma	Red apples, or yellow or green ones with red spots	Cons	Sulfites in preservation	H2O + sulfites
Ni. & Sl.	1928	Nieuwland & Slavin	-	Monotropes	Cons	Sulfites in fixation and preservation	Same or xylene
Eckert	1931	Eckert	-	Green algae (Intended for microscopy)	Fix	-	-
Ad. & Yo. 1	1933	Adriano & Yonzon	-	Green fruits and vegetables	Fix	Sulfites in preservation	H2O + sulfites
Ad. & Yo. 2	1933	Adriano & Yonzon	-	Yellow or white fruits and vegetables	Cons	Sulfites in preservation	Same
Ad. & Yo. 3	1933	Adriano & Yonzon	-	Red or multicoloured fruits and vegetables	Cons	Sulfites in preservation	Same
van Steenis 1	1935	van Steenis	-	Parasitic plants	Cons	Sulfites in preservation	Same
van Steenis 2	1935	van Steenis	-	Orange fruits of Gonocaryum	Cons	?	Same
Blaydes 1a	1937	Blaydes	-	Green plants, slow fluid penetration	Fix	Physical	FAA, 70% EtOH or other
Blaydes 1b	1937	Blaydes	-	Green plants	Fix	Physical	FAA, 70% EtOH or other
Blaydes 2	1937	Blaydes	-	Green plants	Fix	-	Same?
Scully A	1937	Scully	-	Green plants (with yellow flowers)	Cons	Sulfites in preservation	Same
Scully B	1937	Scully	-	Green plants with yellow flowers	Fix	Sulfites in preservation	H2O + sulfites
Johansen 1	1940	Johansen	-	Green plants?	?	-	Same
Johansen 2	1940	Johansen	-	Green plants	Fix	Physical	?
Johansen 3	1940	Johansen	-	Green plants	Fix	-	?
Johansen 4	1940	Johansen	Blaydes	Green plants	Fix	-	?
Johansen 5	1940	Johansen	-	Green plants	Fix	Physical	70% EtOH + 5% glycerol

Code	Date	Author	Source	Type of specimens	Colour retention	Antioxidant	Preservative
Johansen 6	1940	Johansen	Keefe	Green plants	Fix	-	?
Chandrashekar	1958	Chandrashekar	Verma	Green plants and others	Fix	-	Formaldehyde
Wa. & Fi. 1a	1968	Wagstaffe & Fidler	-	Green plants	Fix	Sulfite in fixation	Formaldehyde
Wa. & Fi. 1b	1968	Wagstaffe & Fidler	-	Green plants	Fix	-	Formaldehyde
Wa. & Fi. 1b'	1968	Wagstaffe & Fidler	-	Green plants	Fix	Physical	Formaldehyde
Wa. & Fi. 1c	1968	Wagstaffe & Fidler	Keefe	Green plants	Fix	-	Formaldehyde
Wa. & Fi. 2	1968	Wagstaffe & Fidler	-	Red apples and other red fruits	Cons	-	Same
Wa. & Fi. 3	1968	Wagstaffe & Fidler	-	Yellow apples with red spots	Cons	Sulfites in preservation	H2O + sulfites
Wa. & Fi. 4	1968	Wagstaffe & Fidler	-	Red and blue flowers	Cons	Thiourea in preservation	Same
Knudsen 1	1972	Knudsen	-	Green plants	?	?	?
Knudsen 2	1972	Knudsen	Scully	Flowering plants, mostly yellow	Fix	Sulfites in preservation	H2O + sulfites
Ha. & Di. 1	1985	Hangay & Dingley	-	Ferns and "seed plants"	Cons	-	Same
Ha. & Di. 2	1985	Hangay & Dingley	-	Green plants	Fix	?	?
Ha. & Di. 3	1985	Hangay & Dingley	-	Green plants	?	Physical	?
Ha. & Di. 4	1985	Hangay & Dingley	-	Green plants	Cons	Sulfites in preservation	Same (except Na silicate used alone before)
Ha. & Di. 5	1985	Hangay & Dingley	-	Green plants	Fix	Sulfites in fixation	Formaldehyde
Ha. & Di. 6	1985	Hangay & Dingley	Knudsen	Coloured plants (green with yellow flowers)	Fix	Sulfites in preservation	H2O + sulfites
Viswanathan et al.	1991	Viswanathan et al.	-	Green plants	Cons	-	Same
Moore	1999	Moore		multiple recipes	multiple recipes	multiple recipes	multiple recipes
Verma 1	2008	Verma	-	Green plants	Fix	-	Propionic acid, formaldehyde
Verma 2	2008	Verma	-	Yellow flowers	Cons	-	Same