



# Chapter 133

## Replacement of xylene with extra virgin coconut oil in the clearing step of the histological routine

  <https://doi.org/10.56238/devopinterscie-133>

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### ABSTRACT

Xylol is a compound used in histological processing, and in the clearing step, and is a harmful solvent for public health and the environment. The objective of this study was to present an alternative methodology to the use of xylene, in the diaphanization stage of the histological routine using extra virgin coconut oil. Fragments of the ear, cerebellum, and tongue of *Rattus norvegicus* divided into five groups were

used. The control group was cleared with xylene, a treated group I with xylene and 30% coconut oil, treated II with xylene and 50% coconut oil, treated III with xylene and 70% coconut oil and treated IV only with coconut oil. Slides from all groups and tissues were stained with Harris Hematoxylin and Eosin, cerebellum slides were also stained with Phosphotungstic Hematoxylin and Eosin, tongue slides by Gomori's Trichrome method, and ear slides by Orcein with Harris Hematoxylin. The evaluated tissues did not show differences between the groups in the process of clearing, blocking, and staining, the clearing action of the oil maintained the morphology of the tissues and did not interfere with the staining. We can conclude that coconut oil is a promising substitute for the use of xylene in the clearing step, being a safer and low-cost alternative, not compromising the morphology of the tissues and without interfering with the different stains used, in addition to minimizing the risks to public and environmental health.

**Keywords:** Animal structures, Histology, Histological techniques, *Cocos nucifera*, *Rattus norvegicus*.

### 1 INTRODUCTION

Tissue samples that must be submitted to histological or histopathological examinations, when a method with paraffin is chosen, need to undergo a sequence of procedures whose objective is to guarantee that the fragment can be studied and in the histological routine, soft tissue to become a slide and be observed under optical microscopy, goes through several stages, ranging from its collection and fixation, passing through dehydration, clearing (clarification), impregnation (embedding in paraffin), microtomy, deparaffinization, staining and mounting, these steps provide support to the tissue, in addition to enabling its visualization under the microscope. In this context, xylene is used in the steps of clearing, dewaxing and final assembly of the slides (Bancroft & Gamble, 2008; Mohammedsaleh, 2014; Patraquim, 2015; Camillo

et al., 2017; Do Nascimento et al., 2020), the protocols for these steps still depend on the biological specimen, the type of tissue, among other factors (Souza et al., 2010).

Xylol or xylene is an aromatic compound derived from petroleum that makes up the BTEX group (benzene, toluene, ethylbenzene and xylenes) of Volatile Organic Compounds (VOCs) as well as benzene, toluene and ethylbenzene. Its isomers (ortho, meta and para) are solvents, used in paints, varnishes, rubber, dyes, dyes, pharmaceutical preparations, plastics and oil industries, in the manufacture of some acids and solvents in laboratory analysis (Costa et al. ., 2007; Cetesb, 2016; Hernandez et al., 2017).

The use of protective equipment is necessary when dealing with xylene, as it can cause damage to health related to time and the concentration resulting from exposure to this compound. Irritation of the eyes, mucous membranes and skin, gastric discomfort and headaches are the most common reports of excessive solvent exposure (United States, 2007; Cetesb, 2016; Hernandez et al., 2017; Cetesb, 2021). Conama (National Council for the Environment) classifies xylene residue in Group B, that is, it poses a risk to public health and the environment (Brasil, 2005). Xylol waste must be chemically incinerated (Cetesb, 2021) or, if it comes from a histology and/or pathology laboratory, it can be reused through fractional distillation of approximately 70% (Schwarz, 2017).

There are several reports in the literature of vegetable oils as substitutes for xylol in the histological routine, Rasmussen et al. (1992) used olive oil (olive oil) in the clearing step and coconut oil in the impregnation step and did not consider them less suitable for the histological diagnosis when compared to xylene. Udonkang et al. (2014) evaluated palm oil as a clarifying agent and found small differences compared to xylene, without compromising tissue quality; Sermadi et al. (2014) compared coconut oil with xylene as a clarifying agent and did not show impairment of histological quality; Indu et al. (2014) proposed cedar oil as an effective, ecological and safe alternative to xylene as a deparaffinization agent in the histopathological laboratory; Swamy et al. (2015) successfully used carrot oil, pine oil, rose oil and olive oil (olive oil) in histological processing, and found that the four oils have a capacity to clear tissues similar to xylene, being economical oils and that do not compromise histological staining; pine oil was superior in its physical and whitening properties; Digala et al. (2017) report that the results obtained with coconut oil and peanut oil are better than xylene in the processing of tissues without health risks; Ashitha (2018) used coconut oil and palm oil in the clearing step and found that both oils, with an advantage over coconut oil, have potential as a substitute for xylene; Chandraker et al. (2018) reported difficulties replacing xylene with coconut oil, but stated that the histological quality is similar and without risk to health; Ravindran et al. (2018) evaluated palm oil and found similarity with xylene, with good histological results, being a product free of toxicity, without risks, non-flammable, biodegradable, economical, easy to handle and readily available, in addition to staining characteristics with hematoxylin staining and eosin (H/E) showed longevity without fading.

Recently, Sermadi et al. (2019) used olive oil in the clarification step, without reporting differences in the use of xylene; castor oil was proposed by Carreira et al. (2019) as a good substitute for xylol in

histological diaphanization processing, given the results obtained in their study; Abreu et al. (2019) demonstrated that in the clearing step, the 1:1 solution (xylene and coconut oil) was satisfactory and meets the specificities of the histological routine; Akpulu et al. (2021) recommended eucalyptus oil as an effective agent in the dewaxing process without compromising the staining step and Tsamiya et al. (2021) stated that olive oil, clove oil, and peanut oil can clear tissues when compared to those clarified with xylene, also considering their reduced cost, availability, beneficial effects on health and safety for the environment.

The bay coconut or simply coconut is the fruit of the coconut tree (*Cocos nucifera* L.) and is the only species of the genus, one of the most important families of the class Monocotyledoneae. It is a monocotyledonous flowering plant of the Arecaceae family. The fruit has a smooth epidermis or outer epicarp, a thick and fibrous intermediate mesocarp, and an inner endocarp with solid white albumen and liquid albumen (Cuenca et al., 2021; Lima, 2015). Coconut farming is a sector of great socioeconomic importance, as it generates a range of direct and indirect jobs, and this is due to the versatility of all components of the tree and especially the fruit. Oil is one of these coconut products extracted from solid white albumen, is the main source of lauric acid for the industry (Embrapa, 2019). Coconut oil is used in the production of foods, soaps, cosmetics and in the manufacture of biopolymers and biodiesel (Araújo et al., 2009; Carpiné, 2015; Bressan et al., 2017).

Because it is an extremely volatile compound, xylene requires people involved with its use to be aware of its risks, ways of prevention, disposal and the need for individual and collective protection equipment, and in this context, research on means of total or partial replacement of xylene become necessary. Thus, coconut oil can be a low-cost and non-toxic alternative for replacing xylene in the histological routine. Coconut oil is easily found commercially and has no relevant history of toxicity to the population. It is a product of vegetable origin, which has a wide production chain and is present in several regions of the country, used in human and animal food, and explored as an alternative to other petroleum-derived compounds, such as wax. The objective of this study was to present an alternative methodology in the histological routine, with reduced cost and biological risk for the diaphanization process, using extra virgin coconut oil.

## **2 METHODOLOGY**

This is a bibliographic and experimental study (Gil, 2017), whose object of study was an alternative to replace xylol in the histological routine. The study was carried out at the Laboratory of Histological and Embryological Techniques of the Institute of Biological Sciences of the University of Pernambuco, at Campus Santo Amaro, Recife (PE).

To carry out this work, five fragments of different tissues of *Rattus norvegicus* (cerebellum, tongue, and ear), previously fixed in 10% buffered formalin and maintained (preserved) in 70% alcohol, were used.

The experimental histological processing consisted of the steps of:

1. Dehydration: The fragments (control and treated), preserved in 70% alcohol, underwent similar protocols of dehydration in alcohol for 1 hour [Alcohol 90%, 100% (I) and 100% (II)], differentiating in the diaphanization step.

2. Diaphanization: The fragments were distributed into five groups according to an adaptation of the research carried out by Ofusori et al. (2009). The control group had the fragments subjected to routine histological treatment, using two 1-hour baths in xylene (room temperature), while the treated groups were subjected to two 1-hour baths (room temperature) using the following protocols: treatment I – 70% xylol and 30% coconut oil; treatment II – 50% xylene and 50% coconut oil; treatment III – 30% xylene and 70% coconut oil; IV treatment – 100% coconut oil.

3. Impregnation and preparation of paraffin blocks: The tissues of the groups (control and treated) underwent two baths in paraffin for 1h:00 and 30m in paraffin/beeswax at 65oC (Do Nascimento et al., 2020), in blocks were then made in liquid paraffin in the form of silicone, labeled and taken to cool.

4. Microtomy: Blocks (control and treated) were cut to 5 µm using the Leica® RM2165 micrometer. Then the slides were identified and placed to dry in an oven at 60oC for 25 minutes and stored.

5. Staining: The organs (cerebellum, tongue and ear) of the control and treated groups were stained with the Harris Hematoxylin with Eosin (H/E) technique. Ear slides were stained in Orcein and Harris Hematoxylin, tongue preparations were stained by Gomori's Trichrome method and cerebellum slides were stained by Phosphotungstic Hematoxylin and Eosin. To be stained, all slides were routinely dewaxed with xylol and submitted to the staining process (Tolosa et al., 2003).

6. Mounting: The final stage of histological processing followed the methodology proposed by Cazari et al. (2013) with modifications, which consisted of submitting the slides to an oven at 65oC for 10 minutes for drying and mounting with a coverslip in Entellan®.

The analysis and recording of the images were carried out using the Olympus® CX31 microscope and the images were obtained using a photographic camera from the same manufacturer. The organs used in this study had prior approval from the Ethics Committee for use with animals, of the University of Pernambuco / UPE (CEUA: 02/14).

### **3 RESULTS AND DISCUSSION**

The different tissues evaluated (ear, tongue and cerebellum) did not show differences, regardless of the treatment used (T-I to T-IV) in the clearing, impregnation, blocking and staining process. It has been demonstrated that associated or pure coconut oil is a good substitute for xylene in the clearing step.

Figures 1 and 2 show ear sections stained with H/E and Orcein with Harris Hematoxylin, respectively. In all slides, it is possible to identify the same histological structures, connective tissue and cartilaginous tissue. In the coloring used in Figure 2, the elastic fibers are highlighted.

In Figures 3 and 4, slices of tongue were stained with H/E and Gomori's Trichrome, respectively. It is possible to identify adipose tissue, striated muscle fibers and peripheral nerves on histological slides.

Figures 5 and 6 show sections of the cerebellum stained with H/E and Phosphotungstic Hematoxylin with Eosin, respectively. The structures shown in the cuts are the meninges, molecular layer, Purkinje cells, granular layer and white matter.

After the fixation and/or conservation process, the next stage of histological processing is dehydration, considering that the substance used before inclusion in paraffin does not homogenize with the water existing in the tissues. Ethyl alcohol is commonly used in increasing proportions up to absolute alcohol (Timm, 2005; Camillo et al., 2017; Moreti et al., 2019; Do Nascimento et al., 2020). The alternative use of coconut oil replacing xylene did not interfere with the clarification process and maintained the histological structures of the tissues. Diaphanization is the stage of histological processing where the previously dehydrated piece has its dehydrating agent replaced by a substance that has an affinity with paraffin, allowing its penetration into the tissue. In this step, tissue bleaching also occurs, which is why it is also called clarification (Nunes & Cinsa, 2016). In addition to xylol, substances routinely used in histology are toluol, benzene, cedar oil and universal solvents such as butyl acetate, methyl benzoate, and diethylene dioxide (Michalant, 1988; Tolosa et al., 2003; Ofusori et al. , 2009; De Souza Junior, 2010; Piculo et al., 2014; Nunes & Cinsa, 2016; Gartner & Hiatt, 2017), other agents have been reported as capable of replacing xylol, such as kerosene, chloroform, methyl salicylate, hydrocarbons long-chain aliphatics (Tolosa et al., 2003; Ofusori et al., 2009; Nunes; Cinsa, 2016).

A characteristic of the clearing or clarification phase in organs subjected to xylene is the translucent appearance of tissues exposed to the solvent (Tolosa et al., 2003; Timm, 2005, Nunes & Cinsa, 2016). The tongue, ear and cerebellum fragments used in our study were routinely cleared using xylol, pure coconut oil and coconut oil in association with xylol at room temperature. This methodology meant that the organs were not completely translucent. This may be related to the melting point of coconut oil, which according to Martins and Santos is around (24.4 - 25.6 °C), while that of xylene is around 13.3 oC (Cetesb, 2021).

According to Chandraker et al. (2018) fabrics processed with coconut oil showed lower translucency and lower stiffness when compared to xylol, low translucency was a characteristic of fabrics treated with coconut oil, while the stiffness of the fabrics was similar to fabrics treated with xylol. These difficulties were not reported by Sermadi et al. (2014) and Chandraker et al. (2018) who used only coconut oil, Rasmussen et al. (1992) who tested olive and coconut oil, Digala et al. (2017) who tested coconut oil and peanut oil, Swamy et al. (2015) who tested pine, carrot, rose and olive oils, Indu et al. (2014) who used cedar oil; Udonkang et al. (2014) who evaluated palm oil, Ashitha (2018) who used coconut oil and palm oil, Carreira et al. (2019) who used castor oil, Ravindran et al. (2018) who evaluated palm oil, Tsamiya et al. (2021) who tested clove, peanut, and olive oils, and Akpulu et al. (2021) who evaluated eucalyptus oil .

Chandraker et al. (2018) using coconut oil and Tsamiya et al. (2021) testing clove, peanut and olive oils, diaphanized their tissues at room temperature, unlike Rasmussen et al. (1992) and Digala et al. (2017) who, after tissue dehydration, performed clarification in olive oil at a temperature of 50oC, and coconut oil at 60oC, respectively, following with the fabric impregnation step. Pure coconut oil or combined with



xylene, in our study, did not differ from the procedure performed with xylene alone; vegetable oils can be used in routine histology and have great potential as a substitute for xylene in the clearing step (Rasmussen et al., 1992; Indu et al., 2014; Udonkang et al., 2014; Swamy et al., 2015; Digala et al., 2017; Ashitha, 2018; Chandraker et al., 2018; Ravindran et al., 2018; Carreira et al., 2019; Akpulu et al., 2021; Tsamiya et al., 2021).

Despite the little translucency of the tissues, we were able to verify that the slides obtained by processing with pure coconut oil or in association with xylene were similar to those made with xylene only, in addition to not having interfered in the next phase, the impregnation (paraffin bath in the greenhouse) and blocking (Rasmussen et al., 1992; Premalatha et al., 2013; Chandraker et al., 2018; Akpulu et al., 2021). The adoption of extra virgin coconut oil without the need for association with xylene as a clearing agent minimizes the risks of neurological, gastrointestinal, respiratory, renal, cardiovascular and dermal problems (United States, 2007; Cetesb, 2016; Cetesb, 2019) as is the case of technicians of laboratories that use this solvent, as well as the environment (Brasil, 2005).

After the clearing step, in our work, the organs were transferred to an oven at 65oC to undergo the impregnation process, the tissue is initially soaked in liquid paraffin at 60-65oC and, subsequently, included in a mold that is also filled with paraffin. Once solid, the block can be cut and the slides obtained stained (Tolosa et al., 2003; Timm, 2005; Nunes & Cinsa, 2016; Camillo et al., 2017; Tsamiya et al., 2021). Proper performance of the dehydration, clarification and impregnation phases are essential for satisfactory block preparation, facilitating microtomy (Nunes & Cinsa, 2016). In our study, the organs also underwent paraffin impregnation in a histological oven at 65oC. Regardless of this fact, we can state that xylene has good homogenization with coconut oil and paraffin, which had repercussions on the quality of the blocks, microtomy and tissue staining.

All groups tested in our study showed ease in cutting, although group IV, treated only with coconut oil, showed greater ease. This is important, as the methodology proposed in this work does not require the additional use of xylene for the success of this histological step; Rasmussen et al. (1992) state that the miscibility of coconut oil with paraffin is similar when compared to xylene and that there are no differences in the stages of histological processing; Chandraker et al. (2018) state that despite the good homogenization between coconut oil and paraffin, when compared to xylol, paraffin blocks from coconut oil present difficulties when cutting the microtome, without compromising, however, the histological result.

The different stains used in this study (Hematoxylin/Eosin, Orcein/Hematoxylin, Gomori Trichrome and Phosphotungstic Hematoxylin/Eosin) did not suffer interference due to the use of coconut oil as a clarifying agent. According to Nunez and Cinsa (2016) the staining process is a step important, and fundamental since it dyes the tissue components and consequently allows its visualization. To carry out the staining of the tissues, it is essential to remove the paraffin, which is usually preceded by xylene and alcohol baths (Tolosa et al., 2003; Timm, 2005 and Nunez & Cinsa, 2016). In our study, paraffin removal was also performed with xylol, as well as Udonkan et al. (2014) who used palm oil. For this step Rasmussen et al.

(1992) removed the paraffin on the slides, with coconut oil at 60 o C. Regardless of the methodology used, the miscibility of oils with xylene and paraffin is high and does not interfere with staining with H/E (Rasmussen et al., 1992; Premala tha et al., 2013; Sermadi et al., 2014; Swamy et al., 2015; Indu et al., 2016; Ashitha, 2018; Chandraker et al., 2018; Abreu et al., 2019; Sermadi et al. 2019; Career et al. 2019 Tsamiya et al. 2021; Akpulu et al., 2021). Regarding other histological staining techniques, Rasmussen et al. (1992) showed no differences in histochemical and immunohistochemical staining, a fact corroborated by Sermadi et al. (2014) who found that staining with PAS (periodic acid Schiff) is not compromised by the use of coconut oil or olive oil (Sermadi et al., 2019).

After the staining step, the slides were immersed in alcohol baths, growing and then placed in an oven at 65oC for 10 minutes, as proposed by Cazari et al. (2013) with adaptation, so in this stage of histological processing, xylene was not used. Regarding the assembly of histological preparations, Rasmussen et al, 1992, used Pertex® and Udonkang et al. (2014) used (DPX - Distrene Plasticiser Xylene), both are synthetic resins miscible in xylene. In the histological routine, the recently stained slide is usually subjected to alcohol and xylene baths and then a liquid resin (veniz) miscible in solvent is added to the stained tissue and a glass coverslip is placed over it (Timm, 2005; Paiva et al. al., 2006, Cazari et al., 2013), there are other examples of varnishes, among the most used are Euparal®, Entellan® and Permount® (Kraus & Arduin apud Paiva et al., 2006) and vital varnish 500® (Paiva et al., 2006).

#### **4 CONCLUSION**

The use of coconut oil is a safe, low-cost option capable of minimizing the use of xylene in the histological routine, not compromising tissue morphology or the development of different stains, in addition to reducing risks to public health and to the environment. The divergences found in our work with some studies that made use of oils, show that the replacement of xylene still requires studies, after all the technique in use with this solvent and paraffin is old, being worldwide disseminated and consolidated in histology and pathology laboratories, even so, with occasional differences between the numerous teaching and research centers. The present study used soft tissues, as well as the numerous studies consulted in the literature, which supported this work, however, we can prove that it is possible to replace xylol with coconut oil as a solvent.

#### **Suggestions for future work**

Analyze how hardened tissues, bone and tooth, behave using coconut oil, since their histological processing usually requires the use of acids to remove minerals and this acid-oil-paraffin interaction needs further investigation.

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