THE INFLUENCE OF CLARIFYING AGENTS ON RESIDUAL WATER CONTENT IN PARAFFIN EMBEDDED TISSUE SAMPLES

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The degree of histological processing of tissue samples was determined by measuring the amount of water extracted through the processing solutions and of the amount of residual water left in the final paraffinated samples. Two processing protocols employing different clarifying agents (xylene and n-butanol) and processing durations (6 hours and 11 hours) were analyzed. The amount of water in the processing solutions was determined by Karl Fisher coulometric titration. The residual water in the paraffinated samples was initially determined by desiccation and validated using the destructive method of thermogravimetric analysis.

The results of this study showed that by using n-butanol instead of xylene dehydration will continue throughout clarification. No major differences between the two protocols used can be observed, but the residual water in the tissue after infiltration with paraffin was reduced after the butanol-based protocol. The findings of this study recommend the use of butanol as a routine clarifying agent, but further studies are required.

Keywords: histology, residual water, tissue processing, dehydration of tissue samples

1. Introduction

One of the most useful methods of analyzing biological samples for identifying and confirming the diagnosis of a disease is the preparation of histological sections that can be examined under an optical microscope. Tissue

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samples harvested from the patient (biopsies, explants, aspirates, etc.) are dissected and stabilized in such a way as to preserve the biological and chemical characteristics of the organ from where it originates. For this, the tissue samples are passed through a series of solutions that prepare the material for microscopic analysis. The first stage is fixation of the tissue, a crucial stage that must be done as soon as possible because it helps preserve the cellular architecture, the chemical composition and prevents cellular autolysis. To fulfill this function, a series of conventional fixatives (formaldehyde, glutaraldehyde, ethanol, etc.) or alternatives (picric acid, chromic acid, Zenker's solution, Bouin's solution, etc.) can be used. The most common fixative is 10% formaldehyde in 50 mM phosphate buffer aqueous solution at pH 7.2-7.4: "neutral buffered formalin" (NBF). Formaldehyde reacts with amino acids and proteins, and has the highest affinity for lysine, cysteine, histidine, arginine, tyrosine, and the hydroxyl groups of serine and threonine. Formaldehyde reactions are reversible if the tissue is intensely hydrated (hydration of the tissue for 24 hours removes 50% of the reactive groups formed). If the tissue has been stored for a long time in formaldehyde, oxidation reactions occur and reversing fixation is becoming more difficult [1,2].

The next step in preparing the tissue for microscopic analysis is replacing the water present in the tissue with paraffin. Since water and paraffin are not miscible, intermediate steps and solutions are necessary: the dehydration step and the clarification step. The dehydration step is done progressively, with solutions that have the role of removing the fixing agent and free water from inside the tissue. Dehydration is preferably done slowly, with increasing concentrations, to avoid deformations of the tissue. The most common dehydrating agents used in histology are ethyl alcohol, isopropyl alcohol, methyl alcohol, acetone, *n*-butanol, isobutanol, etc. [2]. To facilitate the diffusion rate of solvents in the tissue various techniques can be used: mechanical agitation (reducing the processing time by up to 30%), heat (a temperature limited to 45 °C can increase the diffusion rate and diffusion exchange), selecting reagents with low viscosity (the lower the viscosity of the solutions, the faster they penetrate into the tissue - Table 1) or negative pressure (vacuum helps in the removal of solvents from within the tissue only if this is more volatile than the solvent with which is being replaced) [1] .

Since alcohol and paraffin are not miscible, the next stage involves replacing the dehydrating agent with a transitional solvent, which is miscible with both ethanol and paraffin. In histology these solvents are colloquially named clarifying agents (xylene, toluene, benzene, butanol, chloroform, etc.) because they render the tissue transparent. After the clarification stage the tissue samples are infiltrated with a support medium (paraffin) to allow the tissue to be sectioned and mounted on a glass slide for microscopic examination.

The best known and routinely used clearing agent in histology is xylene. Due to its structure (aromatic ring on the inside and hydrogen bonds on the outside) xylene is

miscible with both ethyl alcohol and paraffin. In histology, a mixture of isomers (*ortho*-xylene, *meta*-xylene, and *para*-xylene) is used because it displaces the alcohol faster [3, 4, 5].

Studies have shown that people working in histology laboratories exposed for prolonged periods to xylene showed generalized toxicity. Different clarifying agents have been tried as xylene replacements, such as limonene, aliphatic hydrocarbons, different mineral oils, but it was found in most cases that these substitutes are more expensive and less effective [6,7].

Butanol is a primary alcohol (C_4H_9OH) with four isomers (*n*-butanol, *sec*-butanol, isobutanol, *tert*-butanol). Butanol is gentler and slower acting than xylene, requiring a longer processing protocol, but generates significantly less tissue distortion. Butanol can also be used to complete dehydration, since it is partially miscible with water (depending on the isomer - Table 1), as well as an intermediate between ethyl alcohol and paraffin. Isobutanol has properties similar to *n*-butanol, but it is less expensive. *Tert*-butanol is largely used in plant processing. A major disadvantage of *tert*-butanol is that it solidifies at 26 °C [3,4].

Table 1

Physico-chemical properties of some solvents used in histological tissue processing [8-15]

Solvent	Isomers	Viscosity (cP) (15 -25 °C)	Solubility in water (25 °C)
Xylene	*ortho*-xylene	0.76 cP	167-221 mg/L
	meta-xylene	0.581 cP	134-206 mg/L
	para-xylene	0.603 cP	130-215 mg/L
Butanol	*n*-butanol	2.544 cP	68 g/L
	Isobutanol	4 cP	66.5-90.9 g/L
	tert-butanol	4.41 cP	1x10+6 mg/L
	sec-butanol	4.21cP	181 mg/ml
Ethyl alcohol	-	1.074 cP	1000 mg/L

The quality of the processed tissue sample is determined on the one hand by the reagents used in histological processing and on the other hand by the temperature and time of exposure. These two variables must be standardized and adapted for both the type of tissue to be analyzed and its size, to avoid obtaining incompletely processed or over-processed samples. If the tissue is not completely dehydrated and clarified, water and/or ethanol will remain in the sample, and the paraffin can no longer infiltrate, making the tissue difficult to section and resulting in processing artifacts (tissue tears and folds). Bleuel et al., 2012 showed in his studies that after processing with fresh solutions, up to 6% water remains in the

tissue samples after the dehydration step, 5% after clarification and up to 3% after paraffin infiltration [15]. Residual water in insufficiently processed paraffin blocks is incriminated as the main cause of progressive deterioration of histologically processed tissues (along with atmospheric oxygen). The most sensitive biological compounds affected by incorrect processing are the antigenic determinants (glycoproteins in particular) and messenger RNA, while nuclear DNA seems to be the least sensitive. On the other hand, biological samples collected, fixed, and processed correctly can allow accurate diagnosis even after tens, if not hundreds of years of storage [2].

Water content is a good predictor of the stability over time of paraffin-embedded tissues. The residual water from the paraffinated samples participates in hydrolytic degradation reactions, facilitating the alteration and degradation of the embedded tissues.

In our previous study, we observed that the quality of the paraffin blocks and hematoxylin-eosin slides have been influenced by the protocol used for dehydration, clarification, and infiltration of the sample. Tissue artifacts were observed in tissue samples incompletely infiltrated with paraffin, due to an insufficient processing protocol. Our experimental data suggested that the clarifying agent employed had a very important influence on the outcome of the processing protocol [16] and that a more detailed analysis is warranted.

In the present study, we investigated the effectiveness of different clarifying solvents, such as xylene and butanol, in removing traces of ethanol and water from biological samples. For this, we employed 2 different processing protocols (Table 2): one of 6 hours total duration using xylene as the clarifying solvent (Protocol 1) and another one 11 hours long, using *n*-butanol instead (Protocol 2). The amount of water extracted from the tissue by the processing solutions was quantified by Karl Fisher coulometric titration whereas the residual water in paraffinated samples was determined by desiccation and validated by thermogravimetric analysis.

2. Materials and methods

2.1 Tissue samples

For this study we used New Zealand rabbits from the "Cantacuzino National Military-Medical Institute for Research and Development. The animals were euthanized under general anesthesia with an overdose of Tanax®: a mixture of embutramide, mebenzonium iodide and tetracaine hydrochloride used for euthanasia in veterinary medicine, (T-61). The tissue samples were harvested by a veterinary surgeon and fixed immediately in 10% neutral buffered formalin. The warm ischemic time before collecting the samples was, on average, 2.5 minutes while the cold ischemic time (between harvesting the tissue sample and initiating fixation) was virtually nil. All experiments were approved by the ethics commission

Cantacuzino National Military-Medical Institute for Research and Development, Bucharest, Romania and were performed in accordance with the rules for the care and use of animals used for scientific purposes established by national and European regulations and the application of the 3R principles of replacement, reduction, and refinement. Organ harvesting took place in the "Băneasa" Animal Facility, Preclinical Testing Unit, part of the "Cantacuzino" National Military-Medical Institute for Research and Development and authorized as a user unit by the competent national authority.

Fourteen testicular biopsies (from a total of 7 rabbits) were collected with an 8 mm diameter dermatological punch (Kai Industries co., ltd., Seki City, Japan), with an average weight of 0.8 ± 0.1 grams. All samples were fixed in 10% neutral buffered formalin and divided into two groups. Half of the biopsies were subjected to Protocol 1 and the other half to Protocol 2 of processing.

2.2 Solvents

Fixation was performed in 10% neutral buffered formalin (BIO-Optica S.p.a, Milano, Italy) at room temperature. Anhydrous denatured ethyl alcohol (Tunic Prod SRL, Bucharest, Romania), xylene – mixture of *ortho*-, *meta*- and *para*- isomers (Lachner, Neratovice, Czech Republic), and *n*-butyl alcohol (Chimreactiv SRL, Bucharest, Romania) were used for tissue processing according to the protocols in Table 2. For vacuum paraffin infiltration after dehydration and clarification we used Paraffin Surgipath Paraplast (Leica Biosystems, Wetzlar, Germany) which is a blend of highly purified paraffins containing plastic polymers and with regulated molecular weights (melting point 56-58 °C).

2.3 Tissue processing

Table 2 shows the two processing protocols evaluated in this study. Protocol 1 employed xylene as the clarifying agent and was 6-hour long, while Protocol 2 lasted for 11hours and used butanol instead.

Dehydration in both protocols was done with increasing concentrations of ethanol (70%, 80%, 95%, 100%). For the last two dehydration steps, two changes of 95% ethanol and two changes of 100% ethanol were performed for Protocol 1. For Protocol 2, the second change of absolute ethyl alcohol was replaced by butanol and more time was allotted for clarification and infiltration since butanol is a slower solvent, unlike in the xylene protocol where the total clarification time was 1h and the total infiltration time was only 1h30min long. Processing was done manually, under agitation; dehydration and clarification took place at a constant temperature of 37 °C, and the paraffin infiltration was performed under vacuum, at a constant temperature of 60 °C.

Table 2

Tissue processing protocols

Reagent	Protocol 1			Protocol 2		
	Time (minutes)	Vacuum	Temperature (°C)	Time (minutes)	Vacuum	Temperature (°C)
70% Ethyl alcohol	30	NO	37	30	NO	37
80% Ethyl alcohol	30	NO	37	30	NO	37
95% Ethyl alcohol (1)	30	NO	37	30	NO	37
95% Ethyl alcohol (2)	30	NO	37	30	NO	37
100% Ethyl alcohol (1)	30	NO	37	30	NO	37
100% Ethyl alcohol (2)	30	NO	37	-	-	-
Xylene (1)	30	NO	37	-	-	-
Xylene (2)	30	NO	37	-	-	-
n-butyl Alcohol (1)	-	-	-	30	NO	37
n-butyl Alcohol (2)	-	-	-	30	NO	37
n-butyl Alcohol (3)	-	-	-	60	NO	37
Paraffin (1)	30	YES	60	60	YES	60
Paraffin (2)	30	YES	60	60	YES	60
Paraffin (3)	30	YES	60	120	YES	60
Paraffin (4)	-	-	-	120	YES	60
TOTAL TIME	**6 hours**			**11 hours**		

2.4 Determination of water content in the processing solutions

The determination of water content in the processing solutions was performed at the Research and Development Institute for Chemistry and Petrochemistry – ICECHIM, Bucharest, using an 831KF Coulometer (METROHM, Herisau, Switzerland) for Karl Fisher coulometric titration according to SR EN ISO 12937: 2001 (Petroleum products. Determination of water. Karl Fischer coulometric titration method) and SR ISO 760:1994 (Determination of water content. Karl Fischer method Fischer - General method) [17, 18].

The method consists of coulometric titration. In this technique the following two reactions take place:

In the first reaction, an alcohol (usually methanol), sulfur dioxide (SO_2) and a base (RN - which is usually pyridine or imidazole) react to form alkysulfite salt:

$$CH_3OH + SO_2 + RN \leftrightarrow [RNH]SO_3CH_3$$

In the second reaction, the alkylsulfite reacts with iodine (I_2) and water from the sample and form an alkylsulphate salt:

$$H_2O + I_2 + [RNH]SO_3CH_3 + 2RN \leftrightarrow [RNH]SO_4CH_3 + 2[RNH]I$$

The necessary iodine is generated directly from the electrolyte by electrochemical means. The quantitative relationship between the electric charge and the amount of iodine generated is used for a high precision iodine distribution. One mole of I_2 consumed corresponds to one mole of H_2O. In other words, for every mole of water, two moles of electrons are consumed.

2.5 Determination of water content in tissue samples by desiccation

Desiccation is a simple and effective method and provides a direct estimate of the amount of water present in the tissue sample. In this study, 14 paraffin-embedded tissue samples were subjected to drying and weighing to obtain estimated values of the amount of residual water after the two processing protocols. The 14 samples were weighed initially (after paraffin infiltration) and after 4 days of drying. The amount of water was calculated by subtracting the weight of the sample after drying from the initial weight (prior to drying), assuming a density of 1g/ml. The tissue samples were dried under vacuum in a desiccator filled with activated Zeolite 3A (Grace GmbH & Co., Worms, Germany), at a temperature of 25 °C. Tissue weight was assessed with a Kern ADJ 200-4 (Kern & Sohn, Germany) analytical balance having a weighing accuracy of 0.0001 g. A one-way ANOVA test was used to compare the amount of water in the tissue from the two dehydration protocols. Differences were considered significant if $p<0.05$.

2.6 Determination of water content in tissue samples by thermogravimetry

After the tissue samples were dehydrated with increasing concentrations of ethanol, clarified with xylene, respectively butanol, and infiltrated with paraffin, they were grinded with a mortar and pestle and subjected to thermogravimetric analysis at the Research and Development Institute for Chemistry and Petrochemistry - ICECHIM in Bucharest, to determine the residual water in the paraffinated samples. The method involves changing the mass of the sample (loss or increase in weight) depending on the temperature at which the sample is heated. The following conditions for performing thermogravimetric analysis were used: heating from 25 °C to 700 °C; heating speed: 20 °C/min; determination atmosphere (purge gas): air. Quantitative analysis is resolved from the thermal curve by measuring the mass loss.

3. Results and discussions

3.1 Determination of water content in the processing solutions

Fig. 1 shows the water concentrations in the solutions used in the two processing protocols (as percentages of water extracted from the tissue). For coulometric analysis we analyzed 100% ethanol (1 and 2) and xylene (1 and 2) for Protocol 1 and 100% ethanol solution (1), and butanol (1, 2 and 3) for Protocol 2, respectively.

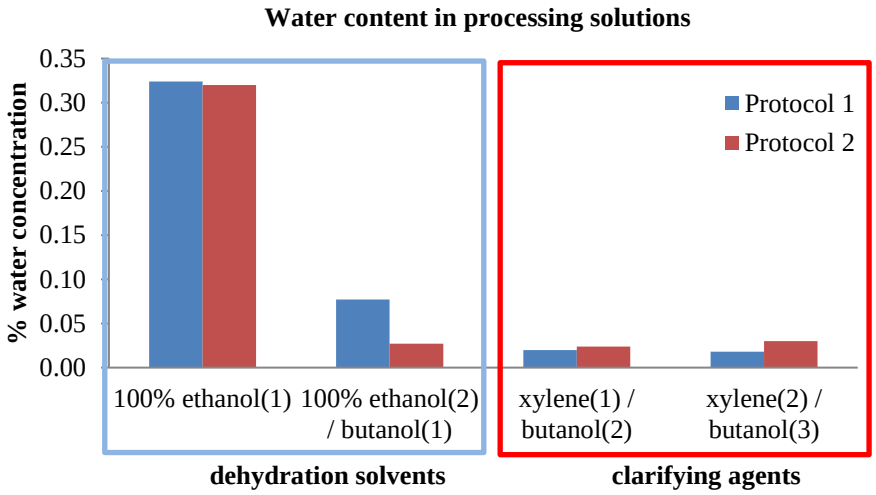

Fig. 1. Determination of water content in the processing solutions by Karl Fisher coulometric titration

In the dehydration stage, absolute ethyl alcohol has the role of extracting residual water from the tissue. It can be observed that in the first change of 100% ethanol the same amount of water was extracted during both protocols. For the last stage of dehydration, two different solutions were used for the two protocols: in Protocol 1, the dehydration continued with 100% ethyl alcohol, and for Protocol 2, the dehydration was completed during the first change of butanol, since this hydrocarbon is miscible with water. At the clarification stage, no notable differences were observed between the two protocols, except for the presence of a minute amount of water in the clarifying agent for Protocol 2.

The total amount of water extracted from the tissue during the dehydration stage (prior to clarification) by the two solutions is different, the use of absolute ethyl alcohol making it possible to dehydrate the sample much faster than butanol. On the other hand, after the last change of absolute ethyl alcohol in Protocol 1, the little amount of water remaining in the tissue will no longer be extracted by the clarifying agent due to the extremely low miscibility of water with xylene. In Protocol 2, which uses butanol as a clarifying agent, dehydration will persist

throughout clarification, because *n*-butanol, being a universal solvent, will continue to slowly extract even the last traces of free water from within the tissue. It has been well known for over 150 years that, especially when it comes to fragile tissues (mammalian brain, cephalopods, plants, etc.), universal solvents allow the best histological preparations, particularly because the dehydration is slower, delicate, but at the same time complete. Although ethyl alcohol or acetone, for example, allow much faster dehydration, their osmotic aggressiveness can lead to severe artifacts, sometimes incompatible with precision histopathology. It is interesting to mention that while ethyl alcohol (or any other aggressive dehydrating agent) cannot be used for the long-term preservation of animal tissues, in the case of butanol (or other universal solvents: dioxane, tetrahydrofuran, etc.), the long-term preservation (decades) of pre-fixed tissues generates virtually no artifacts and is also compatible with high-precision molecular assays [3, 19].

3.2 Determination of water content in the tissue samples by desiccation

Table 3

Water content of tissue samples (rabbit testes) determined by desiccation

Sample	Water content (ml/g)	
	Protocol 1	**Protocol 2**
1	0.022	0.016
2	0.010	0.017
3	0.017	0.009
4	0.012	0.012
5	0.012	0.025
6	0.010	0.012
7	0.016	0.012
Average	**0.014**	**0.015**
SEM	**0.002**	**0.002**

The amount of residual water in the tissue samples processed with the two protocols was initially determined by desiccation (Table 3).

To reach a constant weight, the samples were left in the desiccator for 4 days. The results showed that the statistical differences between the two categories of values were not significant ($p > 0.05$). On average, the amount of water in the tissue after processing the samples with Protocol 1 is 0.014 ± 0.002 ml/g, and for Protocol 2 the average amount of water is 0.015 ± 0.002 ml/g.

3.3 Determination of water content in the tissue samples by thermogravimetry

After determining the amount of water in the tissue samples by drying and weighing, a second determination was made by thermogravimetric analysis. As a result of this analysis, a one-step thermogram showing decomposition was obtained for each sample. In the temperature range 100 - 120°C, free water and any volatile compounds are eliminated from the tissue sample. The mass losses recorded in the range 100-120 °C following the thermogravimetric analysis are centralized in Table 4.

Table 4

Mass losses in the range 100-120°C

Sample	% mass loss		% inorganic residue	
	Protocol 1	**Protocol 2**	**Protocol 1**	**Protocol 2**
1	0.02	0.02	5.5	3.4
2	0.05	0.01	4.7	2.9
3	0.03	0.01	7.5	3.2
4	0.05	0.01	1.7	5.6
5	0.05	0.01	1.3	4.8
6	0.04	0.01	2.6	1.2
7	0.04	0.01	4.5	4.4
Average	**0.040**	**0.011**	**3.971**	**3.643**
SEM	**0.004**	**0.001**	**0.842**	**0.546**

At higher temperatures, a decrease in mass is recorded, due to the burning of the sample. In scientific literature, thermogravimetric analysis for different paraffins has demonstrated that the weight starts to decrease at 150°C and continues until it evaporates completely at 238 °C – 320 °C, depending on the type of paraffin used. At the same time, the addition of additives can increase this range of temperatures [20, 21, 22]. Above 700 °C, only inorganic substances remain present in the samples. Fig. 2 shows the thermogram established for the samples subjected to Protocol 1 and Fig. 3 shows the thermogram for the sample processed with Protocol 2. No major differences between the two protocols used can be observed with regards to thermogravimetry.

As in the case of desiccation, the thermogravimetric results revealed that the tissue samples from Protocol 2 contain smaller amounts of residual water: 0.011±0.001 % vs. 0.04±0.004 from Protocol 1 ($p < 0.05$, values are significant).

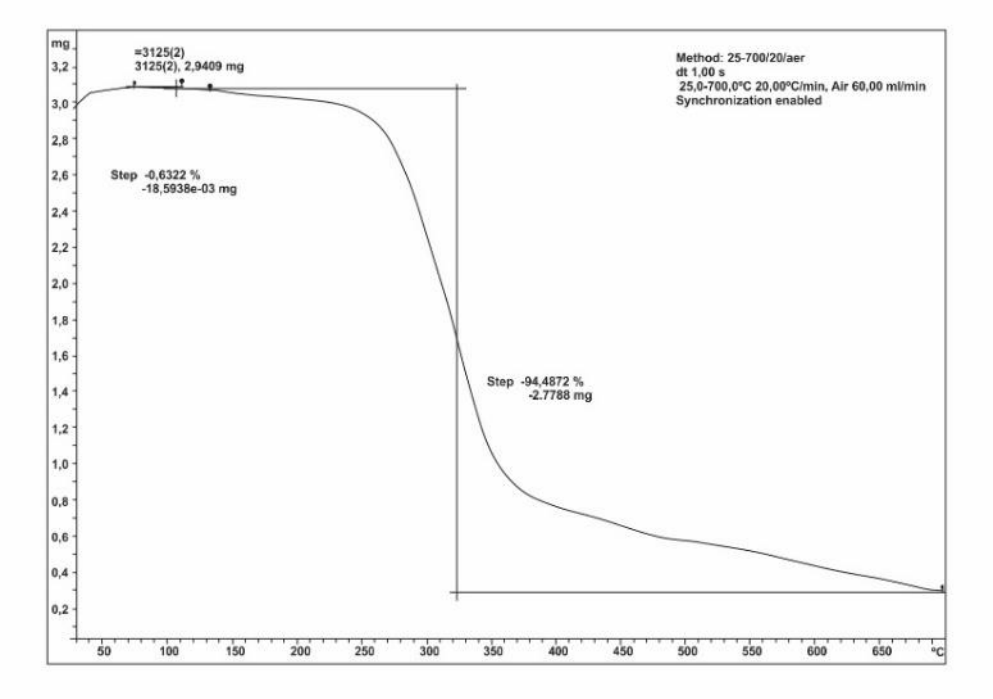

Fig. 2. One-step thermal decomposition of tissue samples from Protocol 1; thermogravimetric analysis; method: 25-700/20/air

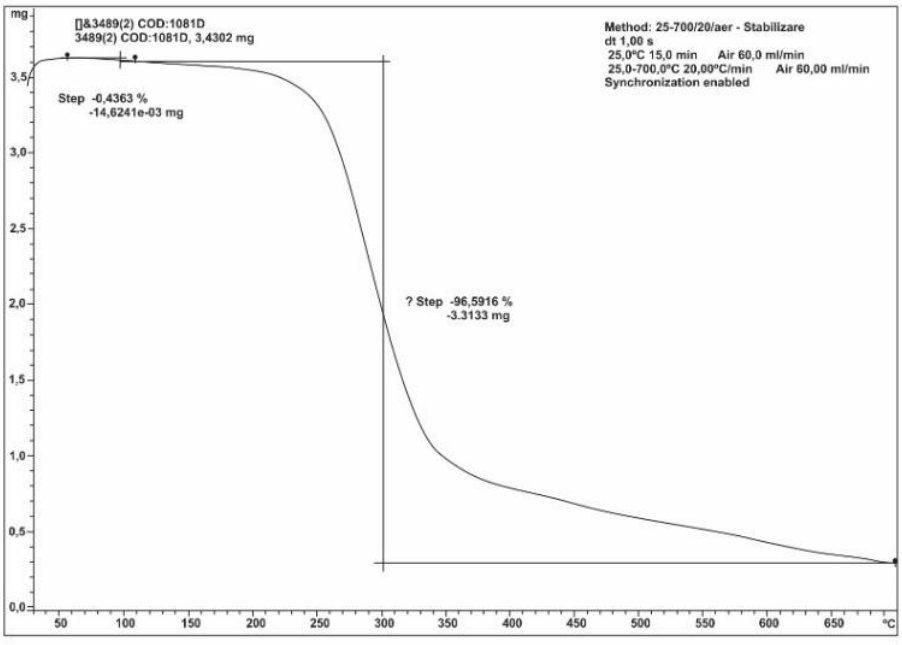

Fig. 3. One-step thermal decomposition of tissue samples from Protocol 2; thermogravimetric analysis; method: 25-700/20/air.

The thermogravimetric results support the premise that the water was displaced in the dehydration step by the graded ethanols, that butanol contributed to the further elimination of water during clarification and at the same time to the infiltration of the paraffin in the sample.

Testing performed on rabbit tissue (testes) proved that in paraffin blocks generated by regular tissue protocols (employing xylene as a transitional/clarifying agent), a small quantity of water remains within the tissue. This can result in an inadequate infiltration with clarifying solvents and/or paraffin. The quantity of residual water can be further decreased by using, as transitional/clarifying agent, a solvent that is miscible with both paraffin and water. By virtue of this dehydrating quality, universal solvents such as dioxan, tetrahydrofuran and butanol have been historically used in histology for performing both as dehydrants and transitional solvents prior to paraffin infiltration. However, while the first two solvents (dioxan and tetrahydrofuran) were abandoned due to their toxicity, butanol felt in disfavor because it is a slow acting dehydrant [3]. By using butanol only to complete the initial dehydration while concomitantly removing the last traces of ethyl alcohol in preparation for paraffin infiltration we achieved both a better-quality paraffin block using only a slightly longer protocol. The protocols evaluated in this study were performed on testes, a tissue not only with unusually high-water content, but also with a long history of faulty histological processing (due to its very high fat content). Water left in the processed tissue sample signifies “under processing” and faulty infiltration with paraffin. Even if we are talking about a relatively small amount of residual water, this can affect long-term storage of the resulting paraffin blocks. Environmental conditions during storage can further influence tissue quality (particularly high humidity can result in accelerated paraffin block degradation). Residual water in processed tissue samples, up to 3% after paraffin infiltration [15] can lead to diagnostic errors and for this reason it is very important to choose the right protocol. This fact was also demonstrated by Lerch et. al. in 2019, when they were able to monitor all the phases of tissue processing cycle by ultrasound time-of-flight (TOF) and they correlate the poor histomorphology of tissue samples with incomplete dehydration [23].

4. Conclusions

The results of this preliminary study provide some novel information on the impact of tissue processing protocols on the dynamic of water extraction from biological samples. The quantity of residual water can be decreased by using, as transitional/clarifying agent, a solvent that is miscible with both paraffin and water. The present study concludes that n-butanol can be used as a better clearing agent instead of xylene because will continue to extract the last traces of water from the tissue throughout clarification. Despite the constant pressure for faster turnaround

time, a proper processing protocol should never be overlooked because the unsatisfactory diffusion of solvents into the tissue can affect the storage of the tissue paraffin blocks. Among the two different protocols tested, Protocol 2 (11-hour long protocol, using butanol for clarification) is recommended. It is obvious that this preliminary analytical study of histological processing must be followed by a more thorough analysis using more specific and sensitive techniques, while a similar strategy should be employed for a larger variety of processing protocols and types of tissue.

R E F E R E N C E S

[1]. *S.K. Suvarna, C. Layton*, J.D. Bancroft, Bancroft's Theory and Practice of Histological Techniques (Eighth Edition), Elsevier, 2019, pp.73-83.

[2]. *D. Gologan, A.E. Ștefan, R. Stan, M. Militaru, S. Mușat,* Factors influencing the quality of formaldehyde fixed paraffin embedded tissue samples–review. Rev Rom Med Vet, **vol. 31**, no. 4, 2021, pp. 87-92.

[3]. *J.A. Kiernan, Histological* and Histochemical Methods. Theory and Practice, 5th Edition, Scion Publishing Ltd, UK, 2015, pp. 52-71.

[4]. *L. Winsor,* Laboratory histopathology, a complete reference. (Ed.) Woods A.E., Ellis R.C., Churchill Livingstone, New York, 1994, pp. 4.2-7/4.2-19.

[5]. *C.M. Chapman*, (2019). Troubleshooting in the histology laboratory. Journal of histotechnology, **vol. 42**, no. 3, Aug. 2019, pp. 137–149.

[6]. *C.L. Bush, G.E. Nelson*, Xylene—Dangers of its use in the Histology and Cytology Laboratory. Laboratory Medicine, **vol. 8**, no. 4, April 1977, pp. 16.

[7]. *R.J. Buesa, M.V. Peshkov*, Histology without xylene. Ann Diagn Pathol., **vol. 13**, no. 4, August 2009, pp. 246-256.

[8]. National Center for Biotechnology Information. PubChem Compound Summary for CID 263, *1*-Butanol. https://pubchem.ncbi.nlm.nih.gov/compound/1-Butanol Accessed Dec. 6, 2022.

[9]. National Center for Biotechnology Information. PubChem Compound Summary for CID 6386, *tert*-Butanol. https://pubchem.ncbi.nlm.nih.gov/compound/*tert*-Butanol. Accessed Dec. 6, 2022.

[10]. National Center for Biotechnology Information. PubChem Compound Summary for CID 6568, *2*-Butanol. https://pubchem.ncbi.nlm.nih.gov/compound/*2*-Butanol. Accessed Dec. 6, 2022.

[11]. National Center for Biotechnology Information. PubChem Compound Summary for CID 6560, Isobutanol. https://pubchem.ncbi.nlm.nih.gov/compound/Isobutanol. Accessed Dec. 6, 2022.

[12]. National Center for Biotechnology Information. PubChem Compound Summary for CID 7809, p-Xylene. https://pubchem.ncbi.nlm.nih.gov/compound/*p*-Xylene. Accessed Dec. 6, 2022.

[13]. National Center for Biotechnology Information. PubChem Compound Summary for CID 7237, o-Xylene. https://pubchem.ncbi.nlm.nih.gov/compound/*o*-Xylene. Accessed Dec. 6, 2022.

[14]. National Center for Biotechnology Information. PubChem Compound Summary for CID 7929, m-Xylene. https://pubchem.ncbi.nlm.nih.gov/compound/*m*-Xylene. Accessed Dec. 6, 2022.

[15]. *E.P. Bleuel, T.P.C. Roebers, E. Schulting, W.F.A. den Dunnen,* Solvent-free tissue processing using supercritical carbon dioxide. Histopathology, **vol. 61**, no. 6, June 2012, pp. 1198-1208.

[16]. *D. Gologan, A.E. Ștefan, M. Militaru, A.C. Sanda, S. Arjan, S. Mușat, M.O. Leavitt, R. Stan*, Quality Assurance and Cost Reduction in Histopathology Laboratories Using Tissue Microarrays. *Vet. Sci.* **vol. 10**, no. 4, April 2023, pp. 280.

[17]. SR EN ISO 12937: 2001 (Petroleum products. Determination of water. Karl Fischer coulometric titration method).

[18]. SR ISO 760:1994 (Determination of water content. Karl Fischer method Fischer - General method).
[19]. *M. Gabe*, Histological techniques, (Ed.) Springer-Verlag, New York, USA, 1976.
[20]. *W. Su, Y. Li, T. Zhou, J. Darkwa, G. Kokogiannakis, Z. Li*, Microencapsulation of Paraffin with Poly (Urea Methacrylate) Shell for Solar Water Heater. Energies, **vol. 12**, September 2019.
[21]. TA Instruments, Thermal Analysis of Phase Change Materials - Three Organic Waxes using TGA, https://www.tainstruments.com/pdf/literature/TA405.pdf.
[22]. *R.G. Craig, J.M. Powers, F.A. Peyton*, Thermogravimetric analysis of waxes. Journal of dental research., **vol. 50**, March 1971, pp. 450-4.
[23]. *M.L. Lerch, D.R. Bauer, A. Theiss, D. Chafin, M. Otter, G.S. Baird G*, Monitoring Dehydration and Clearing in Tissue Processing for High-Quality Clinical Pathology. Biopreserv Biobank, **vol. 17**, no. 4, August 2019, pp. 303-311.