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Technical Note

Histology without xylene

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Abstract After the hazardous effects of xylene became indisputable in the 1970s, many potential substitutes became available, some with as many if not more hazards. This article discusses the inadequacy of 5 vegetable oils as substitutes, as well as the characteristics of 22 D-limonene–based substitutes, all less effective in their chemical role, some capable of inducing health problems, and costing more than twice as much as xylene. Some of the 35 alkane-based substitutes discussed are effective for tissue processing, less toxic, with a cost about the same as xylene, but are not very effective for dewaxing and other staining tasks. Isopropanol (2-propanol) alone or mixed with molten paraffin is a technically acceptable and cost-effective substitute for xylene for tissue processing, but in this study, we demonstrate that the best clearing agents from the sectioning quality and diagnostic value point of view, with automated or manual protocols, are mixtures of 5:1 and 2:1 isopropanol and mineral oil, followed by undiluted mineral oil, all at 50°C, making them a safer and cheaper substitute than xylene. Using a 1.7% dishwasher soap aqueous solution at 90°C to dewax before staining and oven drying the stained sections before coverslipping will eliminate xylene from the staining tasks. Tissue processors retorts and conduits can be dewaxed with a 2% solution of a strong glassware laboratory detergent. These 4 methodologies will make the histology laboratory xylene-free but, due to the natural resistance to change, many histotechs will be reluctant to adopt them if they think that their technical expertise could be jeopardized, and the only way these changes will succeed is if the pathologists, as stewards of the histology laboratory, commit to their implementation.

Keywords: Clearing agents; Mineral oil mixtures; Tissue processing; Xylene-free

1. Introduction

The historical use of xylene in the histology laboratory is an example of a failed substitution. Starting as the safest alternative to dangerous chemicals such aniline oil, benzene, chloroform, dioxane, and toluene in the 1950s [1,2], by the late 1970s, there were great concerns about its safety [3] with evidence that its acute neurotoxicity was greater than that of benzene or toluene. Other toxic effects of xylene include heart and kidney injuries, some fatal blood dyscrasias, and other less dangerous problems, such as skin erythema, drying and scaling, and secondary infections, all associated with its use [3-5] and caused by depletion of mitochondrial adenosine triphosphate in the affected cells [6].

It was determined that xylene enters the body via the lungs and is stored in adipose tissue (due to it's solubility in it) with a half life of 1 to 6 days in the subcutaneous fat, with long-term exposure causing permanent disability in many workers [7]. Laboratory workers exposed during 1.5 to 18 years were described as having the equivalent of general poisoning disorders [8] including bone marrow toxicity and pancytopenia caused by a xylene contaminated wound [9]. Monitoring xylene vapors became a practice in some work places and an 8 hours time-weighed average (TWA) exposure limit of 100 parts per million (ppm) was established by the Occupational Safety and Health Administration (OSHA) of the US Department of Labor. Both employees and employers were satisfied if that limit was not reached, but this approach does not reflect its incorporation into the employee's system as measured by the concentration of its major metabolite in urine, methyl-hippuric-acid (MHA),

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with a biological exposure index limit of 20 mg/dL recommended by the American Conference of Governmental Industrial Hygienists.

Exposure to xylene vapor levels between 16 and 7000 ppm of a group of workers (2 histotechs included) showed a correlation between vapor levels and the presence of MHA and demonstrated that the meta-xylene configuration (isomer) was the preferred metabolite over the other two (ortho-xylene and para-xylene) via methyl-benzyl-alcohol by the cytochrome P4502E1 [10].

Even TWA levels of 0.03 to 14.44 ppm (average = 3.36 ± 3.63 ppm), well below the OSHA threshold, led to increments of urinary MHA and creatinine levels in 20 subjects, with a correlation predicting a value of 1.3 g of MHA per gram of creatinine after exposure to the OSHA limit of 100 ppm [11]. The maximum concern was reached when a controlled study of 125 women, including 21 laboratory technicians, research students and researchers showed that malformations in fetuses occurred 13 times more frequently in those exposed to aromatic organic solvents (xylene included), [12] concluding that it was prudent to minimize women's exposure to organic solvents during pregnancy because xylene readily passes through the placental barrier and is even present in the maternal milk. However, in spite of that and of being recognized as a reproductive toxin [13], 31% of US histology laboratories in 2007 still did not protect pregnant employees from xylene exposure [14]. Other documented effects of xylene include bilateral auditory neuropathy (retrocochlear hearing loss) [15], and possibly epithelial and stromatal keratopathy, either as splash contamination or through its fumes [16].

It is also worth noting that xylene is present in many household solvents, air fresheners, stainless steel cleaners, floor polishers, and gasoline and that a total of 2.1 million workers in 42 sectors of the US economy were exposed to it in 1995, according to a projection of the 1981 to 1983 Hazard Surveillance Program of the National Institute for Occupational Safety and Health, with a total of 108 767 workers (69% women) belonging to the health services. Other sectors with considerable female participation are the manufacture of electronic equipment and computer components including silicone wafers and chips (47%) and the apparel and other textile products sector (76%).

Besides these health hazards, in 1980 [17], it was shown that when compared with chloroform and inhibisol, clearing with xylene resulted in a 50% reduction of the immunohistochemical (IHC) signal of plasma cells because of its extracting effects on the cytoplasm as observed ultrastructurally using electron microscopy.

In the histology laboratory, the histotechs are exposed to xylene during tissue processing, dewaxing sections before staining, clearing them before coverslipping, while cleaning tissue processors and recycling. The knowledge of xylene toxicity pushed for its substitution, so much so that 41% of US histology laboratories in 2007 used substitutes for tissue processing and 62% had automated coverslippers, whereas 54% were recycling xylene [14].

This article presents a review of the fundamental groups of xylene substitutes and practices, along with 4 new approaches to its total elimination from the histology laboratory that, if generally adopted, will make a real improvement in the safety of the work environment and reduce operation costs.

2. Materials and methods

Eliminating xylene during processing and staining are validated in this article following the same methodology published elsewhere [18] consisting in the blind evaluation of pairs of sections followed by the statistical comparison of the observed and theoretical frequencies using the null

Table 1
Validation of mineral oil alcoholic mixtures as clearing agents

Information	Mineral oil alcoholic mixture	
	EIM vs X [18]	IM vs WS (tp)
Mineral oil used	Drakeol 7	Lukoil I-20A
Flash point	179°C	180°C
Kinematic viscosity (cS)	11-14 (40°C)	12 (50°C)
Mixtures composition, temperature, and (mineral oil proportion)	EIM 2:3:1 (45°C) (M = 1/6) EIM 1:3:2 (50°C) (M = 1/3)	IM 5:1 (50°C) (M = 1/6) IM 2:1 (50°C) (M = 1/3)
Tissues used for validation	36 types[a]	29 types[b]
Tested HC and IHC procedures	12 HC and 21 IHC	H&E
Pairs of slides for the blind evaluations	92	56
Referees/total evaluations	9/828	5/560
Sectioning quality comparison (χ^2)	NS for $P > .90$	NS for $P > .14$
Diagnostic value comparison (χ^2)	NS for $P > .005$	NS for $P > .001$
Implemented as standard procedure	June 1999[c]	August 2007[d]
HC methods after implementation	48	16
Antibodies (IHC) after implementation	115	4
Tissue processing	Automatic	Manual
Protocol time without fixation (h)	7.5	28
Clearing (% of protocol time)	53	68

E indicates ethanol; HC, histochemical; I, Isopropanol; M, Mineral oil; ns, difference nonstatistically significant; tp, this paper; WS, white spirits; X, xylene.

[a] Adrenal, appendix, artery, artery-vein-fat package, breast, cartilage, colon, epiglottis, Fallopian tubes, gall bladder, heart, hemorrhagic mesentery, hemorrhoid, kidney, lipoma, liver, lung, melanocytic nevus, muscles (skeletal and smooth), nasal septum, parathyroid, pituitary, placenta, pleural fluid, prostate, scalp, sebaceous cyst, secreting skin glands, skin, small intestine, spleen, testicle, thyroid, tonsil, uterus.

[b] Amnion, appendix, bone, brain, brain stem, breast, cerebellum, colon, curettage, epidermal cyst, eye ball, fibrin, gall bladder, heart, kidney, lipoma, liver, lung, lymph node, ovary, pancreas, peritoneum, placenta, prostate, skin, stomach, umbilical cord, thyroid, uterus.

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hypothesis (in which the differences are due to chance) with the χ^2 test of independence, with an α-type error and a minimum accepted significance level of $P_{0.95}$ as is common practice [19].

When the alcoholic clearing mixtures made by adding ethanol (E) and isopropanol (I) to mineral oil (M) or EIM mixtures were going to be tested at the Taganrog laboratory (Russia), the fortuitous fact that affordable ethanol of sufficient quality was difficult to obtain determined changing to IM alcoholic mixtures that resulted in cheaper, simpler to prepare reagents that were equally as effective as EIM. These new mixtures, although very similar to those originally validated [18], require their own validation that is the subject of the first part of this article.

2.1. Clearing with the alcoholic isopropanol and mineral oil (IM) mixture

A total of 36 different tissues were used for the EIM validation [18] and 29 for the IM validation, 14 used in both, for a total of 51 different tissues for both alcoholic mixtures (Table 1). From each type of tissue to be processed (from surgical or autopsy material), there were 2 similar slices placed in individual cassettes and numbered sequentially, one with subnumeral "1" (odd) and the other with "2" (even). One cassette from each pair of cassettes was randomly selected (odd or even) to be assigned to the existing procedure with acetone and white spirits (WS) also known as *paint thinner* or *mineral spirits*, whereas the other cassette was to be processed with the IM mixture. There were a total of 56 pairs of blocks from the 29 types of tissues, with one slide from each evaluated twice after 1-month intervals by 5 referees for a total of 560 evaluation results. There were only 2 options to evaluate the results: either one section was better than the other for diagnostic purposes or both were equivalent. The section quality of the blocks was evaluated by 6 histotechs, also with only 2 options as follows: one block sectioned better than the other or both were equivalent.

2.2. Dewaxing without xylene

The tested procedure is a variation of one published previously [20] that consists in dewaxing the sections with a 1.7% household liquid dishwasher soap aqueous solution (vol/vol) (we used the brand "Fairy") heated at 90°C and then oven drying the stained sections before coverslipping without the chemical dehydration and clearing steps. The data of Falkeholm et al [20] included 3 types of tissues and 3 stains with 90 pairs of sections evaluated by 9 referees and validated statistically, so it was decided to expand the validation to 10 different staining procedures from 13 different types of tissues processed with IM in 33 pairs of sections evaluated twice with 1-month interval by 7 referees for a total of 462 evaluation results with the same 2 options as follows: either one section was better than the other for diagnostic purposes or both were equivalent.

The possible bias of the referees participating in the evaluations (IM tissue clearing and xylene-free sections dewaxing-coverslipping) was studied with the F test (analysis of variance) after the $\sqrt{(x_i + 3/8)}$ transformation of the data [19].

2.3. Theoretical frequency

The χ^2 test compares the observed frequencies (f_o) obtained from evaluating each pair of slides, against the theoretical frequency (f_t) that should be found had the differences been due to chance, using each referee's 2 allowed options as follows: either one section was better than the other, or both were equally useful for diagnosis.

If the existing tissue clearing procedure with WS was equivalent to the new isopropanol-mineral oil mixture (IM), they were the same (S), and IM would be selected as IM or as part of S, and the same would happen with WS in a way that their frequencies had the following theoretical distribution:

$$(S + WS) + (S + IM) = 1.00,$$

and if both procedures were really equivalent, the null hypothesis was not rejected and the χ^2 calculated value could not be higher than the χ^2 value in the tables; for the number of degrees of freedom (*df*) for each comparison,

$$(S + WS) = (S + IM) = 1.00/2 = 0.50,$$

this being the f_t for each procedure.

2.4. Kinematic viscosity

It is known that the flowing speed of the reagents through the processing tissues is an inverse function of their kinematic viscosity (v) in centi-Stokes (cS = mm^2/s) [21], so it was decided to determine it for the reagents used at Taganrog laboratory with a capillary viscometer [22] using the median flow value of 5 consecutive determinations per reagent. The value of v and other physics constants for other substances were obtained from their Material Safety Data Sheets, their descriptions in the Chemical Abstract Service (CAS number) and other recognized sources [23].

2.5. Comparisons with other xylene substitutes

Starting in the late 1970s, there have been many xylene substitutes in the market, but only few have been independently evaluated so the characteristics of some of the 57 brand names included come from the anecdotal information posted in HistoNet, a free World Wide Web list server with more than 3300 members worldwide (http://www.histonet@southwestern.edu), where the issue of xylene substitutes appears sporadically.

3. Results

3.1. Clearing with the alcoholic IM mixtures

The sectioning quality of the blocks processed with IM vs WS was considered equivalent for 91% of pairs without

significant differences between them ($\chi^2 = 1.93^{ns}$; $P > .14$; $df = 5$).

The diagnostic value of the sections showed no statistical differences ($\chi^2 = 20.74^{ns}$; $P > .001$; $df = 110$), and the referees' evaluations were unbiased ($F(4;8) = 0.24^{ns}$; $P > .10$) meaning that both procedures are equivalent, that IM can substitute WS and by extension can substitute xylene in the same way that EIM can [18], and that both mineral oil alcoholic mixtures (EIM and IM) are equivalent as clearing agents before the paraffin wax infiltration (Table 1).

3.2. Dewaxing without xylene

The sections dewaxed with the dishwashing soap solution (DWS) were diagnostically equivalent to those dewaxed with xylene ($\chi^2 = 47.00^{ns}$; $P > .005$; $df = 64$), and the referees' evaluations were unbiased ($F(6;12) = 0.04^{ns}$; $P > .10$).

The DWS is prepared by dissolving 25 mL of household dishwasher soap in 1500 mL of distilled water for a 1.7% solution, but it could go up to 30 mL of soap for a 2.0% DWS. The protocol is as follows:

1. The sections have to be totally drained before being dried in an oven at 60°C for at least 20 minutes as is usually done before staining.
2. The dried sections are placed in the DWS at 90°C for 1 minute.
3. Transfer to another container with DWS at 90°C for another minute.
4. Wash the slides in tap water at 90°C for 30 seconds.
5. Wash the slides in tap water at 90°C for another 30 seconds.
6. Wash the slides in tap water at 45°C for 30 seconds.
7. Place the slides in distilled water at room temperature and stain as usual.

This dewaxing procedure lasts 3.5 to 4 minutes per batch of slides.

The stained sections are washed with distilled water, drained, oven dried (5 minutes at 60°C will be sufficient), and coverslipped as usual, even when this departs from common histologic technique. This protocol is the one showing no diagnostic differences against the usual dewaxing and clearing with xylene.

4. Discussion

Xylene became the clearing agent of choice when chloroform was declared a carcinogen and a safer substitute was needed, but when xylene was also identified as a health hazard, replacing it with safer chemicals became a major objective of researchers and manufacturers alike. However, all focused on its use as a clearing agent even when the exposure is greater during dewaxing and staining.

4.1. Clearing without xylene

The proposed substitutes included vegetable oils, terpenes, alkanes (aliphatic, isoparaffinic, naphthenic, and paraffins of several molecular weights), and even a trend to process tissues going directly from the dehydrating agent into the paraffin wax, each with different levels of success in their substitution goals.

4.2. Clearing with vegetable oils

Olive and coconut oils were evaluated in 1992, and the results showed only minor differences in comparison to xylene in a minority of cases from a total of 232 specimens stained with hematoxylin and eosin (H&E), some histochemical and IHC procedures, but the long-term preservation of the processed tissues was not determined [24]. The 2 oils tested have kinetic viscosities (v) 3.1 to 4.3 times higher than molten paraffin wax, and this is a disadvantage at the moment of the latter trying to displace the oils during infiltration. Also olive oil and coconut oil are 1.78 and 2.21 times more expensive than xylene, respectively.

Later (1994), 3 clearing-infiltrating mixtures made with vegetable oils and paraffin at a ratio of 1:1 were tried for 1 hour after dehydrating for 8 hours with isopropanol [25]. Peanut oil, a mixture of soybean with cotton seed oils, and coconut oil were used with a total of 99 blocks from 3 types of tissues, and it was concluded that the best results were obtained if after the dehydration with isopropanol the tissues were treated with a mixture of isopropanol and paraffin (1:1) at 50°C (2 hours) followed by a final infiltration with pure paraffin (4 stations with 1 hour each). The oils used have a v of 3.5 to 4.2 times higher than molten paraffin posing the same infiltration difficulty stated before, but, except for coconut oil, they are between 0.05 (soybean oil) and 0.32 (peanut oil) to 0.54 (cotton seed oil) times the price of xylene.

Vegetable oils are a poor alternative to xylene because of their high kinematic viscosity and immiscibility with alcohols as was concluded in one of the trials [25].

4.3. Clearing with terpenes

Terpenes or terpenoids are isoprene polymers found in essential oils from plants. They were the first clearing agents used in histology and include turpentine, initially known as *terpentine* (after which the whole group was named), and oils of bergamot, cedar wood, clove, oregano, terpineol, and thyme, among others [2].

Limonene, obtained by steam distillation of the liquid slurry resulting from citrus fruit peel pressings, also belongs to this group. Chemically consisting of 2 isoprene units, exists as dextrorotatory (D or +) and levorotatory (L or −) racemic mixtures with the "D" or (+) form being the main

component in the citrus peel oil (CAS no. 5989-27-5) and has been "generally recognized as safe" by the US Federal Drug Administration.

Limonene is produced in technical, refined, and food grades and when in the late 1970s xylene dangers became an issue, many manufacturers started to produce D-limonene–based alternatives because it was readily available, cheaper than other terpenes, and had already been incorporated in many household dewaxing and cleaning products. All these new substitutes had the property of having a strong and, for some, nauseating citrus odor, sometimes causing skin reactions because it readily oxidizes with the air producing allergens [26]. It is also oily, irritant to the eyes, has a low evaporation rate and is considered by some able to cause as many if not more problems than xylene, although of a less dangerous nature. Occupational Safety and Health Administration has not established exposure limits for limonene, but the American Industrial Hygiene Association has an exposure standard of 30 ppm. It is difficult to eliminate from the paraffin and some recommend using toluene before the infiltration, defeating the purpose of use. It usually requires processing schedule modifications, and the stains tend to fade when exposed to strong light. It is biodegradable, having an average density (ρ) of 0.84 to 0.85 g/mL, a ν = 1.07 cS, both at 25°C, and a dielectric constant (ε) of 2.3 very similar to paraffin wax. Sometimes, it is sold mixed with aliphatic hydrocarbons or diluted with water and is between 0.93 and 7.44 (average of 2.19) times the price of xylene.

Table 2
Clearing with terpenes (D-limonene)

Xylene (X) substitute: name and evaluation	Price[a]
AmeriClear: 60% of the quality of X [27]; citrus smell in 20-year-old blocks; produces a "metallic taste" in some histologic technicians; requires process modification; does not fade H&E	4.12
CitriSolve-hybrid: aliphHC (50%) + D-limonene + emulsifier + BHA	2.31
CitroClear: better and faster than others, strong smell, tendency to turn yellow and throw out oily deposits	2.22
CitroSolve, CitraSolve, CitriSolve: mostly water and surfactants (91%) + D-limonene (9%) making it water soluble	1.40
Citrus Clearing Solvent: fruitlike odor	1.63
Citrus Natural Solvent: D-limonene; irritant	1.11
Clearene: redistilled d-limonene, contains antioxidant to prevent stains fading	1.27
D-limonene: possible health hazards, nonrecyclable [27]; not user friendly [28]; can be oxidized with histologic reagents causing dermatitis [26]; has caused respiratory problems (asthma) in many histologic technicians	1.98
Hemo-De: from less effective [29], to 61% [30], to 97% [31], to similar to X [27,32,33], contains an unknown amount of BHA	2.44
Histoclear, Histolene: behaves similar to X [27,34], poor dewaxing for IHC sections; fades hematoxylin; needs a special mounting medium; hardens brain, liver, and spleen; causes skin problems and headache	1.47
Histoclear II: aliphHC (70%-90%) + D-limonene (30%-10%)	1.46
Histolemon: 90% D-limonene, strong odor, results similar to X	2.62
Histosolve X, Bio-Clear: flammable waste, needs special mounting medium, dries tissues, and produces wrinkles difficult to open [35], some have used it satisfactorily for 17 years, absorbs too much water	2.07
K-Clear: contact dermatitis on histologic technician [26] needs to use L-Mount (DDK, Milano, Italy)	N/A
Master Clear Clearing Agent: technical grade d-limonene; strong odor	0.93
Pro-Clear: D-limonene	7.44
Roti-Histol: mandarin fragrance, classified as dangerous to the environment, to be disposed as hazardous waste, skin irritant, needs special mounting medium	1.42
Safety-Solv: mild citrus odor, nontoxic or flammable, biodegradable	1.36

aliphHC indicates aliphatic hydrocarbon; BHA, butylated hydroxy anisole (CAS no. 25013-16-5) is a suspected carcinogen; N/A, not available.
[a] Price = times the price of Xylene.

From a total of 161 HistoNet postings commenting about xylene substitutes, 32% refer to D-limonene containing products of which AmeriClear, Histoclear, and Histosolve X (Bio-Clear) account for 67% but because of their use results, the personnel safety concerns they raise and their cost (Table 2) are not good alternatives to xylene.

4.4. Clearing with alkanes

Alkanes are saturated hydrocarbons with a variable number of carbons arranged in straight line (aliphatic hydrocarbons), branched (isoparaffinic hydrocarbons), or with one or more cycloalkene carbon rings (naphthenic hydrocarbons), with physical and chemical properties dependent on their structure and number of carbons in the molecule, none containing benzene.

Knowledge that naphtha [2], gasoline (petrol) [36] and specially the so-called WS or Stoddard solvent (CAS no. 8052-41-3) had been used for years as clearing agents in some European histology laboratories motivated the development of proprietary blends of alkanes to substitute xylene.

In general, they have low odor level, are not very oily, are recyclable, with low hazard levels (TWA up to 600 ppm), and render tissues less brittle than xylene and D-limonene but are less effective at dewaxing during staining, and if used to clear sections are usually incompatible with xylene or toluene-based mounting media, some being skin irritants and toxic if inhaled. As a general advantage their cost is between 0.12 and 2.56 (average of 0.94) that of xylene or 2.3 times cheaper than D-limonene–based substitutes. Their number of carbons determine their properties (ρ = 0.74-0.88 g/mL; ν = 0.8-2.6 cS) and with $\varepsilon < 20$ are nonpolar substances miscible with paraffin.

Of a total of 161 comments posted in HistoNet dealing with xylene substitutes, 68% were about alkane-based products, and of them, 76% referred to Clear-Rite, Formula 83, and ProPar. The information summarized in Table 3 shows that 60% are either pure or combined naphtha formulations (CAS no. 64742-48-9) and generally speaking are better xylene substitutes than D-limonene but still have some shortcomings when compared with xylene.

Table 3
Clearing with alkanes

Xylene (X) substitute: name and evaluation	Price[a]
Clear-advantage: (naphHC) low odor level, recyclable	0.92
Clearify: (naphHC) low hazard	0.49
Clear-Rite 3: (aliphHC) oily, dries and makes tissues brittle, sections wrinkles difficult to open [37]; fades stains; recyclable; needs processing modifications and changes more frequently; allergen; used for 10 y satisfactorily	0.87
EMS: (isopHC) can cause skin, eye, and respiratory tract irritation; absorbs moisture; needs processing modifications; flammable	N/A
Envirene: (naphHC) methanol and most mounting media incompatible	2.56
Formula 83: (naphHC) cannot be used in coverslippers, recyclable, irritating, used for 20 y satisfactorily	0.67
Isopar-L: (naphHC) C_{11-15} hydro heated heavy naphtha	N/A
K-Clear Plus: (isopHC) needs Ecomount-K mounting medium	N/A
Kerosene: (isopHC) combustible; oily (ν = 3.4 cS at 20°C)	0.12
MCS-2806 process fluid: (naphHC) (ν = 1.28 cS at 38°C)	N/A
Microclear: (isopHC) from 88% [38], to 98% [31] to equal to X [39] good for processing and staining, needs MicroCover mounting medium, X required to clean tissue processors, biodegradable	0.80
Naphtha: (naphHC) causes only minor tissue shrinkage	1.48
Neo-Clear: (StodSol) only for TP	1.07
P-4: (isopHC/naphHC) only for TP (ν = 0.8 cS at 25°C)	N/A
Paraclear: (naphHC) only for TP, cannot clean the tissue processor	0.97
Pro-Par: (isopHC + PGE) from 60% [27] and 88% [38] to equal to X; needs an additional container for TP and staining, tissues less dry than with X, needs changing more frequently	0.67
Roticlear: (naphHC) for TP and staining (ν = 1 cS at 20°C)	0.65
S1-Histo: (isopHC/naphHC) for dewaxing sections	N/A
S3-Histo: (isopHC/naphHC) for clearing stained sections	N/A
SafeClear: (StodSol) only for TP	1.72
SafeClear II: (StodSol + naphHC + isopHC) only for TP	1.91
Shandon xylene substitute: (aliphHC) from 84% [38] to less effective than X [29]; recyclable, less oily, for TP and dewaxing but not to clear stains, used for 13 y satisfactorily	0.99
ShellSol 15: aliphatic naphtha 85% + light aromatic naphtha 1% + ethyl benzene + TMB; vapors can cause drowsiness and dizziness; causes skin irritation; combustible	N/A
ShellSol A 100: (100% naphHC) used satisfactorily since 1980 for TP	0.24
Slide Brite: (aliphHC) from 93% [27] to as good as X [38]; somewhat greasy, with ethanol is not transparent; good for TP	0.84
Slide Brite Elite: (aliphHC) more compatible with regular mounting media	0.65
Solvent 100: (light and heavy aromatic naphtha) (ν = 1 cS at 25°C)	0.47
Sub-X xylene substitute: (naphHC) will not harden tissue, not greasy, slightly water soluble	0.61
Trican XKO: (aromatic and isopHC 60%-100% + aromatic naphtha 5%-10% + benzene <1%); skin irritant; contains X; flammable	0.16
UltraClear: (isopHC) with C_{9-12} replaces X, toluene, and D-limonene as clearing agent in TP; dermatologically inert; odorless	N/A
Waxol: (naphHC) with C_{11-15} for TP (TWA = 171 ppm) (ν = 1.67 cS at 25°C)	1.27
White spirits: (StodSol) mild odor, noncorrosive, good for TP (ν = 0.93-2.06 cS at 25°C)	0.54
XS-3: (naphHC) dewax incompletely; too much water	0.47

Table 3 (*continued*)

Xylene (X) substitute: name and evaluation	Price[a]
sensitive; slow to dry; tissues less brittle than in X; bleeds DAB; can cause dermatitis, dizziness, drowsiness, and headache	
Xylene substitute 2: (aliphHC) as good as X [27] biodegradable; used for 13 y for all steps except cleaning the tissue processor	2.44
Xyless II: (naphHC) only for TP	0.59

aliphHC indicates aliphatic hydrocarbon; isopHC, isoparaffinic hydrocarbon; naphHC, naphthenic hydrocarbon; naphtha (CAS no. 64742-95-6; TWA = 300 ppm); DAB, diaminobenzidine (chromogen in immunohistochemistry). ν, kinematic viscosity in centi-Stokes (cS); N/A, not available; PGE, propylene glycol ether; StodSol, Stoddard solvent (TWA = 500 ppm); TMB, 1,2,4-try-methyl-benzene (CAS no. 95-63-6) a suspected carcinogen; TP, tissue processing.

[a] Price = times the price of xylene.

4.5. Processing tissue without a clearing agent

This rather drastic option certainly eliminates the use of chemicals with different levels of toxicity by going directly to the infiltration step with paraffin wax after the dehydration is completed. Even when it is contrary to most of the protocols found in the specialized literature and the practice of most histology laboratories, paraffin has been described as a clearing agent by itself with a clearing time similar to chloroform [36]. Going directly to paraffin after dehydration is the technology used in one type of tissue processor and in

Table 4
Isopropanol as dehydrating and clearing agent

General processing protocol	Reference
Graded IP (70%-80%-95%-100%) at 50°C (8 h) → mixture IP:P (1:1) at 50°C (2 h) → P at 60°C (4 h) at 1 h per station	[25]
Graded IP (50%-70%-90%-100%) 2 stations each → mixture IP:P (1:1) at 50°C → P at 60°C [ex Romeis]	[55]
Pure IP (6 stations for 5.5 h) → molten P (3 stations for 5.5 h). Validated with 810 paired observations (3 types of tissues and 3 stains) with 74% as good as or better than with X; used as sole method for 6 y before the publication of the results in 2001; evaluation with Wilcoxon and κ measure of agreement tests	[20]
50% E (2 stations) → mixture E:IP (4:1) → Pure IP (3 stations) → P (85°C and vacuum) (2 stations) → P (65°C and vacuum). Brain tissue and 2 stains, with the Peloris instrument; manufacturer reported good results; no independent evaluation	[40]
Manual processing with pure IP with Triton X15 at 1:10 000 at room temperature (8 changes for 30 h) → P at 60°C (for 3.5 h). The surfactant facilitates the tissue transition between water and P; used for >10 years with better results than with acetone, benzene, chloroform, ethanol, and xylene; no quantitative evaluation	[46]
Manual processing with pure IP (5 changes for 26 h) → P at 60°C (for 4 h) with good infiltration results; no quantitative evaluation	[a]

E indicates ethanol; IP, isopropanol; P, paraffin wax; X, xylene.

[a] Tested in 2001 at the Taganrog laboratory (Russia) by MV Peshkov.

several manual protocols, but the dehydrating agent has to be totally eliminated if immiscible with paraffin, which is done by increasing the temperature under low pressure before the paraffin infiltration. Although vacuum and high temperature (up to 85°C) are applied [40], the pieces of tissue are being dried in the cassettes without any type of support other than their microscopic architecture held in place by the hardening effects of the initial fixative and the dehydrating agent.

The Peloris tissue processor uses this infiltration technology after dehydrating with ethanol and/or isopropyl alcohol. The qualitative evaluation of the processing results for this processor used central nervous system stained with H&E and luxol fast blue/cresyl violet [40], 10 other types of tissues (3 porcine) [41], and 5 porcine tissues [42] stained with H&E and were published as the manufacturer's internal documents. This instrument has been mentioned elsewhere [28], but there are no independent performance evaluations. A nonvalidated paraffin infiltration after ethanol dehydration with the help of microwave heating technology was also published [43].

Isopropanol is a known dehydrating agent not used frequently because of a slower, although gentler and less hardening effect, than ethanol [36,44]. It has been used combined with xylene [25] or instead of a clearing agent with heat [25] or at room temperature very effectively for years because in all those manual methods there is always a remnant of isopropanol in the tissue that will mix with the melted paraffin wax allowing a good infiltration.

The initial experiments with the Peloris were done with ethanol, which is a small (46.07 Daltons) polar (ε = 25 at 20°C) [23] primary alcohol absolutely immiscible with paraffin wax requiring its elimination by vaporization (boiling) at low pressure. This technique for vaporizing ethanol was kept as part of the instrument's protocol after isopropanol replaced ethanol as the dehydrating agent. The instrument heats the tissues at above the 82.3°C isopropanol boiling point even when this is unnecessary because isopropanol is a 1.30 times larger (69.11 Daltons) less polar (ε = 18 at 25°C) [23] secondary alcohol within the range of the non polar liquids making it miscible with molten paraffin wax especially at temperatures above 25°C.

Reaching a good equilibrium between low pressure (level of vacuum) and high temperature is claimed to be the key for a successful infiltration, but if there is too high a temperature, the tissue will appear "cooked." If the vacuum is applied too abruptly, there will be clefts in the tissue underlining the structural vulnerability of dried out tissues, and if the time is too short, the infiltration will be incomplete [45] making the whole operation not risk-free from tissue damage. The manufacturers of Peloris claim that their instrument's success resides on being able to raise the temperature to 85°C very quickly and with the adequate amount of vacuum, but there have been no independent validations of this instrument especially when the elimination of isopropanol under vacuum and high temperature raises some concerns about the potential damage to the structural integrity of the tissues.

The use of isopropanol as both a dehydrating and clearing agent is summarized in Table 4 and, except for one procedure whose results were evaluated statistically [20], none other has been similarly validated although some have been used as routine protocols for years [46] making it a viable xylene substitute. Some of these procedures use isopropanol directly before the paraffin wax determining a solubility parameter gradient (δ_{grad}) of 9 Mega-Pascals (MPa), but if mixed with melted paraffin (1:1), the δ_{grad} is reduced to 4.5. This led to a logical next step, such as introducing paraffin of low molecular weight or liquid paraffin, also known as *mineral oil*, mixed with the dehydrating alcohol. This mixture acts as the clearing agent at a lower temperature than molten paraffin and allows a gentler transition between the dehydration and the paraffin wax infiltration steps.

4.6. Clearing with mineral oil alcoholic mixtures

Because of their polarity (expressed by their dielectric constant or ε value), ethanol and isopropanol behave

Table 5
Clearing with mineral oil pure or in alcoholic mixtures

(Year) and processing protocol	Reference
(1994) dehydration with DMP → clearing with M→ P. (No validation)	[47]
(1996) graded E (6.25 h) → M at 45°C (2 h) → paraffin at 60°C (3 h); 12 tissues, qualitative evaluation about cellular detail and antigenicity preservation; blocks 6 mo later were found in good condition	[48]
(1998) patented 1 step dehydrating and clearing reagent composed of E+ I + "a long chain hydrocarbon" ("JFC solution"); 4 types of tissues; using an MWTP (no validation)	[49]
(2000) after graded E (80%-90%-100% × 3) → EIM (2:3:1) mix at 45°C (M = 1/6) → EIM (1:3:2) mix at 50°C (M = 1/3) → M (50°C) → P × 4 (58°C) 4670 blocks with <1% of substandard quality; 37 tissues; 12 HC + 21 IHC procedures (no diagnostic differences between X and M) ($[\chi^2]^{ns}$ $P > .005$)	[18]
(2002) A + I (MW at 62°C) → AIM (×2) mix (MW at 62°C) → M + P (×2) at 65°C → P (×4) all at 65°C (qualitative evaluation by patent holders) (Initial patented protocol for the "Xpress" TP)	[50]
(2003) AIM (25:55:20) mix (M = 1/5) (x2) (MW at 62°C) → M+P (65° with convection heat) → P (65°C convection heat) (qualitative evaluation by patent holders)	[35]
(2004) AIM mix (2 MW retorts at 62°C) → M + P (convection retort at 65°C) → P (convection retort at 65°C) (qualitative evaluation by patent holders) (final configuration of the "Xpress" TP)	[51]
(2008) graded I (70%-80%-90%-95%-pure) → IM (5:1) mix at 50°C (M = 1/6) → IM (2:1) mix at 50°C (M = 1/3) → M at 50°C → P (×4) at 60°C; 29 tissues (no diagnostic differences between WS and M) ($[\chi^2]^{ns}$ $P > .001$)	Tp

A indicates acetone; DMP, 2,2-dimethoxy-propane; E, ethanol; HC, histochemical; I, isopropanol (2-propanol); M, mineral oil; MW, microwaves; P, paraffin wax; TP, tissue processor; tp, this paper; WS, white spirits; X, xylene.

differently when in contact with mineral oil; isopropanol does not mix with mineral oil at room temperature but mixes well in a proportion of 3:2 at 44°C [18] when its ε is less than 18, which is below the upper limit of the nonpolar liquids ($\varepsilon < 20$). Ethanol, on the other hand, does not mix with mineral oil even at more than 60°C [18] because its ε remains more than 20 preventing it from mixing with paraffin also. The use of mineral oil either undiluted or in alcoholic mixtures as a clearing agent is summarized in Table 5.

After dehydration with ethanol, a group of 12 tissues were immersed directly in low viscosity mineral oil at 45°C before infiltration in 2 consecutive changes of paraffin at 60°C. The results were qualitatively graded as good, the blocks filed and reviewed 6 months later and found in good condition [48]. Later (1998) this protocol was tried [18] resulting in 67% of 268 blocks/slides of substandard quality. The Brooke Army Medical Center (Houston, Tex) was contacted and informed us that their article [48] recorded an experimental procedure that was never implemented.

In 2002, this same method (ethanol → mineral oil → paraffin) [48] was used elsewhere [52] and, although the method itself was not evaluated in that article, the 10-color photomicrographs included show signs of a very poor infiltration attributable only to ethanol that was not completely eliminated from the tissue and, being immiscible with molten paraffin, resulted in incomplete infiltration, this being the great disadvantage for this particular sequence.

The next step was to use mixtures of ethanol and mineral oil at different concentrations and temperatures in 6 different protocols, but the overall miscibility was always incomplete and 32% of 467 blocks/slides processed this way [18] were of substandard quality. The ethanol-mineral oil immiscibility was solved when isopropanol was added resulting in completely transparent mixtures at 45°C (EIM at 2:3:1 with $\varepsilon = 17$) and at 44°C (EIM at 1:3:2 with $\varepsilon = 14$), both within the range of the nonpolar solutions. This processing method [18] became the standard at Mount Sinai Medical Center (Miami Beach, Fla) on June 25, 1999, and original blocks, now 10 years old examined on September 18, 2008, are in perfect condition guaranteeing the usefulness of archival material when processed this way.

The "long chain hydrocarbon" included in the dehydrating-clearing solution patented by Milestone as the "JFC solution" [49] is most likely mineral oil based and was intended to be used with one of their initial microwave tissue processors.

Between 2002 and 2004, several mixtures containing isopropanol and mineral oil among other ingredients were developed [35,50,51] and, because they can be heated with microwaves, are used as clearing solutions in the Xpress tissue processor and have been incorporated into several patented procedures.

As explained in Materials and Methods, ethanol quality issues determined that the introduction of the EIM mixtures at the Taganrog Laboratory had to be changed into IM mixtures, one at 5:1 ($\varepsilon = 15$) and the other at 2:1 ($\varepsilon = 13$) with the subsequent statistical validation presented in the Results section in this article. Both types of clearing alcoholic mixtures are equivalent but the isopropanol-mineral oil (IM) mixtures are simpler to prepare and less polar. From the cost point of view the EIM (2:3:1) and (1:3:2) solutions cost 0.51 and 0.50 times the price of X, respectively, but the IM solutions (5:1) and (2:1) are even cheaper (0.43 and 0.34 times the xylene cost, respectively).

Table 6
Characteristics of tissue processing reagents and solutions

Reagent/solution	δ	ε	ν^a	t°C	ρ^a
Neutral buffered formalin	ca.48	ca.75	1.23	18	1.00
IP 70%	32	37	3.62	19	0.85
IP 80%	30	30	3.42	19	0.84
IP 90%	27	24	3.05	18	0.81
IP 95%	26	21	2.81	18	0.79
Pure IP (99.7%)	25	18	2.80	18	0.79
IM (5:1)	23	15	3.19	50	0.79
IM (2:1)	22	13	3.70	50	0.80
Mineral oil	15 [53]	2	11.85	50	0.88
Paraffin wax	16 [54]	3	9.32	61	0.89
Acetone (pure)	20	21	0.47	18	0.79
White spirits (WS)	16	16	1.38	18	0.80
Ethanol (pure)	27	25	1.52		0.79
Xylene	18	2.4	0.74		0.86
IP to IM (5:1) gradient	2	3	0.39		0.00
Acetone to WS gradient	4	5	0.91		0.01
Ethanol to xylene gradient	9	22.6	0.78		0.07

IM indicates mixture of IP and mineral oil (M); IP, isopropanol (2-propanol); acetone and WS was the method substituted at Taganrog; ethanol and xylene was the method substituted at Mount Sinai Medical Center; δ, solubility parameter; standard international (SI) unit, Mega Pascal (MPa); ε, dielectric constant (δ is directly proportional to ε and to solvent strength); ν, kinematic viscosity; unit: centi-Stoke (cS) (square millimeter per second); ρ = density= gram per mL.

[a] Values determined at Taganrog for the IP and M validated protocol.

It is necessary to underline that the mixtures of mineral oil with isopropanol not only act as the clearing agent but also complete the dehydration of the tissues, allowing for the exchange of shorter dehydrating times with prolonged clearing times, ensuring a complete dehydration as well as very gentle clearing. The transition from isopropanol to the first clearing step (IM 5:1, Table 6) has a gentle gradient (δgrad = 2 MPa) contrasting with a $\delta_{grad} = 9$ MPa between pure ethanol and the first xylene in a conventional protocol. The undiluted mineral oil is used to facilitate the transition between the tissues that have been cleared with the IM mixture and the infiltrating paraffin wax. It is also important to underline that isopropanol (TWA = 400 ppm) is one quarter less toxic than xylene (CAS no. 1330-20-7; TWA = 100 ppm) and that mineral oil is not toxic at all except for the airborne mist that can be produced during its industrial use as coolant for high-speed drilling or metal carving machinery (TWA = 5 mg/m^3), something impossible during its use in tissue processing.

It seems evident that clearing tissues with an isopropanol-mineral oil alcoholic mixture followed by undiluted mineral oil is superior to using D-limonene derivates, alkanes, or even isopropanol alone, constituting the safest and cheapest

Table 7
Processing protocols with isopropanol (IP) and mineral oil mixture (IM)

Station	Reagent	Manual processing		Automated processing[a]	
		Time (h)	Temperature (°C)	Time (min)	Temperature (°C)
1	Formaldehyde	[b]	20	25[c]	35
2	Formaldehyde	[b]	20	25[c]	35
3	70% IP	1	20	15	35
4	80% IP	1	20	15	35
5	90% IP	1	20	15	35
6	95% IP	1	20	15	35
7	Pure IP	1	20	15	35
8	IM (5:1)[e]	1.5	50	90	50
9	IM (2:1)[e]	1.5	50	90	50
10	Mineral oil	16[d]	50	60	50
11	Paraffin wax	1	60	45	58
12	Paraffin wax	1	60	20	58
13	Paraffin wax	1	60	20	58
14	Paraffin wax	1	60	50	58
Total time		12 h + 16 h overnight = 28 h		8.3 h	
Clearing + infiltrating as % of the total time		23 h/28 h = 82%		6.25 h/7.5 h = 83%[f]	

[a] Vacuum, pressure, and agitation in all stations also.
[b] The tissues have been fixed in neutral buffered formalin from a few to 2 days before.
[c] The tissues should have been previously fixed.
[d] Specimens remain in mineral oil overnight (16 hours) or during the weekend (64 hours) without any adverse effects. Time permitting this step is 1.5 hours also for a total duration of 13.5 hours.
[e] The IM mixture can offset the effects of short dehydration times.
[f] Formaldehyde stations excluded.

alternative to xylene for clearing tissues without any adverse effects to personnel or tissues.

4.7. Dewaxing without xylene

Xylene is still used as the clearing agent in 59% of all US histology laboratories [14], but the histotech's exposure to it while working with tissue processors is much less than while dewaxing before staining and clearing.

Presently staining and coverslipping are automated in 79% and 62% of US histology laboratories, respectively [14], but some tasks involving exposure to xylene are still completed manually to different degrees, such as doing special stains (87%), recycling (54%), coverslipping (38%), and routine manual staining (21%) sometimes followed by coverslipping outside a fume hood (18%). Reducing the exposure of personnel to xylene should be part of a comprehensive review and not just an effort to develop substitutes for tissue processing alone.

Proper dewaxing, especially before IHC procedures, is a must and when hexane, Histoclear, Trilogy, and xylene were tested simultaneously as dewaxing agents [34], hexane proved to be the best product rendering about 28% stronger IHC signals. Although with an 8 hours TWA = 500 ppm, hexane has been described as posing some dangers to users and, with a price 1.41 times that of xylene, does not constitute a desirable alternative to it.

In trying to eliminate xylene from the dewaxing step some manufacturers have produced proprietary mixtures, such as Aqua-DePar, EZ-Dewax, and Skip Dewax, or have recommended using clearing substitutes (such as UltraClear) at 37°C for better results. Another product (Clearium) is claimed to be able to clear and cover directly from pure isopropanol after staining but, although xylene-free, contains toluene and acrylic resins.

Anecdotal postings on the HistoNet assure that placing the slides in heated standard buffer used for heat-induced epitope retrieval (HIER) with Tween dewaxes very well, and heating slides in aqueous solutions to accomplish dewaxing and HIER simultaneously is done in some IHC autostainers from major manufacturers (Dako, Leica, and Ventana). Placing sections in hot liquids is nowadays a less controversial issue than it was several years ago, especially in any histology laboratory using HIER at up to 98°C during 20 minutes or more for their IHC procedures.

If we assign to each paraffin infiltrated section an area of 1 square inch (6.45 cm^2) and an average thickness of 5 μm, with a ρ = 0.9 g/cm^3 there will be a total of 0.58 g of paraffin to be dissolved for each 200 slides requiring to change 4 L of ethanol and 2 L of xylene at a cost of almost $30 per 100 slides.

On the other hand, if the dewaxing is completed using 2 water baths with a capacity of 4.5 L each, at an average cost of 0.05 cents per milliliter of liquid dishwasher detergent, the cost of replacing 9.0 L of the 1.7% DWS per 200 slides will be just $0.04 per 100 slides, which is 750 times cheaper than using ethanol and xylene and 4 times faster.

The xylene-free dewaxing procedure with the DWS, after being validated at Taganrog Laboratory using the brand "Fairy," was tested successfully during 2 parallel corroborating experiments, one at the Histology Support Unit at the University of Glasgow, Scotland, using the brand "Persil" for routine H&E staining, and another at the Children's Hospital at Westmead, New South Wales, Australia, using the brand "Spree" for dewaxing before IHC, where 25 of 27 antibodies showed the same reaction intensity and 2 (CD10 and CD57) were only one grade weaker (2+ vs 3+) than with BondDewax used in the Bondmax autostainer.

The last task involving xylene in the histology laboratory would be cleaning (dewaxing) the retorts and conduits of tissue processors that can be done using a 2% vol/vol aqueous solution of a laboratory-strength glassware-cleaning detergent such as Alconox.

5. Conclusions

The hazardous effects of xylene on histology personnel are beyond dispute so promoting its elimination from the histology laboratory is an obligation of those in decision-making positions. This includes its elimination not only from tissue processing but also from any other activity where the

personnel are in contact with it. The discussed data show that:

1. 5 vegetable oils reviewed are totally inadequate as potential substitutes;
2. 22 D-limonene–based substitutes pose as many if not more health hazards, are less effective in their chemical roles, and have an average cost 2.19 times higher than xylene;
3. some of the 35 alkane-based substitutes are effective in tissue processing, are less toxic than those D-limonene–based, have an average cost similar to xylene (0.94) but are not very effective for section dewaxing and other staining tasks;
4. isopropanol can be used as a clearing agent alone or mixed with molten paraffin wax, representing a good alternative cost wise (0.40 that of xylene); but
5. this study demonstrates that the best clearing agents from the section quality and diagnostic value point of view that are able to substitute xylene and white spirits alike using automated [18] or manual protocols are alcoholic mixtures of isopropanol and mineral oil at increasing proportions of 5:1 to 2:1 followed by undiluted mineral oil, all at 50°C, assuring a gentle and high-quality paraffin infiltration. The compounds are totally health safe and are 0.34 to 0.43 the price of xylene. The validated processing protocols appear in Table 7.
6. Dewaxing with a 1.7% dishwasher soap solution heated at 90°C has been validated as diagnostically equivalent, 750 times cheaper, and 4 times faster than xylene dewaxing.
7. After staining, the sections are washed, drained off, and oven dried at 60°C before coverslipping without chemical dehydration or xylene clearing.

Clearing with mineral oil, dewaxing with a dishwasher soap solution, and cleaning tissue processors and accessories with a laboratory strength glassware cleaner will totally eliminate xylene from the histology laboratory. However, because less than 30% of the histologic tasks are automated [56], many histotechs are reluctant and even fearful of adopting any procedure that may be seen as capable of compromising their manual expertise, especially section cutting.

The responsibility of transforming each histology laboratory into a xylene-free and safe working environment rests on the pathologists' leadership as stewards of the histology laboratory and its personnel.

A xylene-free histology laboratory could happen, but not without a commitment to change by all concerned in histology, including pathologists.

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