Original Article

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Pathology Section

Colourimetric Detection of Formalin Concentration in Fixing Solutions using Schiff's Reagent: An In-vitro Study

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ABSTRACT

Introduction: Formalin is a widely used tissue fixative in histopathology. Maintaining a precise formalin concentration in fixing solutions is essential, as both over-fixation and under-fixation can compromise tissue quality and pose health risks to laboratory personnel and Pathologists. Existing techniques such as gas chromatography and spectrophotometry are accurate but expensive and impractical for routine use. Hence, a simple, affordable, and reliable method for monitoring formalin concentrations in routine laboratory procedures is required.

Aim: The present study aimed to develop a method to detect and quantify formalin concentration using Schiff's reagent and to further validate it using a colour palette.

Materials and Methods: The present in-vitro study was conducted in the Department of Oral Pathology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from July 2024 to November 2024. Formalin solutions with concentrations of 2%, 5%, 10%, 20%, 25%, 50%, 75%, and 100% were prepared. Schiff's reagent was added to each solution, and the resulting colour changes were recorded. A colour palette was generated based on this gradient, covering the full spectrum of formalin concentrations from 2 to 100%. This colour palette was patented and published in the Official Journal of The Patent Office (202541045779). Fifty filter paper strips (10 for each concentration) soaked in Schiff's reagent were immersed in the different formalin solutions. Two Oral Pathologists independently assessed all 50 strips and compared them with the colour palette to confirm concentration-dependent colour changes. Kappa statistics were calculated using Statistical Package for Social Sciences (SPSS) software version 23 to assess inter-rater reliability.

Results: A gradient of purple colour intensity corresponding to formalin concentration was observed, confirming the specificity of Schiff's reagent for formalin detection. Higher concentrations of formalin produced a more intense dark violet, while lower concentrations resulted in lighter shades. Kappa statistics demonstrated excellent inter-rater agreement, with a κ value of 0.92.

Conclusion: According to the present study findings, Schiff's reagent reliably detects formalin concentrations, providing an accessible, cost-effective colourimetric method for the safe and effective use of formalin in routine laboratory practice.

Keywords: Colour palette, Fixation, Formaldehyde, Histopathology, Health equity, Healthcare innovation, Good health and well being

INTRODUCTION

In histopathology, tissue fixation is an essential process that preserves the cellular and structural integrity of biological samples, thereby enabling accurate morphological and biochemical analysis [1]. Among the various fixatives available, formalin remains the most commonly used in pathology laboratories due to its effectiveness in cross-linking proteins [2,3]. This chemical reaction stabilises cellular structures, making formalin ideal for preserving tissue morphology for a range of downstream analytical procedures, including histological staining and molecular techniques [4].

The advantages of formalin as a tissue fixative include its rapid penetration and uniform distribution within tissues, ensuring that even large specimens are adequately preserved [4]. Formalin is also compatible with a broad spectrum of staining protocols, which enhances the visibility of cellular components and provides diagnostic clarity. Additionally, its ability to preserve both nucleic acids and proteins makes it a versatile fixative suitable for various applications in histopathology [5].

Despite its widespread use, formalin poses significant challenges and health risks. As a recognised carcinogen, it presents serious risks to laboratory personnel, particularly through inhalation and skin contact [6]. Moreover, improper handling and storage of formalin can lead to hazardous laboratory conditions [7]. From a technical perspective, the concentration of formalin in fixation solutions is critical, as both over-fixation and under-fixation can severely compromise tissue quality [8]. Over-fixation often results in tissue hardening, which can obscure antigenic sites and hinder immunohistochemical analysis [9]. Conversely, under-fixation may lead to suboptimal tissue morphology and poor histological detail, potentially affecting diagnostic accuracy [10].

Accurate assessment of formalin concentration is therefore essential for preserving tissue quality and ensuring the health and safety of laboratory personnel [11]. Formalin's volatility and strong odour can lead to both acute and long-term health effects when exposure is frequent. Acute exposure to high concentrations may cause respiratory distress, skin and eye irritation, and prolonged exposure increases the risk of carcinogenic effects [12]. Maintaining optimal formalin concentrations helps minimise unnecessary exposure to toxic vapours and strengthens laboratory safety protocols [8]. Furthermore, consistent formalin concentration is crucial for standardised tissue fixation, as even minor deviations can affect staining outcomes and compromise diagnostic accuracy [13]. Thus, reliable detection and control of formalin levels are vital for both specimen integrity and occupational safety in pathology laboratories [14].

Several analytical techniques have been employed to estimate formalin concentration, including High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and spectrophotometry [15,16]. Although these methods are accurate and widely accepted, they require expensive instrumentation, complex sample preparation, and trained personnel. Consequently, formalin concentrations are not routinely measured in many pathology laboratories, which may result in inconsistencies over time [17]. These limitations significantly reduce

the practicality of conventional methods for routine application, particularly in resource-limited settings.

To overcome these challenges, the present study aimed to develop and validate a Schiff's reagent-based method for the detection and differentiation of formalin concentrations across a wide range. By refining the assessment of formalin concentration, this approach seeks to ensure optimal tissue fixation, improve the quality of histological analyses, and enhance safety protocols within the laboratory environment.

MATERIALS AND METHODS

The present in-vitro study was conducted in the Department of Oral Pathology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India, from July 2024 to November 2024. The study was approved by the Institutional Ethics Committee (SRB/SDC/OPATH2301/24/201).

Study Procedure

Formalin solutions of varying concentrations were prepared by diluting commercially available 37% formaldehyde with distilled water to obtain concentrations of 2%, 5%, 10%, 20%, 25%, 50%, 75%, and 100%. For each concentration, 100 mL of solution was precisely prepared. For example, 10% formalin was prepared by diluting 10 mL of 37% formaldehyde in 90 mL of distilled water.

Subsequently, 3-4 mL of each formalin solution, along with control solutions including saline, distilled water, and a local anaesthetic, were transferred into separate, clearly labelled test tubes. To each test tube, 0.5 mL of Schiff's reagent was carefully added, and the tubes were observed for any colour change after 3 seconds. The resulting colour changes were recorded, forming a colour gradient corresponding to increasing formalin concentrations, ranging from light magenta to dark violet.

To create a standardised visual reference, a colour palette covering the full spectrum of formalin concentrations from 2 to 100% was generated. Two Oral Pathologists independently evaluated the results, which were subsequently re-evaluated by a third oral pathologist. This colour palette was patented and published in the Official Journal of The Patent Office (202541045779).

Additionally, filter papers were soaked in Schiff's reagent (SRL - Sisco Research Laboratories Pvt. Ltd., 17386) for 12 hours to ensure uniform absorption of the reagent. After soaking, the filter papers were air-dried for 1 hour in a dark environment to prevent light interference during drying. Once dried, the filter papers were cut into 50 uniform strips and individually dipped into the prepared formalin solutions for 1 second (10 strips per concentration). The colour changes observed on the strips after 5 seconds were compared with the established colour palette to assess the consistency and accuracy of the Schiff's reagent reaction across varying formalin concentrations.

STATISTICAL ANALYSIS

Inter-rater reliability was assessed using Kappa statistics with SPSS software version 23.

RESULTS

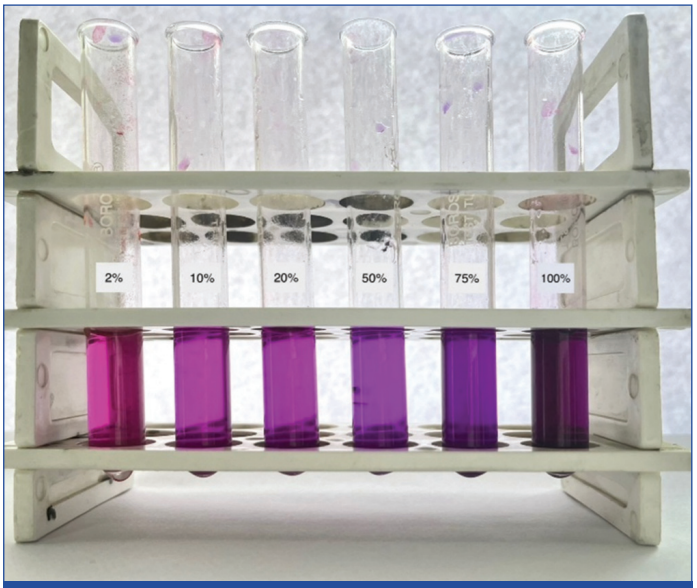
The observed colour changes in the formalin solutions ranged from light magenta to dark violet, with colour intensity directly correlating with increasing formalin concentration [Table/Fig-1]. Higher concentrations produced a more intense dark violet, whereas lower concentrations resulted in lighter shades [Table/Fig-2]. In contrast, no colour change was detected in saline, distilled water, or local anaesthetic solutions, demonstrating the specificity of Schiff's reagent for formalin detection [Table/Fig-3]. Based on these findings, a colour palette was fabricated, as shown in [Table/Fig-4].

The filter paper strips dipped into the formalin solutions mirrored the colour changes observed in the liquid samples, with a progressive intensification of colour from 2% to 100% formalin concentrations [Table/Fig-5]. Notably, the strip immersed in the 2% formalin solution exhibited a significantly lighter colour compared to the strip exposed to 100% formalin, further confirming the direct relationship between formalin concentration and colour intensity.

Validation testing using ten Schiff's reagent-soaked filter strips at each formalin concentration demonstrated consistent colour changes that closely matched the established colour palette [Table/Fig-6]. Kappa statistics revealed excellent inter-rater agreement, with a κ value of 0.92. This uniformity across repeated tests confirms that the method reliably differentiates formalin concentrations based on colour intensity.

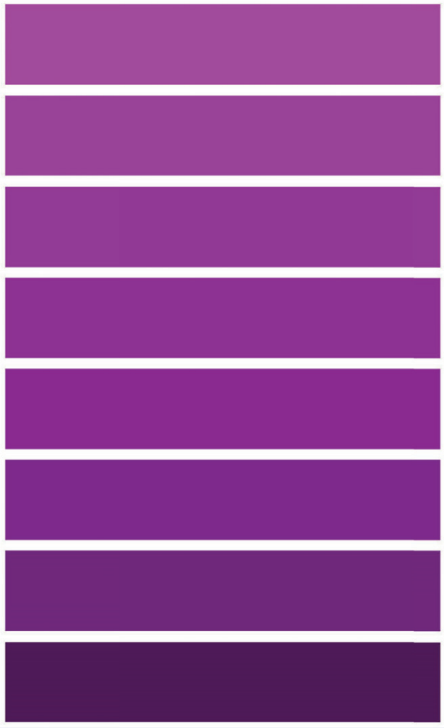
S. No.	Formalin concentration	Colour
1.	2% formalin	Light magenta
2.	5% formalin	Light Pink-magenta
3.	10% formalin	Bright magenta
4.	20% formalin	Medium purple
5.	25% formalin	Purple pink
6.	50% formalin	Standard purple
7.	75% formalin	Deep purple
8.	100% formalin	Dark violet

[Table/Fig-1]: The resulting colour changes were recorded, forming a colour gradient corresponding to the increasing formalin concentrations, ranging from light magenta to dark violet.



[Table/Fig-2]: Test tubes contain formalin-schiff solutions with concentrations of 2%, 10%, 20%, 50%, 75%, and 100%.

[Table/Fig-3]: Test tubes with formalin solutions (2% and 100%) and controls (saline, distilled water, local anaesthetic). Only formalin solutions turned purple, with colour intensity increasing with concentration; controls remained colourless.



[Table/Fig-4]: Colour palette illustrating the gradient from light to dark purple with increasing formalin concentrations (2% to 100%) after adding Schiff's reagent.

[Table/Fig-5]: Filter paper strips dipped into the formalin solutions shows progressive intensification of colour from the 2% to the 100% formalin concentrations.

[Table/Fig-6]: Filter strips produced consistent colour changes that closely matched the established colour palette.

DISCUSSION

Formalin is the most commonly used fixative in histopathology due to its effectiveness in preserving tissue structure and integrity [1,18,19]. Its ability to cross-link proteins and other cellular components makes it essential for maintaining the morphological features required for accurate diagnosis [20]. However, ensuring the appropriate concentration of formalin is critical, as both excessive and inadequate fixation can adversely affect tissue quality and histological outcomes. Therefore, the detection and quantification of formalin concentration are essential to ensure optimal tissue preservation, minimise diagnostic errors, and improve the reliability of histopathological analysis [4].

The results of this study demonstrated a progressive increase in the colour intensity with increasing formalin concentration following the addition of Schiff's reagent to formalin solutions. The colour change, ranging from light magenta to dark violet, directly corresponded to formalin concentrations from 2% to 100%. Schiff's reagent reacts specifically with aldehyde groups to produce a magenta or purple colour, a reaction that occurs due to the restoration of conjugated double bonds within the reagent's fuchsin dye component [21]. Formalin, being an aqueous solution of formaldehyde, contains free aldehyde groups, which explains the observed colour gradient [22]. The findings of the present study are consistent with previous reports highlighting Schiff's reagent as a specific and reliable agent for detecting aldehydes, including formaldehyde, the primary constituent of formalin [23].

Similar observations have been reported in studies that employed Schiff's reagent for aldehyde detection in various chemical and biological applications, further reinforcing its specificity and reliability [22,23]. Research on formaldehyde-based adhesive systems by Solt-Rindler P, et al., demonstrated the strong reactivity of aldehyde groups during cross-linking, a chemical property that underpins the effectiveness of Schiff's reagent for aldehyde detection [22]. Additionally, Thepchuay Y et al., demonstrated the reagent's effectiveness in spectrophotometric analysis of formaldehyde in food samples, where Schiff's reagent, used alongside other chromogenic reagents, exhibited high sensitivity and clear selectivity over structurally related aldehydes [23].

The absence of any colour change in the control solutions, including saline, distilled water, and local anaesthetic, further highlights the specificity of Schiff's reagent for aldehyde-containing compounds. As these control solutions lack aldehyde groups, no reaction occurred, supporting the reagent's established role in histological techniques such as the Feulgen reaction. In this method, Schiff's reagent binds specifically to aldehyde groups generated by acid hydrolysis of nucleic acids, enabling the selective visualisation of Deoxyribonucleic Acid (DNA) [24].

In the present study, the use of filter paper strips soaked in Schiff's reagent provided additional confirmation of the liquid-based findings. The colour changes observed on the filter strips closely matched those seen in the liquid solutions, confirming that Schiff's reagent retains its specificity and sensitivity even when applied in a solid phase. Furthermore, validation testing provided strong evidence supporting the reliability of Schiff's reagent for detecting formalin concentration. The combination of liquid and solid-phase approaches strengthens the robustness of the method across different testing formats. These observations are consistent with previous studies that have successfully employed solid-phase detection systems for aldehyde identification in industrial and food applications [24].

The findings of this study emphasise the importance of maintaining appropriate formalin concentration during tissue fixation, as improper fixation-whether due to over or under-fixation-can result in poor staining quality, distorted tissue morphology, and compromised diagnostic outcomes [25]. Consequently, the ability to accurately assess and verify formalin concentration is critical in pathology

laboratories. The implementation of a simple and reliable method such as Schiff's reagent–based detection could help standardise fixation practices and reduce the risk of diagnostic errors. Surgeons and Pathologists should remain vigilant regarding the proper handling and concentration of formalin to ensure optimal tissue preservation for accurate histopathological analysis [26].

Limitation(s)

One limitation of this study is the reliance on visual inspection to assess colour changes. Although effective, this approach introduces a degree of subjectivity. Future studies could incorporate spectrophotometric analysis to enable more precise quantification of colour intensity and reduce observer bias. Additionally, factors such as reagent storage conditions and the presence of interfering substances, including oxidising agents, may influence the results. While this study focused exclusively on formalin solutions, future research could explore the applicability of this method to other aldehyde-containing compounds, potentially expanding its utility in histopathology and forensic science.

CONCLUSION(S)

The present study successfully demonstrated the ability of Schiff's reagent to detect and differentiate formalin concentrations ranging from 2 to 100%, with colour intensity increasing proportionally with concentration. The specificity of the reagent for aldehydes was confirmed by the absence of colour change in control solutions, while the use of filter paper strips provided a practical and consistent method for visualising these reactions. Although the current approach offers a reliable means of formalin detection, the incorporation of objective, quantitative techniques in future investigations would further enhance its precision. Overall, these findings contribute to the development of a dependable, accessible method for formalin detection with potential applications in both histopathology and forensic sciences.

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