

## Substitutes for xylene in histopathology and the role of temperature

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### Abstract

**Background:** Deparaffinization of wax sections in histopathology requires Xylene which is a harmful and toxic compound. It has been successfully replaced by many common household products such as dishwashing liquid, soaps and mineral oils. However, all these substitutes require higher temperatures of upto 90°C for effective deparaffinization. **Aim:** To evaluate the temperature dependent efficacy of dishwashing liquid in deparaffinization of histopathological sections as a substitute to the routine toxic Xylene. **Method:** 100 tissue sections deparaffinised by conventional Xylene and stained by Hematoxylin and Eosin method were objectively scored against 300 matched sections deparaffinised using dilute dishwashing liquid method at 65°C/75°C/90°C (100 each) by a single blinded pathologist. **Results:** Based on Wilcoxon signed ranks test, at 90°C the Xylene free method stood on par with the conventional method (p=0.422, p>0.05) proving Xylene free sections were as good or better than the matching conventional sections in 76% of the comparisons. Xylene free sections at 65°C and 75°C were found to be inferior to the conventional method in 80% and 69% of the comparisons respectively and also statistically significant from the conventional method (p=0.002 and 0.019 respectively). **Conclusion:** The new Xylene free method using dishwashing liquid is highly temperature sensitive and effective only at higher temperatures.

**Keywords:** Deparaffinization, Dishwashing liquid, Histopathology, Xylene, Temperature

### Introduction

The backbone of daily histopathological diagnostic work is the paraffin section usually stained with Hematoxylin and Eosin. Paraffin sections are still prepared by methods largely unchanged for over 150 years. The process of deparaffinisation of the slides using Xylene is an important preliminary step before the staining process, which makes the tissue sections to uptake the Haemotxylin and Eosin stain properly. This makes Xylene an inevitable compound in histopathology due to its paraffin solvent action. However it is a toxic compound that is hazardous for human use and the environment in which it is disposed [1]. Therefore, any substitute that minimizes the use of Xylene in experiments, reduces tissue staining time and does not compromise its quality will be efficient for diagnostic reasons and valuable for maintaining a safe laboratory environment.

The innovative concept of using dish washing liquid at 90°C to dewax tissue sections was first experimented by Falkeholm *et al* in 2001 [2]. Since then various laboratories have done pilot trials with successful outcomes.

However, the results of these trials were varied with regard to individual parameters like nuclear staining, cytoplasmic staining and clarity. There were also other substitutes that showed promising results like diluted lemon water, mineral oil and hot distilled water.

The common factor among all the substitutes was they had to be used at high temperature (90°C) to be effective. The role of temperature is further favored by studies that showed promising results by substituting Xylene with hot plain distilled water and conducting the process in a microwave. Hence, we designed the experiment using dish washing solution as a substitute to Xylene at three different temperatures 65°C, 75°C and 90°C to implore the effect of temperature.

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## Materials and methods

**I. Preparation of 1.7% Diluted liquid dish washing solution:** 25ml of locally available 'Vim<sup>®</sup>' dish washing solution + 1500mL of distilled water [3].

This product contains sodium laureth sulphate, sodium dodecyl benzene sulphonate, cocamidopropylbetaine and nonionic surfactants. In our study we diluted 25 mL of dish washing solution with 1500 mL of distilled water which brings the level of these compounds well below their reported toxic levels. Additionally, their concentrations in the product are strictly monitored by the manufacturer.

The study group included tissues such as epithelium, connective tissue, glands, bone, muscle, and cartilage. Four sections of 4 microns each were cut from 100 paraffin embedded tissue blocks.

One section was deparaffinised with the conventional Xylene method and the other three with Xylene free method using 1.7% dish washing solution under different temperatures 65°C, 75°C and 90°C. All the 4 deparaffinised sections were stained using conventional Hematoxylin and Eosin method.

### I. Preliminary tissue sectioning and paraffinization:

**1. Fixation:** Square tissue blocks (about 1cm in each dimension) are fixed by chemical fixation. The fixative used is a 4% aqueous solution of formaldehyde, at neutral pH. The chemical added binds to and cross-links some proteins, and denatures other proteins through dehydration. This hardens the tissue, and inactivates enzymes that might otherwise degrade the tissue. Fixation also kills bacteria.

**2. Dehydration and clearing:** The tissue is embedded in paraffin wax, but wax is not soluble in water or alcohol. However, it is soluble in a paraffin solvent like Xylene. Therefore, the water in the tissue needs to be replaced with Xylene. To do this, first the tissue is dehydrated, by gradually replacing water in the sample with alcohol.

This is achieved by passing the tissue through increasing concentrations of ethyl alcohol (from 70% to 90%). Finally, once the water has been replaced by 90% alcohol, the alcohol is replaced with Xylene, which is miscible with alcohol. This final step is called 'clearing'.

**3. Embedding:** The tissue is placed in warm paraffin wax, and the melted wax fills the spaces that used to have water in them. After cooling, the tissue hardens, and is cut into sections.

Upto this, the procedure is same for both the methods – conventional and Xylene free staining.

**4. Sectioning:** The tissue block is trimmed and mounted on the microtome. Thin sections of 4 microns are cut, which are deparaffinised, stained and mounted on a microscope slide.

**5. Deparaffinisation, Staining and Mounting:** *Conventional H & E Method:* Most staining solutions are aqueous, so to stain the sections, the wax is dissolved (deparaffinised) using Xylene and then replaced with water (rehydration). For this the sections are passed through decreasing strengths of alcohol (90% to 70%) and then stained with Haematoxylin and Eosin stains. Once stained, the section is then dehydrated once again, and placed in Xylene. It is then mounted using a mounting media and a coverslip is placed on top, to protect the sample.

**Xylene Free Method:** Deparaffinisation was carried out using the 1.7% diluted dish washing liquid at 65/75/90 °C instead of Xylene followed directly by Haematoxylin and Eosin staining. In this method the process of rehydration before staining and dehydration before mounting using graded alcohols also becomes unnecessary as suitable temperatures are employed. Staining follows immediately after deparaffinization.

Oven drying the Xylene alcohol free sections at 60°C prior to cover slipping are sufficient as depicted in **Table 1**(Comparison in methodology between conventional and Xylene free method)

**Table- 1: Comparison in methodology between conventional and Xylene free method**

STEPS	CONVENTIONAL METHOD		XYLENE FREE METHOD		
	Compound	Duration	Compound	Temp	Duration
Deparaffinization	Xylene I	5 min	1.7% Dil.Dishwash Liquid	65/75/90°C	1 min
	Xylene II	5 min	1.7% Dil.Dishwash Liquid	65/75/90°C	1 min
	90% alcohol	5 min	Distilled water	65/75/90°C	30 secs
	70% alcohol	5 min	Distilled water	65/75/90°C	30 secs
	Water wash	10 min	Wash in distilled water	65/75/90°C	30 secs
				Wash in distilled water	Room Temp
Nuclear Staining	Harri's hematoxylin	8 min	Harri's hematoxylin	Room Temp	8 min
	Water wash	2 min	Water wash		2 min
	1% acid alcohol	1 dip	1% acid alcohol	Room Temp	1 dip
Differentiation	Water wash	10 min	Water wash	Room Temp	10 min
Cytoplasmic Staining	1% eosin	1 min	1% eosin	Room Temp	1 min
			Water wash	Room Temp	1 min
Dehydration	90% alcohol	30 secs			
	70% alcohol	30 secs	Oven dry the sections	60°C	10 min
	Xylene I	5 min			
	Xylene II	5 min			
Approximate time		70-75 min			30-35 min

**III. Assessment of Stained Tissues:** 100 sections were stained with conventional Hematoxylin and Eosin staining and 300 sections were stained with Xylene free method (100 in 65°C, 100 in 75°C, 100 in 90°C). A randomized set of 400 sections matched into 100 quadruplets were scored and analyzed by a single blinded pathologist. Our study eliminates bias and inter-observer variability that may arise due to analysis using multiple pathologists [2]. Each slide was scored objectively based on a standard scoring system used by Ankle and Joshi 2011 [4], graded based on the parameters of nuclear staining (Adequate = score 1, Inadequate = 0), cytoplasmic staining (Adequate = score 1, Inadequate = score 0), clarity of staining (Present = score 1, absent = score 0), uniformity of staining (Present = score 1, absent = score 0), and crispness of staining (Present = score 1, absent = score 0).

**Table- 2: Final Score evaluation and cut-offs**

0-3	Poor; Inadequate for diagnosis
4-5	Average
6-8	Good; Adequate for diagnosis
>8	Excellent; All criteria well seen.

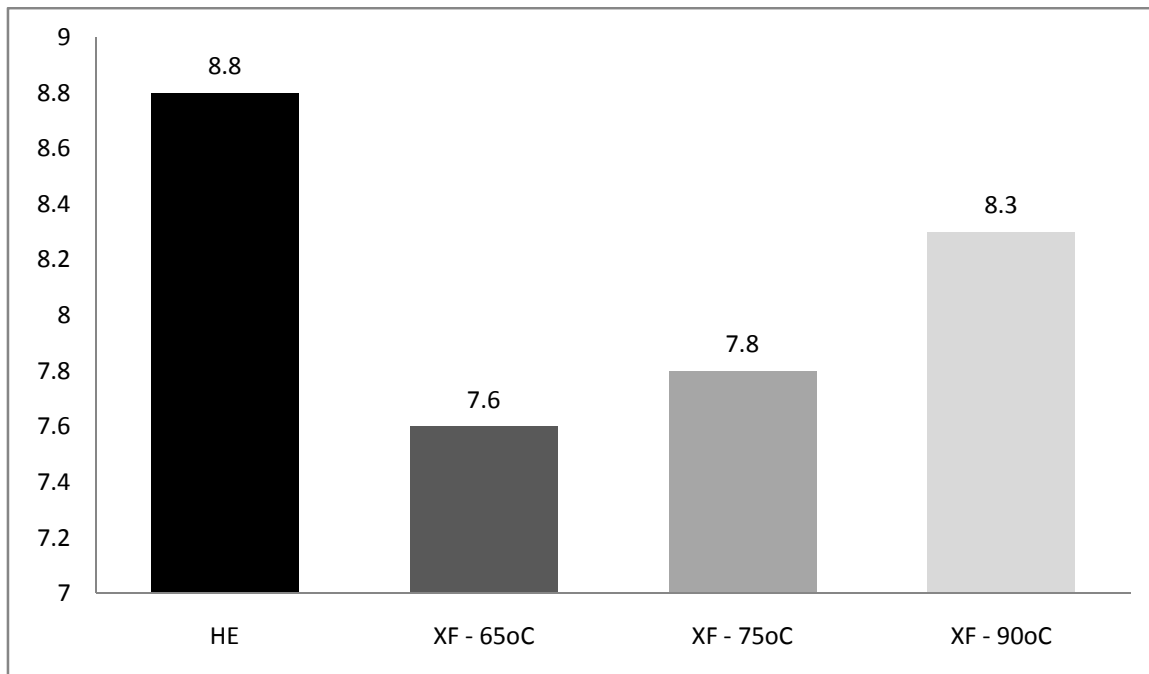
## Results

The total scores from various tissue sections were analysed using Wilcoxon Signed Ranks Test to compare staining efficiency of both methods. The advantage with Wilcoxon Signed Rank Test is that it neither depends on the form of the parent distribution nor on its parameters. It does not require any assumptions about the shape of the distribution. For this reason, this test is often used as an alternative to t test whenever the population cannot be assumed to be normally distributed. Even if the normality assumption holds, it has been shown that the efficiency of this test compared to t-test is almost 95%.

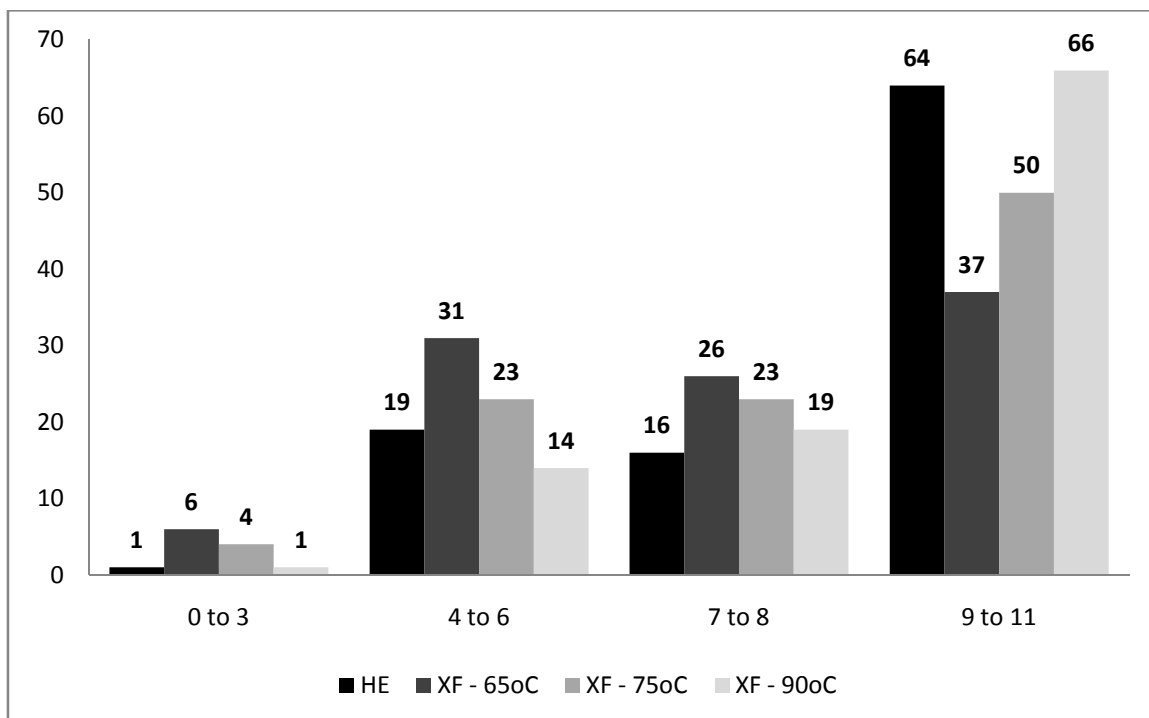
According to the test, the Xylene-free sections at 90°C were judged to be as good as or better than the matching conventional sections in 76% of comparisons and poorer in 24% of the comparisons, which is analogous to results obtained by Falkholm *et al* [2]. Xylene free sections at 75°C and 65°C were found to be inferior than the conventional method in 69% and 80% of the comparisons respectively.

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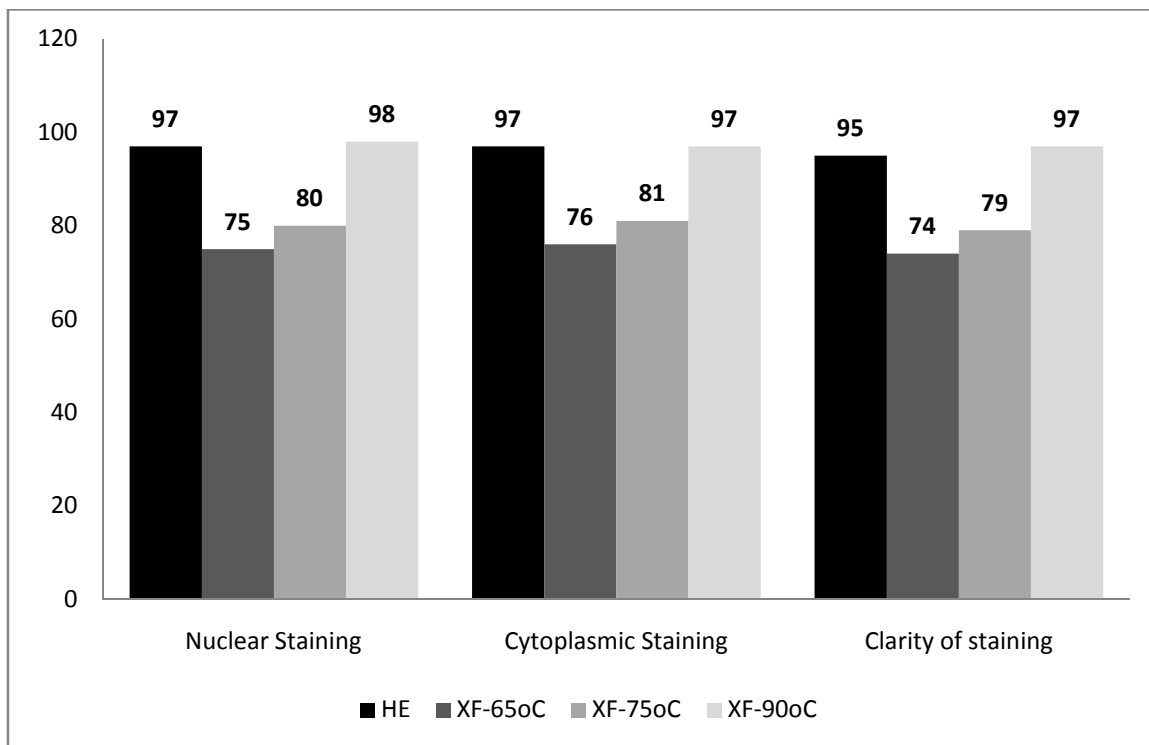
Comparing numerical scores, the maximum score attainable is 11. The mean score among the conventional staining group was 8.77 whereas for xylene-free staining group at 90°C was 8.3. The difference between these two groups was not significant ( $p = 0.422$ ;  $p > 0.05$ ), hence proving no difference in staining characteristics. The mean score for Xylene-free staining group at 65°C and 75°C are 7.6 and 7.8 and the p value being 0.002 and 0.019 respectively. Thus the difference between lower temperatures and the conventional method is statistically significant from each other.



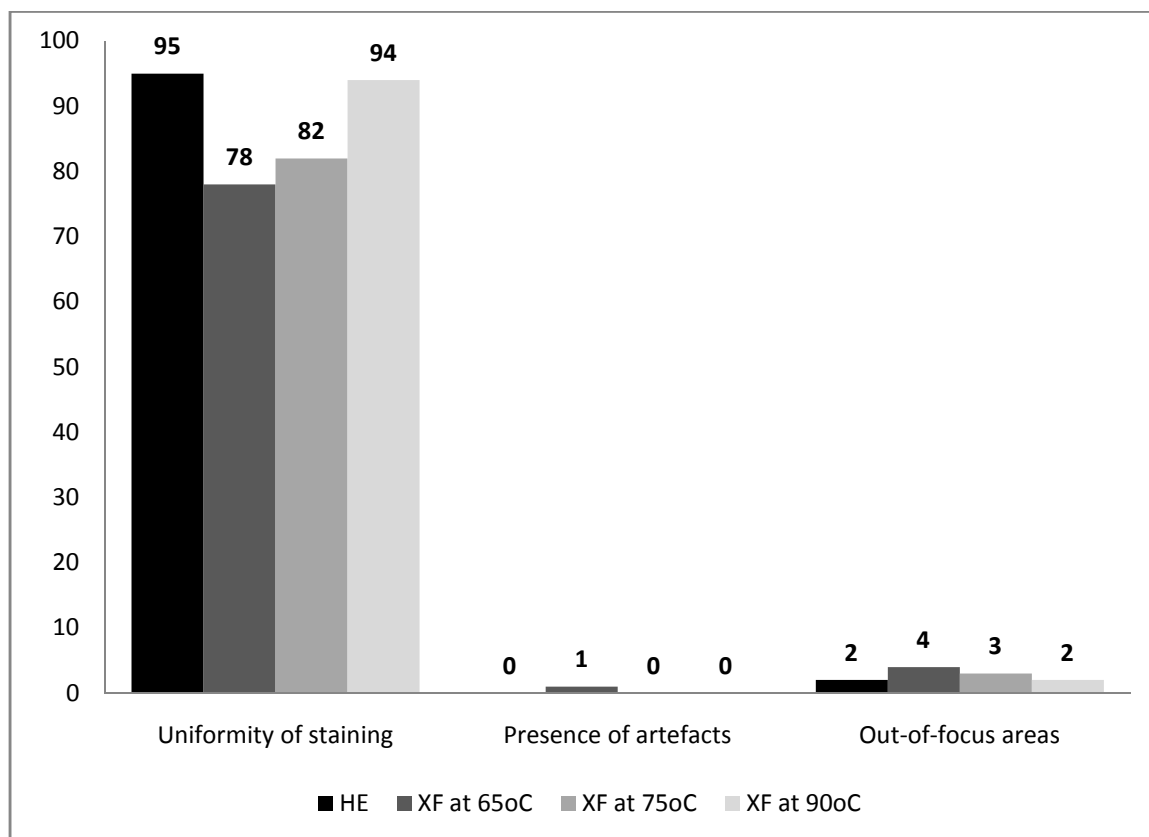
**(Figure 1:** Comparison of average scores between conventional Hematoxylin and Eosin (HE) method and Xylene free (XF) method at three different temperatures)



**(Figure 2:** Average scores by different methods. HE- conventional Hematoxylin and Eosin; XF – Xylene free method)



(Figure 3a: Analysis of components of staining between the two methods: HE- conventional Hematoxylin and Eosin method; XF- Xylene free method)



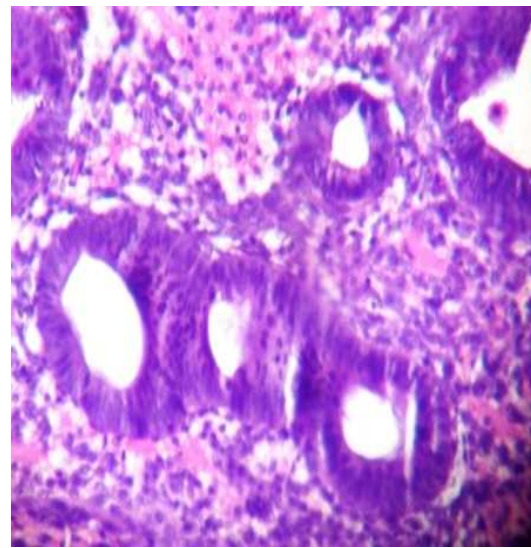
(Figure 3b: Analysis of components of staining between the two methods: HE- conventional Hematoxylin and Eosin method; XF- Xylene free method)



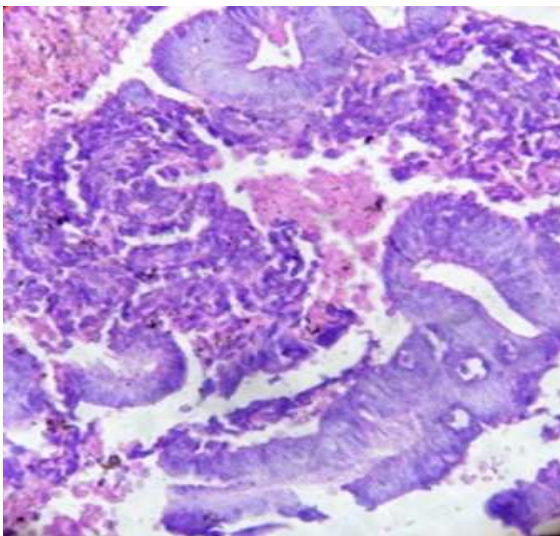
(**Figure 4a-d:** Histopathological comparison of epithelial and stromal elements staining (400x) : a-conventional, b:xylene free 90 °c, c:xylene free 75 °c , d:xylene free 65 °c )



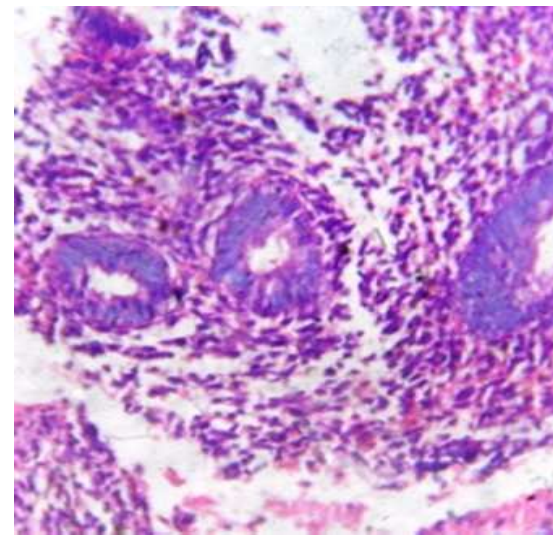
**Figure-4a:** Histopathological comparison of epithelial and stromal elements staining: conventional H&E staining 400x



**Figure-4b:** Histopathological comparison of epithelial and stromal elements staining: Xylene free (90° c)H&E staining 400x



**Figure-4 c :** Histopathological comparison of epithelial and stromal elements staining : Xylene free(75 ° c) H&E staining 400x



**Figure-4 d :** Histopathological comparison of epithelial and stromal elements staining : Xylene free (65 ° c) H&E staining 400x

A cumulative score of  $\geq 6$  was considered to be adequate for diagnosis. The scores for all the components (nuclear and cytoplasmic staining, clarity of staining and uniformity of staining) were equivalent between conventional Hematoxylin & Eosin and Xylene free method at 90°C ( p value of 0.320, 0.456, 0.367, 0.543 respectively).

The score for cytoplasmic staining at 75°C alone, was equivalent to the conventional Hematoxylin & Eosin method (p=0.070).

The scores for rest of the staining components done at 75°C and 65°C Xylene free method showed statistical significance from the conventional Hematoxylin & Eosin method and hence is not reliable as an effective substitute and inferior to the

performance at 90°C. Thus, the new Xylene free method is on par with the conventional Hematoxylin and Eosin method only at 90°C. (Table 4: Statistical comparisons between conventional Hematoxylin and Eosin method and Xylene free method).

**Table-3: Statistical Comparison between conventional hematoxylin and eosin (HE) method and Xylene Free (XF) method**

Staining parameter	HE method	XF method			P value		
		65°C	75°C	90°C	65°C	75°C	90°C
<b>Nuclear Staining</b>							
• Adequate	97	75	80	98	0.002	0.003	0.320
• Inadequate	3	25	20	2	S	S	NS
<b>Cytoplasmic Staining</b>							
• Adequate	97	76	81	97	0.003	0.070	0.456
• Inadequate	3	24	19	3	S	NS	NS
<b>Clarity of staining</b>							
• Adequate	95	74	79	97	0.002	0.003	0.367
• Inadequate	5	26	21	3	S	S	NS
<b>Uniformity of staining</b>							
• Adequate	93	78	82	94	0.001	0.001	0.543
• Inadequate	7	22	18	6	S	S	NS

S-Significant difference; NS- Non significant difference; Adequate – score  $\geq 6$

## Discussion

After the hazardous effects of Xylene became indisputable in the 1970s, many potential substitutes became available in order to make a Xylene-free environment in laboratories, such as limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbons, olive oil, vegetable oils, and mineral oil substitute.

However, these chemicals were used to substitute Xylene as a clearing agent during routine processing, while the exposure and handling of Xylene is maximum during deparaffinization of the tissue sections.

Falkeholm *et al.*, for the first time experimented to use liquid dish washing soap to dewax the tissue sections by eliminating both xylene and alcohol from H and E staining procedures [2].

Liquid dish washing detergent effectively deparaffinizes wax through its surfactant action. The surfactant contains molecules with hydrophilic and hydrophobic portions.

They are absorbed into the surface of oil/grease and remove it from the fabric. After removal, the surfactant molecules remain surrounding the oil to prevent its re-deposition onto the cleaned surface [5]. There are two categories of detergents: automatic machine dish

washing detergents and hand dishwashing detergents. Automatic dishwashing detergents used in automatic dish washers are known to produce skin irritation and are poisonous if swallowed.

Hand dishwashing detergents routinely used in the household are more mild to the skin and if swallowed, may cause mild irritation to the mouth but no dangerous effects [6]. In this technique, since liquid hand dishwashing detergent is highly diluted (1.7%) it has less chances of being toxic to histotechnicians when compared to the harmful effects of Xylene.

An important finding observed in our study was Xylene free staining method is highly temperature sensitive. Temperatures less than 90°C yielded comparatively lower scores and statistically significant changes when compared with the conventional Hematoxylin & Eosin staining.

The diluted 1.7% liquid dish washing liquid has to be maintained strictly at 90°C. Even the slightest increase in temperature resulted in washing away of the tissue sections from the slide and on the other hand, if the temperature was less than 90°C or even if the sections were kept for lesser time than recommended, the sections were not completely deparaffinized leaving

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areas of residual wax, resulting in out-of-focus areas. Similar findings were also demonstrated in another study [9].

The important role of temperature is also evident in studies that used dilute lemon water [10] and hot distilled water [11] which had no surfactants and purely relied on high temperature to melt the wax. As the

deparaffinization of wax is highly dependent on temperature, it is important to standardize the wax used in different labs for accurate comparison of results between various studies.

Experiments by Kayser and Bubbenzer 1990 [12] and Temel *et al* 2005 [13] minimized the staining duration to 5-10 minutes by increasing the temperature during deparaffinisation using a simple kitchen microwave and achieved the same results.

With every innovative substitute like the latest cedarwood oil [8] and mineral oil [9], being coupled with high temperature, it makes us doubt whether temperature potentiates or acts as a confounder in all these studies. More standardized large scale studies, involving various types of tissue sections and different types of stain is needed in the future to derive best results and the ideal Xylene substitute.

A minimum of 30 minutes was required for the Xylene free method whereas a minimum of 70 minutes was needed for the corresponding conventional steps.

Thus in concrete terms the saving in time for deparaffinisation and staining using the Xylene free dish washing solution is of significance especially while handling large number of slides like in a tertiary care center.

The only known substance in literature that doesn't require high temperature for effective wax clearing is a mixture of 86% of white oil No.2 and 14% of N-heptane. It has a high boiling point (188°C) and flash point (144°C) coupled with scentless and decreased volatility [14].

This trial demonstrates that Xylene free histological sections are qualitatively on par with conventional paraffin sections for routine diagnostic work. In view of the savings in time, money and the working environment, the Xylene free method at appropriate temperatures meets today's needs.

It is high time to accept such newer techniques and thereby promoting the elimination of hazardous elements in the routine preparation of slides and thus provide a safe working environment.

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