NATURAL HISTORY WET COLLECTIONS: OBSERVATIONS ON PH READINGS FROM THE USE OF DIFFERENT ETHANOL AND LABEL TYPES

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Abstract.—We examined the effects of different types of specimen labels and tags on pH of different concentrations of ethanol typically used for fluid preservation in natural history collections. Labels were immersed in three different concentrations of ethanol, 96% pure undenatured ethanol (EtOH), 96% EtOH denatured with methyl-ethyl ketone (MEK), and 99.8% pure undenatured EtOH, with or without the presence of insect specimens, and the solutions were evaluated after 26 months for changes over time in pH reading. In general, pH readings of all label trials with 96% and 99.8% ethanol increased over time, except for trials of denatured alcohol, which demonstrated lower pH readings in almost all treatments, regardless of label type. Samples that contained labels with ordinary, nonstandardized, not explicitly acid-free printing paper had higher pH readings compared after the trial. Our observations are a good starting point for further experiments to answer research questions related to chemical interactions with labels in ethanol-preserved specimens, including tissue samples for molecular analyses, which can guide collection staff in their daily work.

Key words.—ethanol, denaturant, fluid preservation, insect, label, MEK, natural history collection, pH.

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Introduction

Which sort of labels or tags should be used in wet collections? This surely is one of the key questions for staff managing natural history collections (NHCs) aiming to maintain a stable chemical environment inside specimen containers. Wet collections of natural history museums comprise fluid-preserved specimens (either fixed or not) stored inside containers with ethanol or isopropyl alcohol as the most commonly used fluid preservatives. Ideally, preservation fluids and/or chemicals should not interact with or alter the condition of the preserved specimen over time. Specimen labels included with specimens serve a key function in keeping relevant specimen metadata (e.g., collection date, locality, collector, tissue ID, curation information, voucher ID) linked with collection objects (Bentley 2004; Zala et al. 2005; Moore 2008; Range et al. 2017). Traditionally, either external or internal labels have been used widely, and both have advantages and disadvantages. In this study, we focus on the interaction between internal labels, preserving fluids, and denaturants such as butan-2-one or methyl-ethyl ketone (MEK) on the chemical equilibrium inside storage containers, with or without the presence of insect specimens. In principle, placing internal labels into specimen containers with preserving fluids has two potential consequences. First, the preserving fluid may reduce the usefulness of labels, for example, by causing fading (Rose 1991; Simmons 1991; Duckworth et al. 1993), decreasing legibility, or affecting structural stability of the label, which risks data loss and threatens the scientific value of the specimen (Zala et al. 2005). Second, addition of a label may introduce or trigger secondary chemical reactions between the label and the preserving fluid, altering chemical equilibria such as the fluid pH. Tags and labels can also chemically interact with fixatives such as formalin used to stop specimen deterioration and decay. Range et al. (2017) offer a comprehensive overview on a variety of media and methods for label printing and printing technologies commonly

used in NHCs, and Bentley (2004) was the first to test the suitability of thermal-transfer labels for wet collections.

The molar concentration of hydrogen ions in an aqueous solution, referred to as the pH value of a solution or simply pH, and the resulting acidic or alkaline equilibrium in a fixative or preservation fluid is one of the most important factors to consider for storage of specimens in fluids. Even though a few studies have investigated the detrimental effects of low or elevated concentrations of hydrogen ions (as high or low pH, respectively) on fluid-preserved specimens (Hargrave et al. 2005; Carter 2009), our knowledge of potentially damaging effects of shifts in pH is still fragmentary (Waller and Simmons 2003; Simmons 2014; Kotrba and Schilling 2017). Moreover, as the pH of a solution is defined as the decimal logarithm of the reciprocal of the hydrogen ion activity H^+ in an aqueous solution, the problems of measuring pH values in alcohol mixtures with low concentrations of water are obvious (Simmons 2014; Kotrba and Schilling 2017). Several internal and external factors can induce pH shifts in a solution: lipids and/or acids that specimens release into the media, chemicals used during fixation or fixative residues released from specimens in storage, containers and closures (e.g., alkaline ions diffusing from glass containers into preservation fluids or gases such as oxygen or carbon dioxide conveyed into the storage fluid by poorly sealed lids), chemicals that may be released from tags and labels, additives such as denaturing agents in the preservation fluid or water used to dilute the ethanol, and last but not least shifts of the physical parameters in the storage environment (e.g., light, temperature, and humidity) (Stoddard 1989; Hargrave et al. 2005; Kotrba and Golbig 2009; Simmons 2014, 2015; Kotrba and Schilling 2017).

Multiple materials have been used historically for specimen tags and labels, e.g., paper, parchment, wood, metal, plastic, and textiles (Hawks and Williams 1986; Simmons 2014, 2015). Moreover, a wide variety of different inks must be anticipated in collections with complex history. Some materials have turned out to be inappropriate as specimen labels because they deteriorate over time. Labels, in general, can interact with preservation fluids and specimens by releasing potentially damaging chemicals (Hawks and Williams 1986) or by interfering with the condition of the preservative. This may include effects on pH. For example, labels made from cellulose typically contain acid-producing compounds such as lignin, residual bleach, dyes, and traces of metals that have been used as catalysts during paper production (Ritzenthaler 1983; Hawks and Williams 1986). Moreover, "acid-free" paper usually contains an alkaline reserve, such as calcium carbonate, to neutralize acidic compounds. It is worth noting that there is no agreed ISO standard production and composition of paper. The same applies for inks used in printers. Desktop inkjet or laser printers are widely used in NHCs. A commonly observed phenomenon is the detaching of the imprint from the label substrate when ink or toner used for respective labels is submerged into preservation fluids (Simmons 2014). Use of a thermal printer on spun-bonded polyethylene stock may be an acceptable alternative for tags and labels for fluid collections (Bentley 2004) as these seem to be resistant to damage in preserving fluids, but labels with a rigid synthetic thread can cause more damage to specimens than cellulose thread (Waller and Simmons 2003). Furthermore, as a result of aging of plastics, the polyester substrate of these labels eventually breaks down (the exact lifespan, still unknown, may exceed 20 years) or may be affected by chemical compounds including denaturants such as MEK (Range et al. 2017).

Specimens in fluid collections are very typically stored in 70–80% ethyl alcohol (EtOH) in order to preserve their structures while keeping them flexible for morphological work (e.g., Martin 1978; Nagy 2010; Doorenweerd and Beentjes 2012), even though the final

concentration inside jars after initial filling may drop to 60–75% EtOH (Simmons 2015). Pure, undenatured EtOH above 96% is preferred for the storage of tissues for molecular studies (King and Porter 2004; Nagy 2010; Moreau et al. 2013; Bressan et al. 2014; Short et al. 2018) to prevent hydrolysis of the DNA molecule (Dingerkus 1982; Simmons 1995, 2014; Hargrave et al. 2005). Highly concentrated ethanol, however, dehydrates and hardens soft tissues, which affects morphological investigations, especially of invertebrates (Häussermann 2009).

Further physical and chemical processes such as evaporation, oxidation, or unwanted secondary reactions may also influence the integrity of the preservation fluid and the chemical equilibrium inside storage containers. This may affect the quality of specimens, making them useless for morphological or molecular studies or both. Previously, researchers have studied the effects of these changes on specific biomolecules such as DNA (Post et al. 1993; Vink et al. 2005; Zimmermann et al. 2008; among others), which is of vital importance in the genomic era (e.g., Delsuc et al. 2005), and for the Barcode of Life initiative (Schindel and Miller 2005). Besides potential hydrolysis, the pH is of vital importance for genomic material because shifts in the pH to acidic or alkaline conditions can destabilize the DNA helix. Moreover, pH shifts in fixatives and/or preservation fluids can cause decalcification of specimens, breakdown of proteins, and specimen damage or may promote unwanted secondary chemical reactions in the holding fluid (Simmons 2014, 2015). Therefore, key factors influencing fixation and preservation of specimens and long-term storage have been intensively studied (e.g., Steedman 1976; Dingerkus 1982; Stoddard 1989; Carter 2009; Simmons 2015). However, we know much less about the long-term stability of chemical equilibria inside storage containers, about potential pH shifts induced by chemicals leaching out of specimens or internal labels, and about how the interaction of these processes may affect the integrity of specimens and their suitability for morphological and molecular research.

Following Simmons (2014), preservative solutions should be slightly acidic to provide protection against bacteria and mold. However, acidic conditions below a pH of 6.4 decalcify bony structures and otoliths, harden tissues of specimens, and lead to embrittlement and dissociation of proteins (see Kotrba and Golbig 2009; Kotrba and Schilling 2017 and references therein). Alkaline conditions can clear soft tissues, as proteins and lipids are leached out of specimens (Dingerkus 1982; Hargrave et al. 2005). Even though the acidity/alkalinity of the preservation fluid affects the integrity of the specimen, pH measurement is not directly interpretable in alcohol solutions for several reasons (Frant 1995; Waller et al. 2003; Simmons 2014; Kotrba and Schilling 2017). The pH value is a measure for proton activity in water-based solutions and indicates the molar concentration of hydrogen ions. Therefore, the pH electrodes are designed for measurements in aqueous solutions, but not for hydroalcoholic mixtures or ethanol above 99.8%, which basically contain no free water molecules.

This study was carried out with the aim of investigating the influence of different label materials on various ethanol solutions frequently used as preservation fluids in tissue collections in NHCs or biobanks (biorepositories that store biological samples for molecular analysis). The pH measurements were used as proxies to indicate shifts in the chemical equilibrium of the storage fluid. Previous studies investigated the viability of DNA in wet arthropod collections (Dawson et al. 1998; King and Porter 2004; Vink et al. 2005; Frampton et al. 2008; Schiller et al. 2014). The present work is to our knowledge the first study to measure the variation of pH in fluid-preserved insect samples, previous work having focused on vertebrates (e.g., Hargrave et al. 2005).

Table 1. Different types of labels used in each experimental treatment.

Description	Abbreviation	Characteristics
Unrated, standard printing paper written with carbon-pigmented pen	PP	Paper: HP® color laser paper, 200 g/m^2, not explicitly acid-free Pen: Staedtler® pigment liner 0.1 mm
Acid-free paper written with carbon-pigmented pen	AF	Paper: Pioneer® CIE 171, 110 g/m^2 Pen: Staedtler® pigment liner 0.1 mm
Laser-printed paper	LP	Paper: HP® color laser paper, 200 g/m^2, not explicitly acid-free Printer: Kyocera® TASKalfa 3051ci
Thermal transfer printed tag	TT	Tag: Datamax nonadhesive polyester label Ink/ribbon: Datamax-O'Neil® SDR-A black resin ribbon Printer: Datamax® M4206 Mark II DT/TT, 203 dpi
Vulcanized fiber tag	TG	Provided by Kay Toledo Tag, 6050 Benore Rd., Toledo, OH 43612, USA

Material and Methods

For our study, we used five different types of labels, three different solutions of ethanol, and two different treatments for each: presence or absence of insect tissue. We tested five different labels that are commonly used in our fluid collections at the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK, Bonn, Germany). All labels were trimmed to a similar size of about 15 × 8 mm; details on label properties and composition are summarized in Table 1.

Each label type was placed in externally threaded polypropylene 8 ml tubes (Sarstedt®, Nuembrecht, Germany; art. no. 60.542.007) containing 7 ml of either 96% vol ethanol, 99.8% vol ethanol, or 96% vol ethanol with 1% methyl ethyl ketone (MEK) or butanone used as a denaturant. Each of these ethanol solutions is commonly used in tissue collections.

In order to reflect typical tissue collection storage situations, whole crickets (*Acheta domesticus* [Linnaeus, 1758]) of roughly identical size sourced from pet food retailers were added to the test tubes. Cricket bodies were submerged in the respective preservation fluid after being euthanized by snap-freezing. All preserved cricket samples were initially cooled at +4°C for 3 days, then the initial ethanol was replaced and the labels were added. Samples were stored at a constant temperature of +31°C in an incubator (INCU-Line, VWR, Radnor, PA) for 2 years and 2 months (from April 2016 until June 2018) to simulate accelerated aging and to accelerate potential pH shift.

All five label types were stored in the three different ethanol solutions with two replicates each; controls of the three ethanol solutions without any label were also created and stored as duplicates. In total, three controls and 15 different combinations of label and ethanol solution were generated with two replicates each. This sample set was created with and without insect tissue, totaling $(3+15) \times 2 \times 2 = 72$ samples with 30 different preservation treatments and six controls, all with replicates. All tubes were additionally sealed with parafilm on the outside to minimize evaporation and to prevent concentration shift during the experiment.

The pH readings of respective storage solutions for all trials were obtained using a Mettler Toledo FiveEasy pH device, equipped with an InLab Ultra Micro-ISM electrode and the electrolyte Friscolyt B (Mettler Toledo, Columbus, OH). The pH of all replicates was measured prior to addition of labels and tissues and 26 months later at the end of the experiment. After each individual measurement, the pH electrode was rinsed with distilled

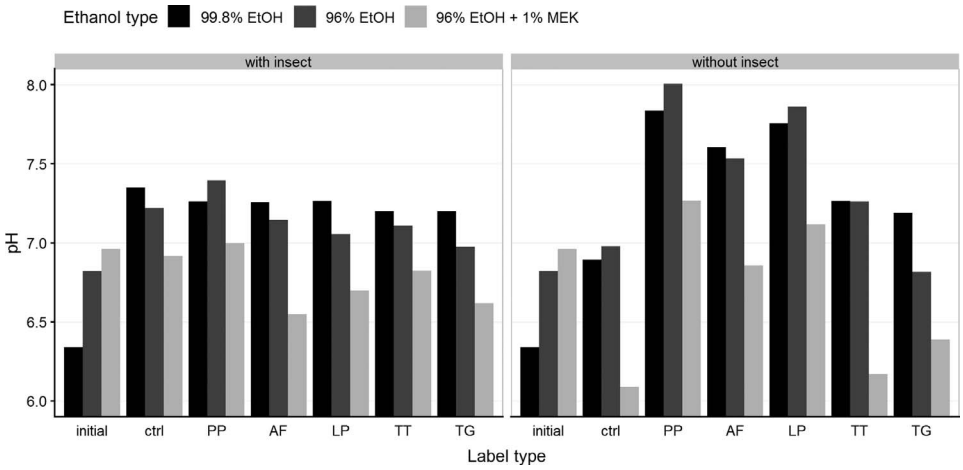

Figure 1. Initial pH readings and pH readings after 26 months (trials = ctrl, PP, AF, LP, TT, TG) at +31°C in samples with and without insect tissue, grouped by label type.

water and reconditioned in KCl 3 mol/L for 2 minutes. Standard buffer solutions (VWR; pH 4 and pH 7) were used for recalibration. For each trial, the pH readings for the two trial replicates were averaged to determine the deviation between initial and posttrial pH values.

Results

The pH readings of the two replicates for each trial were similar after 26 months (see Supplemental Material Table S1), but we admit the low number of replicates. In general, averaged pH values for most trials increased over time both in the control tubes and in tubes with a label and/or insect treatment, showing an increase in pH ranging from 0.08 to 1.5 compared to the initial reading at the beginning of the test. The exceptions were trials of MEK-denatured 96% ethanol, most of which showed a decrease in average pH between −0.04 and −0.87 in comparison with the initial pH reading (Table S1).

Figure 1 compares the average initial pH and pH values after 26 months at +31°C for all treatments grouped by label type and the presence or absence of insect tissue. In the absence of insect tissue, pH values varied considerably across label types and ethanol solutions. Trials with label types PP, AF, and LP showed higher pH values for all ethanol solutions, and trials with 96% ethanol +1% MEK averaged much lower pH across all label types. The presence of insect tissue appeared to result in lower and less variable pH values across most treatments.

Figure 2 summarizes the average pH values after 26 months at +31°C for trials of the three different ethanol solutions, grouped by label type and the presence or absence of insect tissue. The dashed line represents the initial pH reading at the start of the experiment for each ethanol type. The pH readings in pure 96% or 99.8% EtOH increased in all treatments, with little difference between both alcohol concentrations under the same treatment. The pH readings were considerably lower for the MEK-denatured alcohol, except with PP, irrespective of the insect tissue.

Within the group of trials with 99.8% EtOH, pH readings increased over time in all replicates, including the control (Fig. 2). For trials in 99.8% ethanol, the samples with label paper (PP, AF, and LP) show a markedly higher pH reading when insect tissue is present

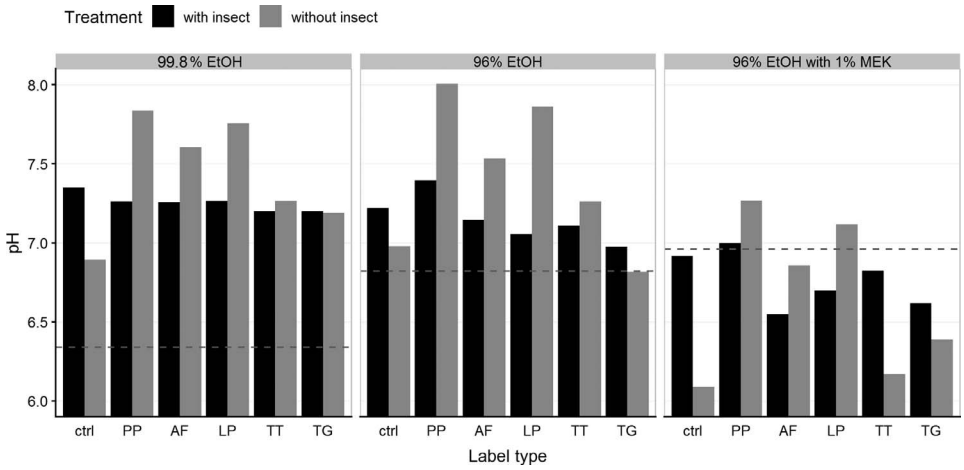

Figure 2. pH readings after 26 months for all trials at +31°C in samples with different ethanol solutions, grouped by label type. The dashed line represents the initial pH reading at the start of the experiment for each ethanol type.

(see Table S1). In contrast, treatments with tags (TT and TG) in 99.8% ethanol had similar pH readings with and without insect tissue (divergence in pH reading less than 0.1; see Table S1).

In the group of trials with 96% EtOH the pH readings emulate those of the group of 99.8% ethanol with an overall increase in the pH readings. The initial pH reading for the group of 96% EtOH was higher than the initial pH reading for 99.8%, and the increments on the pH readings were less pronounced. Again, paper treatments (PP, AF, and LP) had a higher pH value without insect tissue compared with the trials with insect tissue. Also tag treatments (TT and TG) showed minor changes in the pH reading, especially the TG without insect tissue and 96% ethanol, which showed a small decrease in the pH reading from the initial value (Fig. 2).

In the group of trials with MEK there was a notable drop in pH readings compared to initial values across most trials, with the exception of PP treatments (with and without insect tissue) and LP without insect tissue, where the readings increased. The recorded pH values for the control group without insect tissue decreased by almost 1 pH unit (−0.865; Table S1), while the control with insect stayed rather stable (−0.04; Table S1). All other printing media in MEK showed reduced pH values, with trial TT without insect tissue having pH readings nearly as low as in the control without insect tissue (−0.79; Table S1).

Discussion

Each storage container will maintain a specific equilibrium influenced by the preservative, the specimen (which may act as buffer), the labels, and the container, together with the external storage environment. Until this equilibrium is reached, the highest pH shifts can be observed soon after the initial deposition of the sample in its storage container. For long-term storage, stable pH conditions are desired. The reliability of pH measurements of preservative fluids (especially alcohol solutions) is often questionable (Waller and Simmons 2003; Carter 2009), and it is problematic to compare the results (in terms of absolute values) of different studies (Simmons 2014). Moreover, measuring the pH in ethanol solutions is difficult, error-prone, and by no means comparable to values obtained from aqueous

solutions. While the pH scale describes the degree of dissociation of H^+ ions in aqueous solutions, highly concentrated ethanol (e.g., 96% or 99.8%) virtually does not include any free H^+ ions, and the behavior of pH electrodes (designed and calibrated for measurements in aqueous solutions) is unreliable in hydroalcoholic solutions (Kotrba and Schilling 2017).

Because of the relatively low volume of ethanol in tissue tubes in relation to the amount of tissue, tissue collections are especially vulnerable. The influence of poor-quality plastic tubes/containers on the quality of tissues for the long-term storage of natural history collections is largely unknown and should be investigated. Plastic containers and jars with plastic lids are permeable to gases such as oxygen, as the container walls are not vapor barriers. Previous authors have reported leaching compounds from plastic labware and/or binding of DNA to the surface of plastic tubes, which affects routine experiments and measures in the lab (Berlotserkovskii and Johnston 1997; Gaillard and Strauss 1998; Ellison et al. 2006; McDonald et al. 2008; Lewis et al. 2010). Even though this may also have interfered with our experimental setting, potential effects of such compounds should have affected the measured values in all groups and trials equally.

Based on our observations, the presence of insect tissue had a notable effect on pH and pH variability, most commonly reducing pH across solutions and label types. All trials showed differences between treatments with and without insect tissue, especially in the trials with paper labels (PP, AF, and LP) (Fig. 2). Release of residual body and cellular fluids and organic and inorganic compounds affect the chemical equilibrium inside the storage container of the test samples. Even though the insect tissue was initially dehydrated in highly concentrated ethanol for three days before the experiment started, and the tissues thus already lost a high proportion of body fluids, there are still some notable differences in pH readings up to 1 pH unit between trials with and without insect tissue.

The presence of insect tissue appears to balance the pH in almost all treatment groups in our study, so trials with insect tissue show less variability in pH compared with the same trials without insect tissue. A notable exception to this general observation is the controls, where the increase in the pH in the trials without insect tissue is less compared to replicates with insect tissue (99.8% and 96% groups), and the decrease in the pH reading was more pronounced in the MEK group. A similar observation is made in the TT and TG trials of the MEK group. Thus, in the absence of any label, trials without insect tissue generally show a lower pH compared with the replicates with insect tissue, the opposite of observations for trials with any kind of label, which tend to show an increase in pH. This could be explained by continued dilution effects from insect cellular fluid, which could have increased the concentration of water molecules and, thus, stabilized the dissociation equilibrium.

A very interesting result from our observations is that in most samples pH increased over time, except in the trials with MEK. Ethanol dissociates to a much lesser degree than water, and it is a very weakly ionized fluid; thus, the ion activity in 99.8% and 96% EtOH is low. Consequently, the dissociated H^+ ions present in the water component of the hydroalcoholic solution may get diluted by the EtOH component, resulting in a higher pH reading, but not as a result of a shift in the dissociation equilibrium (Kotrba and Schilling 2017). Elevated pH readings in this type of ethanol solution do not imply a shift from neutral to alkaline conditions, as the dilution of the H^+ ions is balanced by a correspondingly lower anion concentration ($[OH^-]$), therefore, constituting a neutral solution (Kotrba and Schilling 2017). Moreover, if free dissociated H^+ ions are available, they may be shielded by the less polar EtOH molecules (Kotrba and Schilling 2017) and presumably are inactivated.

Besides fluctuations discussed above, the pH readings in the 96% EtOH and 99.8% EtOH groups remained relatively stable when compared with the controls. Both ethanol solutions contain relatively few water molecules and a low concentration of free dissociated H^+ ions, which are strongly shielded and thus largely inactive. In contrast, the pH values of nearly all trials in the MEK group decreased notably, with three exceptions: PP (with and without insect tissue) and LP without insect tissue, where the pH values were slightly higher compared to the control and initial recordings.

The MEK-denatured ethanol also includes denatonium benzoate (a bitterant, also known by its trade name Bitrex), methanol, and a few other chemical components that could affect the dissociation equilibrium and interfere with recorded pH values. The MEK-denatured ethanol has a low dissociation rate, but it is a highly polar molecule that oxidizes easily and may catalyze secondary reactions that alter the equilibrium. The containers used in our treatments are not gas-proof, and incubation at increased temperatures may have increased not only the activity of (secondary) chemical reactions inside tubes, but also oxygen influx into tubes and subsequent oxidization and breakdown of MEK into formic acid. This could be an explanation of the lower pH readings using MEK. Weigt et al. (2012) recommended avoiding denatured ethanol if DNA extraction is planned in distant future as the DNA molecule could be degraded and the usefulness of respective samples be compromised. Moreover, acidification triggers DNA depurination (Roger and Hotchkiss 1961; Zimmermann 2008), and it is difficult to amplify high-quality DNA from acidic-based fixatives (O'Leary et al. 1994; Longy et al. 1997; Douglas and Rogers 1998). A low pH in preservation fluids can accelerate the degradation of other molecular components such as lipids (Jones 1976) and subsequent breakdown into other compounds such as glycols, which are leached out of specimens (Carter 2003).

Our findings suggest that label type may have some influence on pH in ethanol solutions, but again, this could be masked by the high percentage hydroalcoholic solutions used in our tests. Synthetic tags (TT and TG) have somewhat lower pH readings than those for conventional paper, either laser-printed (LP), acid-free paper (AF), or written with a pen (PP), with the exception of the AF with insect tissue in the MEK group. This result agrees with previous results from Hargrave et al. (2005) and Zala et al. (2005) and may indicate a more neutral behavior of tags compared to tested papers (PP, LP, and AF). TT and TG demonstrate lower pH values than paper labels and controls with insect tissue, but these pH readings are higher than the controls for each alcohol group without insect tissue (except TG without insect tissue in pure 96% EtOH). We need to point out the pH value of TT labels without insect tissue in the MEK trial, which shows a remarkable drop compared with the same trial with insect tissue, a decrease not seen in other trials with TT. The tags and paper types differ considerably in composition. Tags (TT and TG) are polyester-based, and the tested label papers (LP, AF, and PP) are coated in various ways to aid printing quality, so they may contain components such as kaolinite that can potentially act as a buffer and that may explain some pH differences. Moreover, paper labels usually have slightly alkaline fillers, such as calcium carbonate, that may buffer against potential acidification (Pöykiö and Nurmesniemi 2008). While Bentley (2004) advocated for the usage of spun-bonded polyethylene tags and thermal printers, Waller and Simmons (2003) cautioned that labels with synthetic thread can cause more damage to specimens than cellulose thread. Although durable, the polyester substrate will eventually break down and may be sensitive to certain denaturants (e.g., MEK) (Range et al. 2017). Definitely, more research on the behavior of polyester-based tags is needed to assess potential influence in the chemical equilibrium and durability.

The increase in the pH readings in the samples with PP and LP is quite obvious (Figs. 1 and 2). Both PP and LP trials used paper that is not explicitly rated as acid-free. The term "acid-free" refers to the coating layer of the paper, and it does not necessarily mean that the entire paper (especially the pulp and the fillers that have been used) is pH-neutral. Compared to LP replicates, pH readings in PP trials are similar but slightly higher. The only obvious difference between PP and LP trials is the usage of Staedtler® pigment liners in PP compared to toner carbon-based imprinting, which is considered chemically neutral, in LP replicates. The water-based ink of the Staedtler® pens contains a color pigment but also binding agents and additives of unknown composition that may have influenced the recorded values.

Future research should investigate the effects of the label and paper components and long-term use of tags as well as the interactions between pH and DNA quality and study the effect of the container on the pH over time.

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