NONINVASIVE FLUID IDENTIFICATION: POTENTIAL OF MICRO-RAMAN SPECTROSCOPY

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Abstract.—Conservation of the preserving medium is an essential element for the proper preservation of specimens in fluid collections. However, the preservatives can become chemically altered over time or be lost by processes such as evaporation. To combat such changes and properly care for and maintain immersed specimens, it is therefore necessary to know what preservative fluid was initially chosen and how its chemistry may have evolved with time. The present work explores the possibilities offered by Raman spectrometry for a rapid, nondestructive, noninvasive alternative to commonly employed chemical identification tests, which are often limited to the identification of simple fluids. In a first step, fluids were reconstituted and analyzed in small standard glass containers to evaluate the potential of the technique. Then we successfully applied the procedure to real cases and considered its possible use to estimate the concentration of ethanol and to detect small quantities of formaldehyde (down to 1%). The results demonstrate the power of this technique, which opens up new possibilities for the management of fluid collections.

Key words.—fluid collections, Raman spectroscopy, formaldehyde, alcohol, preservative, mixtures.

Introduction

A Wide Range of Recipes

Understanding the composition of preserving fluids is essential for the conservation of wet collections. A considerable number of recipes and processes for implementing tissue fixation and fluid preservation have been tried (Simmons 2014). The first known example of perennial preparation dates to 1662 and was presented by W. Croone (Cole 1944). The most common preparations were composed of alcohol (mainly ethanol, also called ethyl alcohol or spirit, in ever-increasing concentrations thanks to the mastery of distillation over time). After its synthesis in 1858 and the discovery of its fixative properties at the end of the 19th century, formaldehyde also came into use (Blum 1893, Neuville 1899, Trillat 1892). From these favorable beginnings, naturalists and explorers tried to perfect preservation and retard degradation by including additives such as sugar, camphor, alum, sea salt, mercuric chloride (also called "corrosive sublimate"), zinc chloride, picric acid, or arsenic (Herbin 2013, 2017; Simmons 2014). Complex mixtures include the famous Ruysch solution in which cloves, black pepper, cardamom seeds, and camphor were added to spirits consisting of approximately two-thirds ethanol and one-third water (Daubenton 1749, Cole 1944, Down 1989). Another example is Owen's fluid (Douai 1885, Pouchet 1887), a mixture of water, sea salt, and alum (sometimes with mercuric chloride as well) widely used in the comparative anatomy fluid collection of the French National Museum of Natural History during the 19th century, especially for pelagic animals and bulky organs such as brains (Neuville 1917). It is interesting to note that this formula is very similar to the formula of the English Goadby's solution (Baird 1852). Such variety in the makeup of preservation fluids makes it difficult to know a priori what is in a jar. Labels or catalog records rarely mention the

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type of fluid used for a given specimen in a collection. Moreover, when recipes are found in historical sources, they usually mention generic terms and approximate proportions that make them difficult to reproduce using the techniques of modern chemical preparation.

Preservation Issues

The variety of preparation methods would not be a problem if fluid collections were stable in terms of composition. However, alterations in fluid composition can be observed within the very first hours during the preparation of a specimen, especially if the choice of fixation, preserving fluid, or jar sealant is not optimal for the specimen and if the environmental conditions (e.g., relative humidity, temperature, light) are unsuitable and/or variable (Ellis 1987, Waller and Simmons 2003, Simmons 2014). Description of this degradation and advice on best practices are the subject of other works (Waller and Strang 1996; Moore 2007; Collins 2014; Simmons 2014, 2019). Among the types of degradation that may endanger the specimen and make it difficult to access and study are direct changes in the nature and characteristics of the holding fluid. Additionally, the specimen itself might become altered by interaction with the preservation medium. The color of the specimen can change because of the reaction of its pigments with the preserving fluid, discoloration under the effect of UV light, blackening in the presence of mercuric chloride, or shrinkage or swelling of the specimen, resulting in changes in the reflection or refraction of light. Another well-known phenomenon is the evaporation of the preserving fluid due to an imperfect seal on the container. This can lead to total dehydration of the specimen. In the case of alcohol, which evaporates faster than water under certain environmental conditions (van Dam 2000), the concentration of remaining alcohol will gradually decrease, making tissue preservation less effective. Moreover, the entry of oxygen may cause oxidation of ethanol to acetic acid and formaldehyde to formic acid, resulting in acidification of the fluid and alteration of the specimens. Cases of fluid opacification are also reported. These can be linked to various biochemical mechanisms, for example, the polymerization of formaldehyde into paraformaldehyde (white precipitate) or the release of fat (yellowing) or blood (browning) from the specimen into the fluid. In addition, the growth of micro-organisms in the fluid can alter and degrade the preservation environment of the specimen (Moore 1999, 2002; Simmons 2014).

Fluid identification.—The above considerations demonstrate that jars of historic fluid collections may contain, in addition to the specimens themselves, a wide variety of molecules in solid or liquid phase, some of which are toxic. These chemicals may come from the initial fixation and preservative fluid, from subsequent chemical degradation, from outside contamination due to a degraded sealant, or from the interaction of the specimen with the fluid (van Dam 1997, 2000; Simmons et al. 2007). In addition, putting a specimen in another fluid or topping up a jar with a fluid of differing chemical composition (or even a differing concentration of the same chemicals) can have immediate and harmful consequences for the preservation of the specimen (Notton 2010, Simmons 2014). Similarly, the implementation of a rehydration protocol for dried-out specimens can be problematic. The management, maintenance, and conservation of specimens preserved in fluid requires knowledge of the chemical nature of the compounds involved. Several physico-chemical methods have been developed to identify certain fluids in the collections and check their concentration. For example, simple color-detection chemical tests have been identified to distinguish formaldehyde from alcohols. One example is the Schiff's reagent test, also used in histology for periodic acid-Schiff (PAS) staining. An intense violet stain indicates that an

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aldehyde is present in the preservative fluid. Other reagents, such as 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, can be used. Some of these methods involve tests on disposable strips from a single drop of fluid (see Waller 1987 for directions for the preparation of test strips; commercial test strips include MQuant or Quantofix® from Machery-Nagel™). These semiquantitative tests, developed for rapid detection of aldehydes in water, are very sensitive and can detect traces of formaldehyde fixatives in alcohol preserving fluids. Another type of simple chemical test is based on salting out. In this test, the addition of a salt (e.g., potassium carbonate) causes an alcohol solution to separate into two phases, one containing water and the other containing alcohol, whereas formaldehyde does not salt out (Mayfield 2013).

It is also possible to distinguish pure preservative fluids physically by taking advantage of their different densities. The most affordable method consists of immersing a small calibrated object of known density (e.g., plastic pinheads) in the fluid and observing whether the plastic floats or sinks (Moore 1986, Nicol 1994). A commercial system exploiting this property is sold as the Alcomon Indicator System. Other commercial devices are available to measure the density of a liquid (e.g., a digital density meter such as the Densito 30PX from Mettler Toledo or a hydrometer) thus allowing identification and estimation of the precise concentration of ethanol or formaldehyde (Carter 1994, Nicol 1994, Moore 1999).

Limitations of the Current Fluid Identification Methods

Colorimetric methods have the disadvantage that they cannot distinguish mixtures and may not always be reliable. Indeed, false positives may result from chemical interference from the presence of aldehydes with the Schiff test or acetone with the Quantofix strips, or from alcohol concentrations being too low for the salting-out tests. In order to identify the chemical molecules present in a jar, particularly the biomolecules, precisely, more advanced laboratory analytical methods are required. However, these methods take longer to implement and use equipment that is more expensive, requiring access to an analytical chemistry laboratory. For example, gas chromatography coupled with mass spectrometry has been successfully used to determine the nature of preservative fluids and dissolved lipids, and amino acid analysis, based on liquid chromatography coupled with UV detection, has made it possible to identify the peptides or proteins released from specimens into the fluid (von Endt 1994, von Endt et al. 2000, Marte et al. 2003).

However, all these methods have a major disadvantage for conservation as well as for the safety of personnel: They require opening the specimen container and removing a sample of preservative fluid. This operation endangers the specimen, especially through the entry of oxygen into the jar (and possibly other environmental pollutants). It may expose the person collecting the sample to toxic fumes (Burroughs et al. 2006), and it may result in fluid loss or contamination if the container is not properly resealed. In addition, the procedure may result in the loss of historical information about the original sealant.

Raman Spectroscopy as a Promising Technique

A laboratory spectroscopic technique called Raman spectroscopy allows nondestructive analysis of materials, whether solids or liquids, at the molecular level. The sample is illuminated by a monochromatic laser beam that interacts with the vibrating chemical bonds of the molecules of the sample: the measurement of the frequency of the scattered radiation makes it possible to characterize the sample's chemical composition. The equipment is available in many university laboratories or museum conservation institutes (Casadio et al. 2016) because it is used in fields as diverse as biology, geology, forensics, and gemology,

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in the pharmaceutical sector, and in industry for the detection of contaminants or defects and for the authentication of works of art (Lewis and Edwards 2001). In forensic analysis, as well as in the beverage and pharmaceutical industries, Raman spectroscopy is used for chemical identification of liquids through sealed glass containers (Boyaci et al. 2012, de Oliveira Penido et al. 2016). In natural history collections, it is sometimes used to identify salts formed as a result of degradation reactions in preserved specimens (e.g., Rouchon et al. 2012, Schmid et al. 2016). Moreover, the success of Raman spectrometry has over the last decade enabled the development of portable instruments that can be used in collection storage rooms. Given such advantages, we have investigated this noninvasive and nondestructive technique for use in museum collections to identify the chemical makeup of the preserving fluid in sealed jars of fluid-preserved museum specimens.

Experimental Section

The potential of Raman spectroscopy for use in museum fluid collections was evaluated with a step-by-step approach. First, preservative fluids were reconstituted and analyzed after being placed in small, closed glass vials of identical nature. Fluid samples from historical collections were then placed in the same type of closed vials for analysis. Reference spectra of possible additives, purchased as powders and placed on a microscope slide, were obtained for comparison. To determine the influence of glass and plastic containers, historic glass jars and plastic leak-proof canisters filled with pure ethanol were analyzed. Finally, Raman spectroscopy analyses were carried out on unopened jars and containers filled with historical fluids.

Materials

Model solutions were prepared with ultrapure water (Milli-Q®, Merck) and the following chemicals were used: 37% formaldehyde (ACS grade, Sigma-Aldrich), formic acid (HPLC grade, Sigma-Aldrich), methanol (HPLC grade, Sigma-Aldrich), glacial acetic acid (Sigma-Aldrich), 99% palmitic acid (Sigma-Aldrich), 99% potassium acetate (Sigma-Aldrich), absolute ethanol (HPLC grade, Fisher Scientific), isopropyl alcohol (HPLC grade, Fisher Scientific), potassium alum (Acros Organics), sodium chloride (Fisher Scientific), 99.% mercuric (II) chloride (Alfa Aesar), and thymol crystals (Centre de Recherche sur la Conservation [CRC] collection of natural products). The solvents used for this experiment correspond to those pooled at CRC, in particular concerning the degree of purity of the substance (not required for the present study): the "ACS" quality solvents meet the purity requirements defined by the Committee on Analytical Reagents of the American Chemical Society and the "HPLC" quality solvents are sufficiently pure to be suitable for chemical analysis by HPLC (acronym for high-performance liquid chromatography). Additionally, a Bouin's solution which had been prepared in the late 1980s for a museum collecting expedition to conserve marine mammals was used for analysis.

Test containers consisted of glass 17×58 mm clear borosilicate vials sealed with PTFE (Polytetrafluoroethylene), closures (Wheaton Science Products, Millville, NJ, USA).

Samples from Wet-Storage Collections

Fluid specimens were also taken from two French collections of biological tissues preserved in liquids (Table 1): one glass jar from the Dupuytren anatomical collection (the Dupuytren collections, formerly the Musée Dupuytren founded in 1835, are collections of pathological anatomy comprising 3,800 pieces in fluid, kept in Paris at the Faculty of Sciences of Sorbonne Université) and nine glass jars from the national comparative anatomy

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Table 1. List of samples studied from the Dupuytren collections (Sorbonne Université) and from the fluid collection for comparative anatomy of the French National Museum of Natural History (Muséum national d'Histoire naturelle or MNHN), both in Paris. The catalog number, species, fluid color, presumed fluid type (according to the catalog or the person in charge of the collection), and other information is specified.

Collection and catalog number	Species	Fluid color	Catalog information	Presumed fluid	Result of Raman analysis	Conclusion
Dupuytren (Sorbonne Université)						
MD.T.2015.O.1045	*Homo sapiens*	Transparent pink	Tonsil with squamous epithelioma	Kaiserling III	Kaiserling III	Expectation confirmed by analysis
Comparative Anatomy fluid collection (MNHN)						
MNHN-AC-A-5495/1843-107	*Ursus* sp.	Translucent	Brain. Entered the collection in 1843 (ethanol). Fluid changed in 1887 to Owen's fluid and to formalin in 1924.	Formaldehyde	Formaldehyde	Expectation confirmed by analysis
MNHN-AC-A-4851/1873-54	*Felis tigris*	Translucent yellowish	Brain. Entered the collection in 1872 (Owen's fluid or ethanol). Fluid changed in 1887 to Owen's fluid and to formalin in 1924.	Formaldehyde	Formaldehyde	Expectation confirmed by analysis
MNHN-AC-1878-316	*Hipposideros diadema*	Translucent yellowish	Entered the collection in 1878 (ethanol). Jar opened in 1913	Formaldehyde?	Ethanol	Ethanol was used in 1913 for topping up the jar, or formaldehyde was used in 1913 and ethanol was used to top up the jar afterward
MNHN-AC-1883-14	*Chirogaleus rufus*	Translucent, slightly yellowish	Brain. Entered the collection in 1883 (ethanol).	Ethanol	Ethanol	Expectation confirmed by analysis
MNHN-AC-1895-56	*Macrotus waltherhousii californicus*	Translucent yellowish	Brain. Entered the collection in 1895.	Ethanol?	Formaldehyde	Formaldehyde added after 1895
MNHN-AC-1912-94	*Lynchus caracal*	Translucent green	Brain	Formaldehyde	Formaldehyde and sulfate salts	Expectation confirmed and further details provided by analysis
MNHN-AC-1929-211	*Sus scrofa domestica*	Neon yellow	Brain	Bouin's solution	Bouin's solution	Expectation confirmed by analysis
MNHN-AC-1935-169	*Sus scrofa*	Translucent yellowish	Newborn	Mixture with formalde-hyde?	Formaldehyde and ethanol	Expectation confirmed and further details provided by analysis
MNHN-AC-1963-64	*Helarctos malayanus*	Translucent yellowish	Brain. Malaysian bear died at the zoo on 18 February 1963	Formaldehyde	Formaldehyde	Expectation confirmed by analysis

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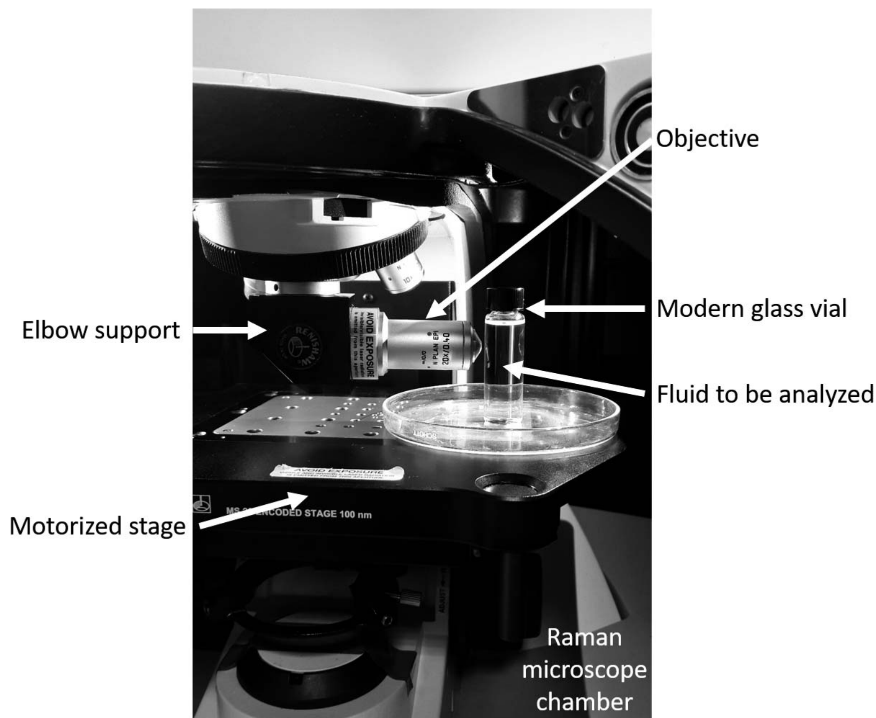

Figure 1. Experimental setup of the Raman analysis.

fluid collection (Muséum national d'Histoire naturelle, Paris). These specimens date from the mid-19th to the early 20th century and are presumed to be preserved in representatives of the different types of fluids found in these collections.

Raman Spectroscopy

Data acquisition was performed with a Renishaw In Via Raman microspectrometer, equipped with two laser sources emitting at 532 nm and 785 nm. It is a confocal Raman microscope, equipped with a binocular viewer and a video camera. Calibration was checked on a silicon wafer (main peak at 520.5 cm^{-1}). The vials and small jars were placed directly under the microscope objective, mounted on an elbow attachment (Fig. 1). For larger jars, the objective was mounted on a remote arm and measurements were performed in the dark (black curtain or lights off). The lattice used had 1,800 lines/mm with the green laser emitting at $\lambda = 532$ nm and 1,200 lines/mm with the near-infrared laser emitting at $\lambda = 785$ nm. Spectral range was between 100 and 4,000 cm^{-1}. Each product (liquid or glass) was analyzed by both the green laser and the near-infrared laser in order to determine which was most suitable to obtain an information-rich chemical signature. The main limitation of Raman analysis lies in the occurrence of fluorescence phenomena that may jeopardize the Raman signal. Fluorescence emission, linked to the absorption of incident radiation, competes with the Raman effect; the corresponding signal is more intense and less informative than the Raman signal. This fluorescence is difficult to predict and can only be assessed through experience. Sometimes, increasing the excitation wavelength limits fluorescence, but in doing so, the Raman signal is also strongly decreased. Therefore, a compromise must be found between reducing fluorescence and increasing the efficiency of the Raman signal.

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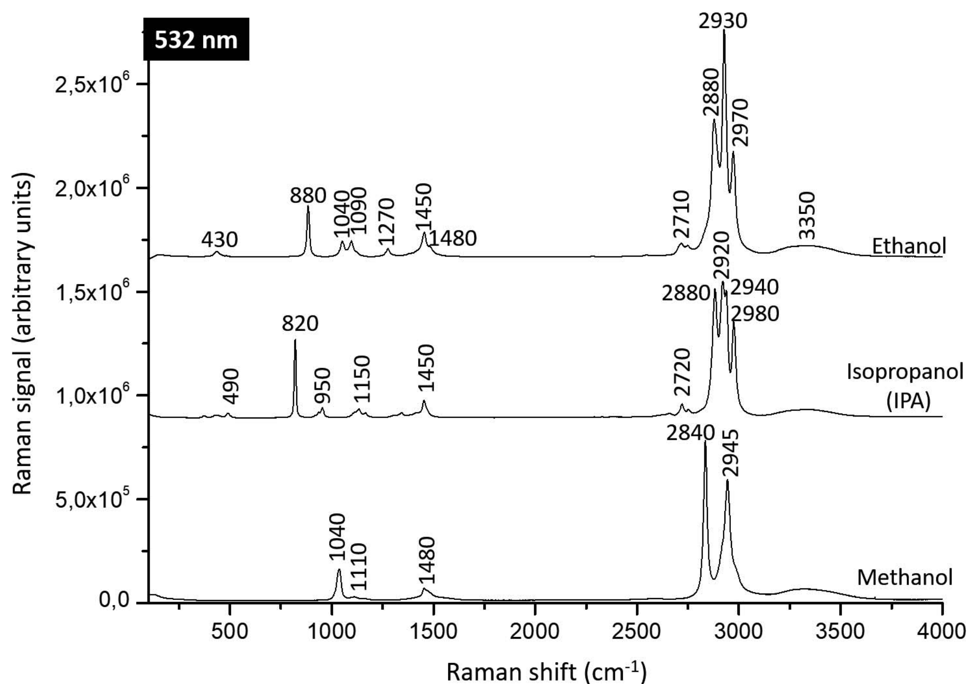

Figure 2. Comparison of the Raman spectra of three pure alcohols used in fluid collections: ethanol, methanol, and IPA, for 532-nm laser excitation with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}.

An additional variable that must be considered is the range of different objectives available (5, 10, 20, and 50×, and 50× with long working distance magnifications), all of which were tested. To optimize the parameters for the acquisition of Raman spectra through the jar, the first tests were carried out on absolute ethanol, which has a particularly complex Raman signal. In this case, the simplest focusing and the best signal-to-noise ratio were obtained with the 50× long working distance objective, for 10 acquisitions of 10 sec at 10% laser power (3.1 mW) for the laser at 532 nm and 100% laser power (50 mW) for the laser at 785 nm. To optimize the focalization of the beam, the vials were first placed in contact with the objective lens, then gradually moved away using the motorized stage of the microscope or by hand (in the case of the remote arm) until the signal with the lowest signal-to-noise ratio was obtained. The laser strength was similarly optimized from the lowest power (1%) to limit any possible damage and to achieve the best signal without saturating the detector.

Data were acquired using Renishaw's WiRE (Windows-based Raman environment) software version 3.4. The cosmic ray interference signals were removed using the dedicated option in this software (cosmic ray remover option). For the estimation of fluid concentration, the data were assessed using the peak analyzer wizard of Origin software (OriginLab Corporation, Northampton, MA, USA) performing a baseline subtraction (with a spline baseline of 9 points) and fitting the peaks with Gaussian function.

Results and Discussion

Identification of Preservative Fluids Through Borosilicate Glass Vials

Alcohols: ethanol, isopropanol, and methanol.—We initially observed that the choice of the incident beam wavelengths had no significant impact on the Raman spectra of these

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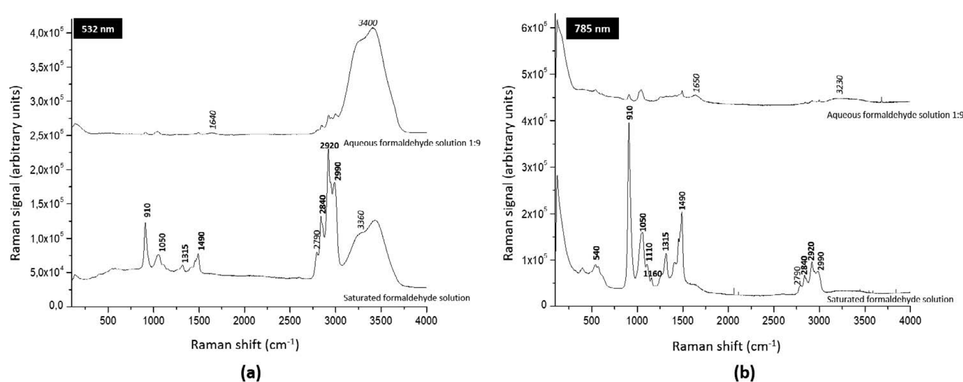

Figure 3. Raman spectra of pure formaldehyde 37% w/w and aqueous formaldehyde solution 1 : 10 for (a) 532-nm and (b) 785-nm laser excitation with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}. Bold annotations indicate the main markers of formaldehyde. Annotations in italics indicate water.

alcohol fluids. Therefore, only data taken at 532 nm are shown in Figure 2, which compares the Raman spectra for three pure alcohols: ethanol, methanol, and isopropanol (IPA).

The ethanol spectrum has a strong band at a Raman shift of 880 cm^{-1}, because of carbon-carbon (C–C) bond symmetric stretching in the molecule, and two small bands at 1,040 and 1,090 cm^{-1}, corresponding, respectively, to carbon–oxygen (C–O) bond stretching and CH_3 rocking or to C–C–O bonds asymmetric vibrations (Boyaci et al. 2012, Pappas et al. 2016). Very strong peaks are observed at 2,880, 2,930, and 2,970 cm^{-1}, corresponding to the C–H bond stretching modes, whereas the broad band around 3,350 cm^{-1} is assigned to the OH bond stretching mode (Numata et al. 2011). Some additional peaks are present in the spectral fingerprint at 430 (C–O deformation), 1,270 (CH_2 twisting), 1,450 (CH_2 scissoring), 1,480 (CH_3 asymmetric deformation), and 2,710 cm^{-1} (Picard et al. 2007, Pappas et al. 2016).

Isopropyl alcohol and ethanol have relatively similar spectral fingerprints, which was expected, because these two molecules are chemically close (Jin et al. 2018). Yet some features make it possible to differentiate these molecules. First, there is a significant shift of the C–C stretching peak from 880 cm^{-1} for ethanol to 820 cm^{-1} for IPA. Second, the C–H stretching peak of ethanol (2,930 cm^{-1}) splits into two peaks for IPA (2,920 and 2,940 cm^{-1}).

The Raman spectrum of methanol contains fewer peaks, which is logical, because it is the simplest of the alcohols. For this fluid, single C–O stretching and CH_3 rocking peaks are observed, respectively, at 1,040 and 1,110 cm^{-1}. Moreover, the CH_3 asymmetric deformation peak and the three C–H symmetric stretching peaks observed, respectively, at 1,455, 2,885, 2,930, and 2,980 cm^{-1} for ethanol, are shifted to two peaks, only situated at 1,480 and 2,945 cm^{-1} (Vaskova 2014).

Formaldehyde.—Figure 3 shows the spectral signatures taken with the 532- (Fig. 3a) and 785-nm (Fig. 3b) laser beams on a saturated formaldehyde solution and a diluted formaldehyde solution in a ratio of 1 : 10 (1 part of saturated formaldehyde solution is diluted in 9 parts of water, corresponding to 3.7% w/w or 4.0% v/v of formaldehyde gas in water). Here the incident wavelength strongly affects the quality of the spectra. The signal of saturated formaldehyde remains rich for both wavelengths, although the peak resolution is improved at 785 nm for low wave numbers (below 1,750 cm^{-1}) and at 532 nm for high wave numbers (over 2,500 cm^{-1}). As expected, the Raman signal of formaldehyde

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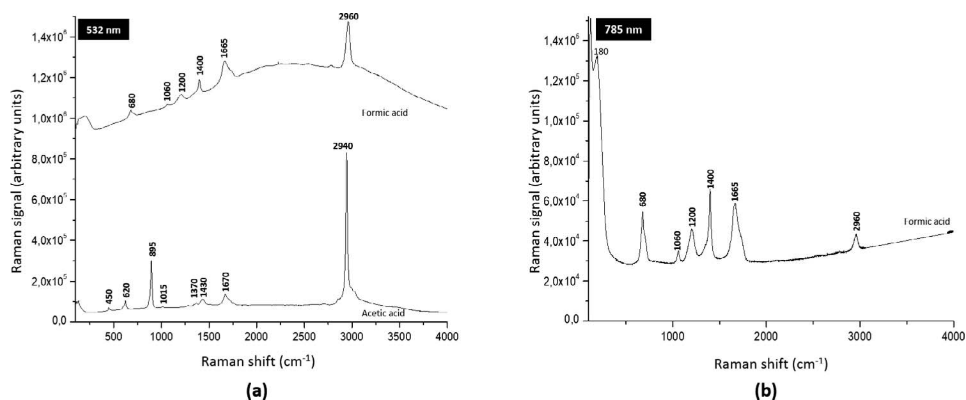

Figure 4. Raman spectra of acetic acid and formic acid (considered as ethanol and formaldehyde degradation product through oxidation) for (a) 532-nm and (b) 785-nm laser excitation with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}.

decreases with the dilution of the solution, and at 532 nm the signal is largely dominated by wide bands corresponding to the presence of water (around 3,400 cm^{-1}).

The Raman spectra of formaldehyde are characterized by the following modes: H–C–H symmetric stretching at 2,840 and 2,920 cm^{-1}; H–C–H asymmetric stretching at 2,990 cm^{-1}, C=O scissoring at 540 cm^{-1}, C=O symmetric stretching at 910 cm^{-1}, C=O asymmetric stretching at 1050 cm^{-1}, H–C–H rocking at 1,110 and 1,160 cm^{-1}, H–C–H deformation at 1,315 cm^{-1}, and H–C–H bending at 1,490 cm^{-1}. The supplementary peak at 1,640–1,650 cm^{-1} for diluted formaldehyde (10%) corresponds to the interaction with water (Lebrun et al. 2003, Delcroix et al. 2010).

Mixtures consisting of 70% ethanol and 10, 5, or 1% formaldehyde were also analyzed. However, the formaldehyde signal was not detectable below the ethanol peaks and all spectra were identical to that of ethanol 70% (data not shown). Therefore, this technique is not able to detect traces of formaldehyde coming from the fixation of the specimen or from previous exposure to formaldehyde.

Degradation products.—The entry of oxygen into a container can promote the oxidation of chemical components (e.g., formaldehyde oxidizes to formic acid and ethanol to acetic acid). To estimate whether such chemical changes could be detected with Raman spectroscopy, vials containing glacial acetic acid and 98–100% formic acid solutions were tested (Fig. 4a, b). Acetic acid gives a good signal for excitation in the visible range (532 nm), but formic acid has a significant fluorescence signal (Fig. 4a) that impairs its detection at this wavelength. Fortunately, its fingerprint is improved with the near-infrared laser (785 nm; Fig. 4b). It is characterized by Raman scattering bands at 1,400 (C–H bending mode), 1,665 (C=O stretching), 1,200 (C–O stretching), 680 (OCO bending), 2,960 (C–H stretching), and 1,060 cm^{-1} (C–H twisting mode; Davies 1971, Jehlička et al. 2010). Furthermore, the fingerprint for acetic acid can be relatively easily distinguished from that of formic acid because of a specific peak at 895 cm^{-1} (C–C symmetric stretching) which is absent from the formic acid signature. Moreover, the peaks of acetic acid appear at different wave numbers than those of formic acid: 2,940 (CH_3 stretching), 450 (H–C–H symmetrical swing and O–H swing), 1,670 (C=O stretching), 620 (O=C–O bending), 1,430 (H–C–H

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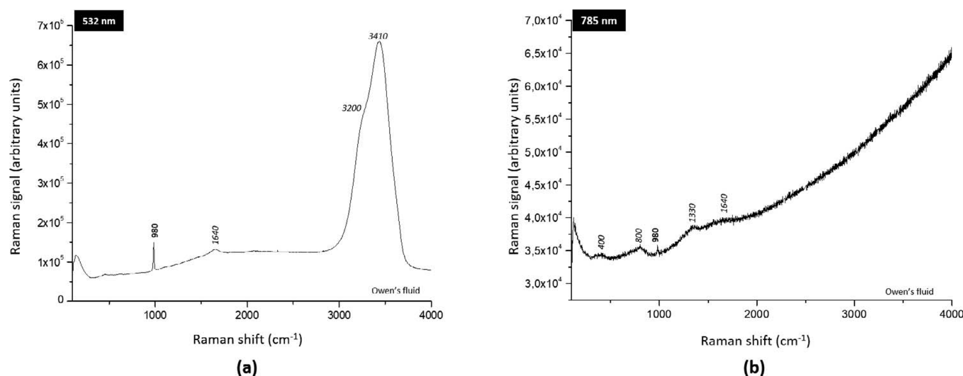

Figure 5. Raman spectra of Owen's fluid reconstituted preservative for (a) 532-nm and (b) 785-nm laser excitation with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}. Bold annotation indicates the main marker of Owen's fluid.

asymmetric swing), 1,370 (H–C–H bending) and 1,015 cm^{-1} (O–C–C asymmetric stretching; Jehlička et al. 2010, Wan et al. 2017).

To simulate fat extraction from the specimen by the preservative, we dissolved palmitic acid, one of the most common saturated fatty acids in animals (Marte et al. 2003), in both the 70% ethanol and diluted formaldehyde solutions until the saturation point was reached. The Raman signal of palmitic acid was characterized separately on pure palmitic acid powder. The palmitic acid signal shows specific signatures of fatty acids with two intense bands at 1,060 and 1,130 cm^{-1} (C–C stretching), one band at 1,290 cm^{-1} (H–C–H twisting), and two bands at 1,440 and 1,470 cm^{-1} (H–C–H wagging and scissoring). The C–C stretching vibration with moderate Raman intensity at 1,100 cm^{-1} distinguishes palmitic acid, but the most prominent marker band is a low-frequency vibration at 375 cm^{-1} (De Gelder et al. 2007). However, the spectra recorded on saturated solutions were identical to those of 70% ethanol and diluted formaldehyde solutions, meaning the palmitic acid signal is obscured by the signals of ethanol or formaldehyde. Therefore, this technique does not allow *in situ* detection of traces of dissolved fats in a common fluid such as ethanol or formaldehyde. However, sometimes, with severely damaged specimens, fatty lobules form in the jar. These have a high concentration of fatty acids and their analysis by Raman spectroscopy is probably feasible. Such a case is beyond the scope of this project but deserves further study.

Mixtures: Owen's fluid, Kaiserling III solution, and Bouin's solution.—The Raman spectroscopy method was tested on reconstituted preservative fluids (using known recipes) to evaluate its potential for the analysis of complex mixtures. The additives used in these recipes were analyzed separately to identify their specific Raman signatures and estimate their ability to induce fluorescence.

Owen's fluid consists mainly of water with added sea salt (100 g/L), potassium alum (50 g/L), and sometimes "corrosive sublimate" (mercuric chloride $HgCl_2$, 0.1 g/L). As expected, its Raman spectrum (Fig. 5) is dominated by large water bands around 1,640, 3,200, and 3,410 cm^{-1} for 532-nm excitation wavelength and 1,640, 800, 400, and 1,330 cm^{-1} for 785-nm excitation wavelength. At 785 nm, the spectrum shows a strong fluorescence signal. For both wavelengths, however, a specific and well-defined peak is present at 980 cm^{-1}. This peak is absent from the fingerprints of the pure products used to make the solution (see Raman spectra in Fig. 6). It is, in fact, specific to molecular configuration changes

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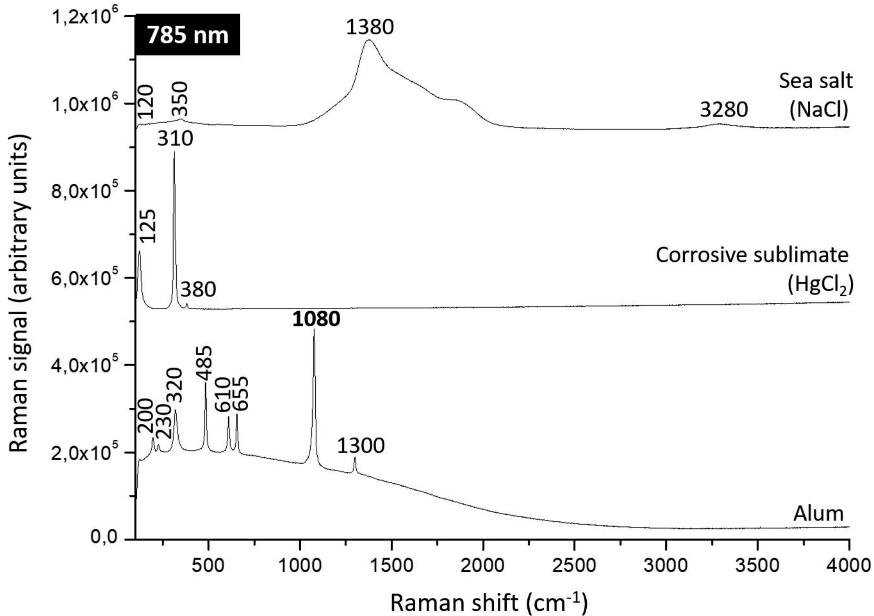

Figure 6. Raman spectra of the main constituents of Owen's fluid recipe: sea salt (NaCl), corrosive sublimate ($HgCl_2$), and potassium alum for laser excitation at 785 nm, annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}.

occurring during the dissolution of the salts. Indeed, the symmetric stretching of the sulfate anion occurring at 1,080 cm^{-1} in the alum crystal is shifted to 980 cm^{-1} when the crystal is dissolved and sulfate surrounded with water (Ishizaka et al. 2013). This peak is therefore a marker of the use of alum in the recipe.

Kaiserling's solutions have been proposed as a preservative mixture to preserve the colors of anatomical specimens better. After the first publication (Kaiserling 1896), the inventor proposed several variants of his method, whether for the fixation solution (known as Kaiserling I), the color-restoration solution (known as Kaiserling II), or the preservative solution (known as Kaiserling III). The latter is a glycerol solution with 83.3 g/L of potassium acetate. Several authors then proposed improvements, including the addition of thymol, camphor, arsenious acid, or sodium hydrosulphite, and these are referred to as modified Kaiserling solutions. The solution prepared in this work corresponds to a modified Kaiserling III solution with the addition of thymol (a few crystals corresponding to about 0.5 g/L) to inhibit mold growth and the adjustment of the pH to 8 by adding 1 N sodium hydroxide. Raman spectra were recorded at 532 nm on this solution. Similar to Owen's fluid, the Kaiserling solution shows wide bands characteristic of water (Fig. 7, peaks labeled with numbers in italics). However, the spectrum is much richer than that of the Owen's fluid because it also includes several additional bands. Many of the bands are related to glycerol (Fig. 7, peaks with numbers in bold; Mendelovici et al. 2000): 2,890 and 2,940 cm^{-1} (C–H stretching from CH_2); 1,460, 1,250, and 980 cm^{-1} (CH_2 deformation, twisting and rocking, respectively); 1,110 and 1,050 cm^{-1} (C–O stretching); 850 and 820 cm^{-1} (C–C stretching); 670 cm^{-1} (C–C–O or O–H deformation); 550 cm^{-1} (C–C–C deformation); 480 and 410 cm^{-1} (C–C–O rocking); 330 cm^{-1} (intermolecular H bonds). In addition, signatures characteristic of acetates can be seen at 930 cm^{-1} (C–C stretching),

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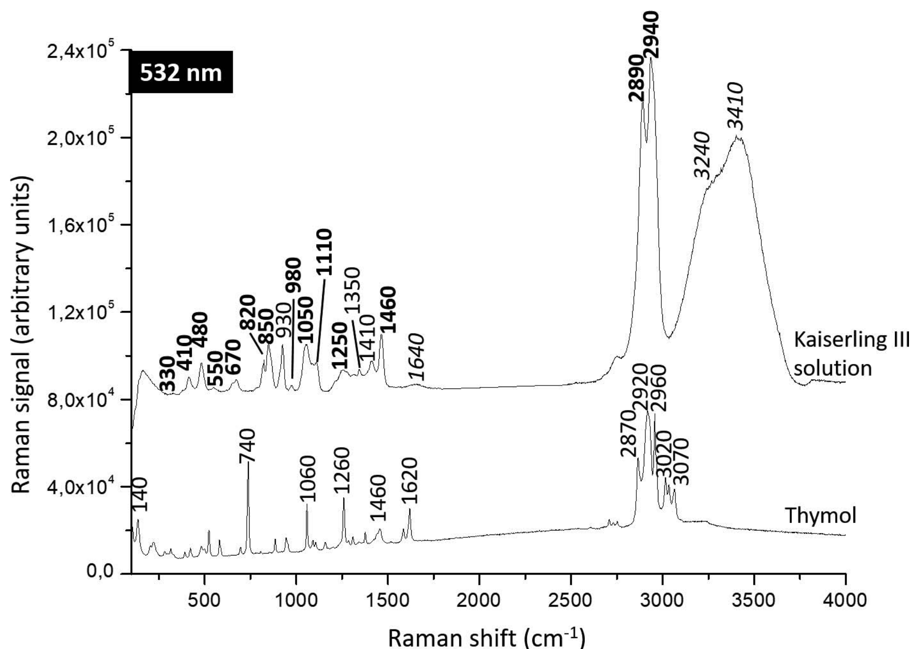

Figure 7. Raman spectra of Kaiserling III solution reconstituted preservative and comparison with the Raman spectra of thymol crystals (supposed to be one of the constituents) for 532-nm laser excitation with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}. The bold annotations indicate the main markers of glycerol. Annotations in italics indicate water.

1,350 cm^{-1} (CH_2 deformation) and 1,410 cm^{-1} (C=O stretching; Frost and Kloprogge 2000), but they remain very low and can be to some extent masked by the glycerol signal. This acetate signature is, moreover, too weak depending on the type of salt (potassium or sodium) that was used. In addition, even though thymol was added to the solution, no thymol peak was identified. As it is a minor compound compared to the other three, its signature is masked or is too weak to be detected. In any case, the spectral signatures of Kaiserling III solutions show glycerol- and acetate-specific features. They can therefore be easily distinguished from the other types of solutions.

Bouin's solution is composed mainly of picric acid (75%), formaldehyde (20%), and a small amount of acetic acid (5%). It has a typical neon yellow color produced by the presence of picric acid. This compound is toxic and irritating on the one hand and explosive on the other, as are the picrates that can be formed by reaction with certain metals (such as those present in metal lids). It is therefore preferable to analyze the Bouin's solution with care and, if possible, without opening the jar. The Raman spectrum for Bouin's shows bands corresponding to these three compounds (Fig. 8a, b). It includes the abovementioned bands specific to formaldehyde (Fig. 8a, bold annotations) and acetic acid (Fig. 8a, italic annotations), as well as bands characteristic of picric acid (Fig. 8, underlined; Srivastava et al. 2017) at 830 (NO_2 scissoring), 1,180 (C–N symmetrical stretching), and 1,345 cm^{-1} (symmetrical NO_2 stretching).

Comparison of Figures 5–8 shows that it is possible to distinguish the different types of fluids, but not the detailed composition of the fluids. This is due to factors such as interference from fluorescence, or because the signal of minor ingredients is not always perceptible.

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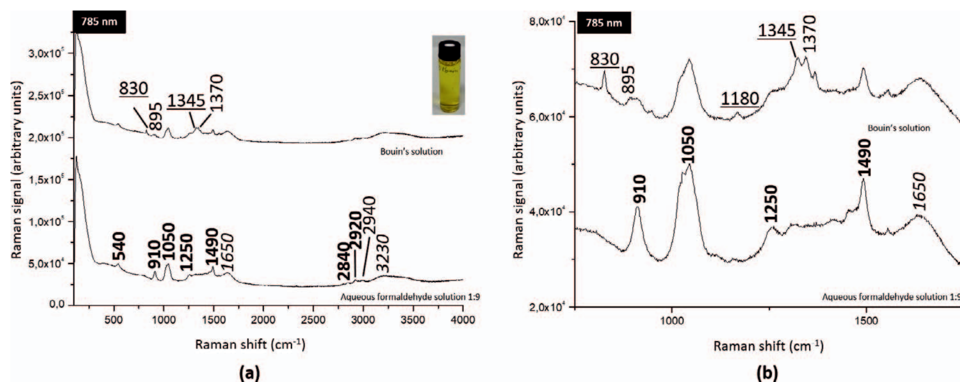

Figure 8. Raman spectra of Bouin's solution reconstituted preservative for 785-nm laser excitation compared with aqueous formaldehyde solution 1 : 10, (a) with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1} and (b) between 750 and 1,750 cm^{-1}. Bold annotations indicate the main markers of formaldehyde. Picric acid markers are underlined. Annotations in italics indicate water.

For example, the signal of thymol is difficult (not to say impossible) to detect, meaning that Raman spectroscopy cannot be used to determine if thymol was used in a recipe. Similarly, fats and degradation products such as acetic or formic acids give too low a signal to be perceptible.

Despite these limitations, Raman spectroscopy unambiguously enables a noninvasive detection of some solution constituents that can, therefore, be considered as markers of the different types of fluids: sulfates (in alum) in the case of Owen's fluid, glycerol in the case of Kaiserling III solutions, and picric acid in the case of Bouin's solution.

Identification of Historical Fluids in Their Historic Glassware

The study method was applied to specimens in original glassware and fluid preservatives from historic collections to check against the presumed fluid composition determined by curators, who must rely on common uses, habits, written recipes, and collection catalogs (Table 1). It should be noted that Raman spectra recorded through older and usually thicker glass are noisier than the test vials and often show a background of fluorescence. It was nevertheless possible to derive information from these spectra.

The analysis of the fluid taken from the Dupuytren collection specimen (MD.T.2015.O.1045) gives the same Raman signal as the Kaiserling III solution (Fig. 9a). This is consistent with a recipe from the 19th century, found in the museum (E. Quétel, pers comm.). The recipe, moreover, indicates the use of sodium acetate (and not potassium acetate). These substances could not be identified by Raman spectroscopy, but this is important additional information regarding the supposed composition of the fluid.

Figure 9b shows the Raman spectrum of the fluid in the jar with catalog number MNHN-AC-1929-211. The neon yellow color suggested a Bouin's solution, a supposition that was confirmed by the Raman spectrum on which characteristic bands of picric acid were detected.

Most of the fluids analyzed from the comparative anatomy collection are formaldehyde. The Raman spectra of fluids MNHN-AC-A-5495, MNHN-AC-A-4851, MNHN-AC-1895-56, and MNHN-AC-1963-64 are shown Figure 10a. Analysis confirms that the relatively recent specimen MNHN-AC-1963-64 was indeed preserved in this fluid during

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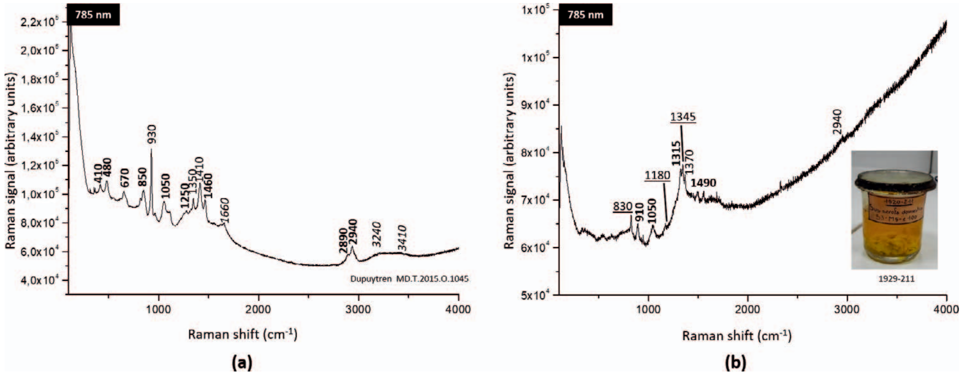

Figure 9. Raman spectra of fluids with annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}, for 785-nm laser excitation from (a) the Sorbonne Université Dupuytren collection (catalog number MD.T.2015.O.1045) and (b) from the comparative anatomy fluid collection of the French National History Museum (catalog number MNHN-AC-1929-211) with a photograph of the jar ©M. Herbin. Bold annotations indicate the main markers of formaldehyde. Picric acid markers are underlined. Annotations in italics indicate water.

preparation in 1963 and that specimens MNHN-AC-A-4851 and MNHN-AC-A-5495 were preserved in formaldehyde in 1924. For specimen MNHN-AC-1895-56, for which the collection catalogs are imprecise, the analysis reveals that, despite its light yellow color, the current preservative fluid is formaldehyde, indicating a change or topping up of fluids after it entered the collection in 1895.

Some fluids in the collection show this signature of formaldehyde but with a few additional bands (Fig. 10b). For example, the MNHN-AC-1935-169 fluid also has bands characteristic of the presence of ethanol (perhaps from housing it in a jar that previously contained ethanol), whereas the fluid from MNHN-AC-1912-94 shows one additional band at 980 cm^{-1}, which is characteristic of a sulfate salt. The formation of copper sulfate, related

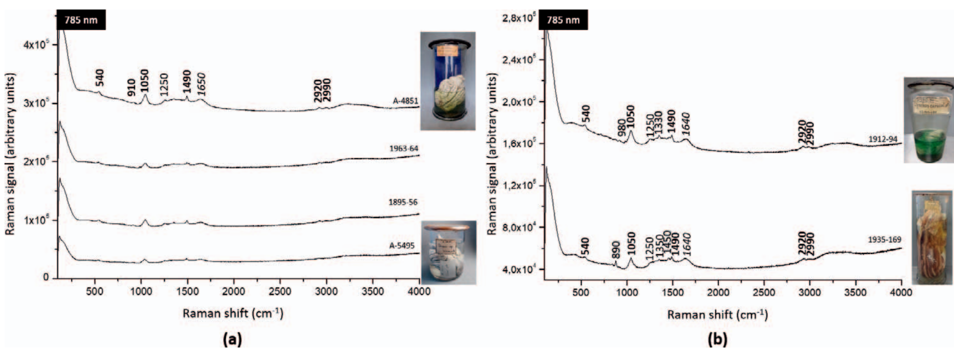

Figure 10. Raman spectra of fluids from the comparative anatomy fluid collection of the French National History Museum. Spectra were recorded with the 785-nm laser excitation. The main Raman shift peaks are annotated between 100 and 4,000 cm^{-1}. Fluids from the jars of catalog numbers (a) MNHN-AC-A-4851, A-5495, 1963-64 and 1898-56, with a photograph of the jars MNHN-AC- A-4851 and A-5495 ©M. Herbin and (b) MNHN-AC-1912-94 and 1935-169 with the corresponding photographs of the jars ©M. Herbin. Bold annotations indicate the main markers of formaldehyde and annotations with an embossed font correspond to minor traces of another fluid. Annotations in italics indicate water.

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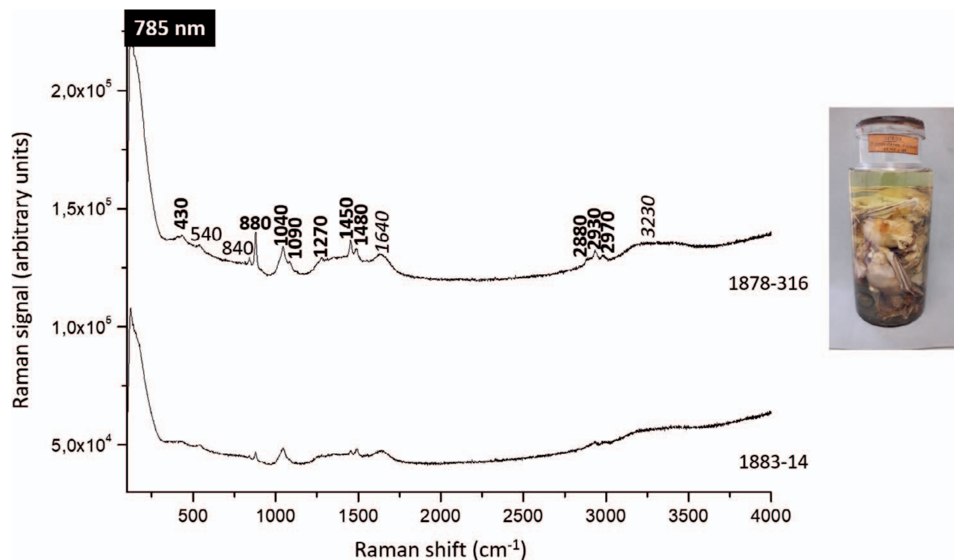

Figure 11. Raman spectra of real fluids for 785-nm laser excitation from the comparative anatomy fluid collection of the French National History Museum annotation of the main Raman shift peaks between 100 and 4,000 cm^{-1}. Fluids from the jars of catalog numbers MNHN-AC-1878-316 and 1883-14 with a photograph of the jars ©M. Herbin. Bold annotations indicate the main markers of ethanol. Annotations in italics indicate water.

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to the degradation of the copper eyelet of an inner label, could explain the green color of the solution.

Two analyzed fluids are exceptions (MNHN-AC-1878-316 and MNHN-AC-1883-14). Despite the influence of glass, the Raman bands of ethanol can be detected in their spectrum (Fig. 11). Some additional bands are present, but could not be attributed (at 540 and 840 cm^{-1}). Although the catalog and the yellow color of the fluid are consistent with the identification of ethanol in the case of the MNHN-AC-1883-14 jar, the catalog indicated a rehousing of the MNHN-AC-1878-316 specimen in 1913, which led to the assumption (based on practices in collections at that time) that the specimen was now in formaldehyde.

Quantification—Concentration of Ethanol and Formaldehyde

In addition to using Raman spectroscopy for fluid identification, we tested whether we could use the technique to determine the concentration of ethanol or formaldehyde in storage solutions.

Raman spectra were recorded for ethanol solutions prepared at several concentrations (Fig. 12a) ranging from 0% (ultrapure water) to 99.8% (absolute ethanol). Result show some bands that vary in intensity, area, and position as a function of concentration. More precisely, water-specific bands around 3,200 cm^{-1} increase with decreasing ethanol concentrations while ethanol-characteristic bands (C–H stretching at 2,880, 2,930, and 2,980 cm^{-1} and C–C stretching at 880 cm^{-1}) are more intense with increasing ethanol concentration (Numata et al. 2011). Quantification of ethanol concentration was attempted based on the bands' intensities. Yet, in the absence of an internal standard to normalize the data, we were unable to calibrate the ethanol concentration using either the intensity, the band area, or the full width at half maximum (FWHM) of the band.

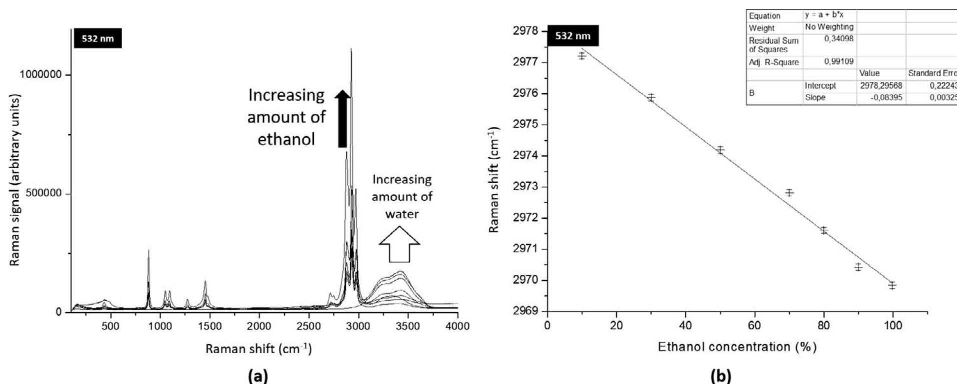

Figure 12. Estimation of ethanol concentration. (a) Raman spectra of standard solutions of ethanol at 0, 30, 50, 60, 70, 80, 90 and 99.8% ethanol for 532-nm laser excitation and (b) calibration curve of the position of the Raman peak around 2,970 cm^{-1} as a function of the ethanol content in the fluid. The insert gives the results of the linear regression. The calibration was obtained after deconvolution of Raman peaks and baseline subtraction.

However, not only were the bands' intensities changing with concentration, but also their positions. For instance, the band appearing at 2,969.5 cm^{-1} on the spectrum of 100% ethanol shifts up to 2,977.5 when ethanol concentration decreases to 10%. The position shifts can be used to determine ethanol concentrations with a calibration curve such as the one shown in Figure 12b. The precision of the measure depends on the quality of the fluid spectrum, which can be compromised by fluorescence or by the signal of the jar glass. In the case of specimens MNHN-AC-1878-316 and MNHN-AC-1883-14, despite some occurrence of fluorescence, we obtained estimates of ethanol concentration between 70% and 80% for MNHN-AC-1878-316 and 40–50% for MNHN-AC-1883-14. These results suggest that specimen MNHN-AC-1878-316 should be monitored more closely because the ethanol concentration in the jar is below 50%.

Formaldehyde appears more difficult to characterize. Figure 13 shows that its characteristic bands are no longer detectable below 1%. In contrast to ethanol, it is not possible to estimate the formaldehyde concentration of a fluid at such low levels (Delcroix et al. 2010). Residues of formaldehyde fixation will remain difficult to identify with *in situ* Raman spectrometry. Yet, if formaldehyde is present at a concentration higher than 1%, it can be detected without opening the jar (bands at 1,050, 1,490, 910, and 540 cm^{-1}) which means Raman spectroscopy could be used to evaluate the risks of formaldehyde exposure for staff and researchers.

Conclusions

Our study shows the power of Raman microspectroscopy for the management of modern and historic fluid collections. It is possible to use this tool to identify a variety of fluids without opening the jars. In the case of pure fluids, such as ethanol or formaldehyde, it is possible to estimate the concentration of the liquid. For more complex mixtures such as Kaiserling's solution or Bouin's solution, the major constituents used in the recipe can be identified.

This preliminary work will be continued to characterize other historic fluids and to detect dissolved biomolecules by further expanding the database of reference products. In addition, specific Raman methods should be tested for more difficult scenarios, such as

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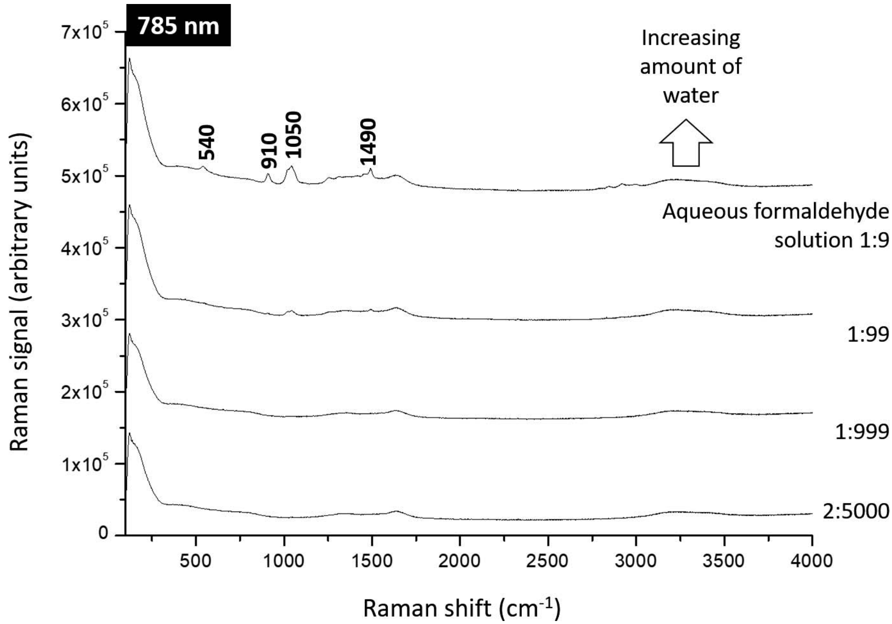

Figure 13. Detection limit of formaldehyde. Comparison of the Raman spectra of diluted aqueous formaldehyde solution for laser excitation at 785 nm, with annotation of the main Raman peaks between 100 and 4,000 cm^{-1}. The peaks in bold correspond to the main markers of formalin. Detection limit of formaldehyde. Comparison of the Raman spectra of 10%, 1%, 0.1%, and 0.04% aqueous formaldehyde solution for laser excitation at 785 nm, with annotation of the main Raman peaks between 100 and 4,000 cm^{-1}. The peaks in bold correspond to the main markers of formalin.

Raman SORS for the detection of fluids through opaque plastic containers (this has already been implemented in the pharmaceutical industry) or FT-Raman (with an excitation source at 1,064 nm) for fluids that become highly opaque over time. In addition, the study of the degradation of glass containers due to interactions with preservative fluids should be developed by parsing the data finely through deconvolution of the spectra.

Finally, we are developing a portable Raman microspectrometer to carry out all the studies described above for use directly in the collections rather than in the laboratory to avoid moving the jars.

Acknowledgments

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Résumé.—La conservation du milieu de conservation est un élément essentiel pour la bonne conservation des spécimens dans les collections fluides. Cependant, les conservateurs peuvent être altérés chimiquement au fil du temps ou être perdus par des processus tels que l'évaporation. Pour prendre soin des spécimens et les entretenir correctement, il est donc nécessaire de savoir quel fluide de conservation a été choisi. Le présent travail explore les possibilités offertes par la spectrométrie Raman pour une alternative rapide, non destructive et non invasive aux tests d'identification chimique, qui sont souvent limités à l'identification de simples fluides. Dans une première étape, les fluides ont été reconstitués et analysés dans de petits récipients en verre standard afin d'évaluer le potentiel de la technique. Ensuite, nous avons appliqué avec succès la procédure à des cas réels et nous avons envisagé son utilisation possible pour estimer la concentration d'éthanol et pour détecter de petites quantités de formaldéhyde (jusqu'à 1%). Les résultats démontrent la puissance de cette technique, qui ouvre de nouvelles possibilités pour la gestion des collections en fluide.

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