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SPECIMEN FIXATION WITHOUT FORMALIN IN NATURAL HISTORY COLLECTIONS: UTOPIA OR REALITY?

Keywords: formalin, ethanol, RCL2, FTIR-ATR, DSC, fluid collection, DNA

ABSTRACT

In the large diversity of natural history preparations, fluid collections are actually widely used by scientists for phylogeny studies through the exploitation of anatomical, chemical or molecular information. The quest for formalin substitutes has been motivated by its recent recognition as a carcinogenic agent; its use is beginning to be limited in museum as well as in university and laboratory preparations. In addition, formalin compromises a complete DNA recovery, essential to molecular biology investigations. The goal of this project was to understand the formalin fixation process on specimens in order to better understand its actions on tissue and biomolecules. In addition, some formalin substitutes were evaluated by studying collagen thermal stability using differential scanning calorimetry (DSC), change in collagen structure with Fourier transform infrared (FTIR-ATR), and DNA extraction yield.

RÉSUMÉ

Dans la vaste gamme de préparations d'histoire naturelle, les collections en fluide sont en réalité largement utilisées par les scientifiques pour mener des études phylogénétiques en exploitant les données anatomiques, chimiques ou moléculaires. La recherche de substituts au formol a été motivée par sa classification récente comme substance cancérigène ; son utilisation commence à être limitée dans les préparations pour les musées ainsi que pour les universités et les laboratoires. De plus, le formol compromet la restitution complète de l'ADN, essentielle pour les recherches en biologie moléculaire. Ce projet avait pour objectif d'étudier le processus de fixation du formol sur les spécimens afin de mieux comprendre son action sur les tissus et les biomolécules. Par ailleurs, cer-

INTRODUCTION

Natural history collections are the single most important source of primary information about the diversity of life on earth. They are massive repositories of information, whose value is increasingly recognized today, not only in biological researches such as systematic biology, phylogeny, inventory of biodiversity, species extinction or DNA barcoding, but also in areas such as environmental conservation, medicine and science education (Suarez 2004). Most specimens come to museums through field collecting. But, whatever their source, all specimens undergo some field and/or laboratory preparation prior to becoming part of a collection. In the large diversity of natural history preparation, spirit collections are actually widely used by scientists for studies. Fluid-preserved specimens are whole bodies of natural history specimens such as plants, invertebrates, small and medium vertebrates, or any parts or biological tissues thereof.

The preparation of a spirit specimen typically undergoes a two-step *modus operandi*. 'Fixation' is the initial stage, where the freshly dead specimen is stabilized by protein coagulation or by the fixative chemical combining with it, which prevents cellular lysis. Then the specimen is immersed in another fluid for 'long term preservation' to maintain tissues in a fixed state. It could be the same chemical type as the fixative. Fluids employed in the past for preparation are generally unknown since preparators have employed a great diversity of personal recipes. During the last decades of the 19th century, formalin has become an unsurpassed standard fixative in museum practice (Fox 1985). Recent health and preservation concerns provide motivation for phasing-out of formalin as a fixation medium for spirit collections, and also for cytopathological and histological preparations. The quest for formalin substitutes is motivated by two fundamental issues. Formalin is toxic; since it has been recognized as a carcinogenic agent, museums as well universities and laboratories have to apply new protective and safety procedures in its preparation. Recent research (Tang 2006) has also shown that formalin compromises a complete DNA recovery, which is essential to many research or molecular biology investigations and is the main focus in museums throughout the world.

In this study, different fluids were investigated by studying collagen thermal degradation by Differential Scannning Calorimetry (DSC), change in collagen structure with Fourier Transform Infrared (FTIR-ATR) and

tains substituts du formol ont été évalués en étudiant la stabilité thermique du collagène par calorimétrie différentielle à balayage, les changements de structure du collagène par spectrométrie infrarouge à transformée de Fourier par réflectance totale atténuée, et le rendement de l'extraction d'ADN.

RESUMEN

En la gran diversidad de las preparaciones de historia natural, los científicos utilizan de manera amplia hoy en día las colecciones de fluidos para estudios de filogenia, a través de la explotación de información anatómica, química o molecular. La búsqueda de sustitutos de formalina ha sido motivada por su reciente reconocimiento como agente cancerígeno; su uso se empieza a limitar en museos, universidades y preparaciones de laboratorio. Además, la formalina compromete una recuperación completa del ADN, esencial en las investigaciones de biología molecular. El objetivo de este proyecto era entender el proceso de fijado de la formalina en los especímenes, para entender mejor sus efectos en tejidos y biomoléculas. Se evaluaron además algunos sustitutos de la formalina estudiando la estabilidad térmica del colágeno con calorimetría diferencial de barrido (DSC, en sus siglas en inglés), cambios en la estructura de colágeno con infrarrojo con transformada de Fourier (FTIR-ATR), y la cantidad de extracción de ADN.

DNA extraction yield. Fluids were selected based on their historical use in museums: ethanol has been employed since the 16th century under the name of 'spirit of wine' to fix and preserve collections; formalin was widely used as a fixative in museums (Simmons 1995), and a recent French commercial formalin substitute named RCL2® CS100 has given good results in histology and DNA studies (Delfour 2006).

MATERIALS AND METHODS

Preparation of study collections

As natural history collections and their preparation are often not documented, a reference collection was prepared for this study to control all parameters of preparation and conservation. Rats were chosen as specimens because of the availability of a company which ensured the standards of breeding, preparation and delivery of rodents, and which certified the consanguinity of rats, facilitating comparisons with results obtained from other research fields in both collagen and DNA aspects.

For this study, entire fresh rats were defrosted and their skin was removed to assure the total penetration of the fixative. The fixation was done on a set of three rats for each fluid: formalin 10% in MiliQ water (3.7% formaldehyde by weight), pure ethanol and RCL2 for 24 hours at room temperature. For each treatment, the set was then transferred to a sealed opaque jar (polytetrafluoroethylene, or PTFE) in ethanol 80% (ethanol and MiliQ water 80:20 (v/v)) to be artificially aged at various temperatures (45, 55, 65, 75°C) (Figure 1). To observe the stability of the specimens for the treatments used, the skin was sampled before treatment (fresh skin, Time 0-'T_0') and after 24 hours fixation (Time 1-'T_1'), and finally during ageing (2-'T_{2h}', 4-'T_{4h}', 6-'T_{6h}' and 24 hours-'T_{24h}') to observe collagen thermal stability (DSC), change in collagen structure (FTIR-ATR) and to compare DNA yield.

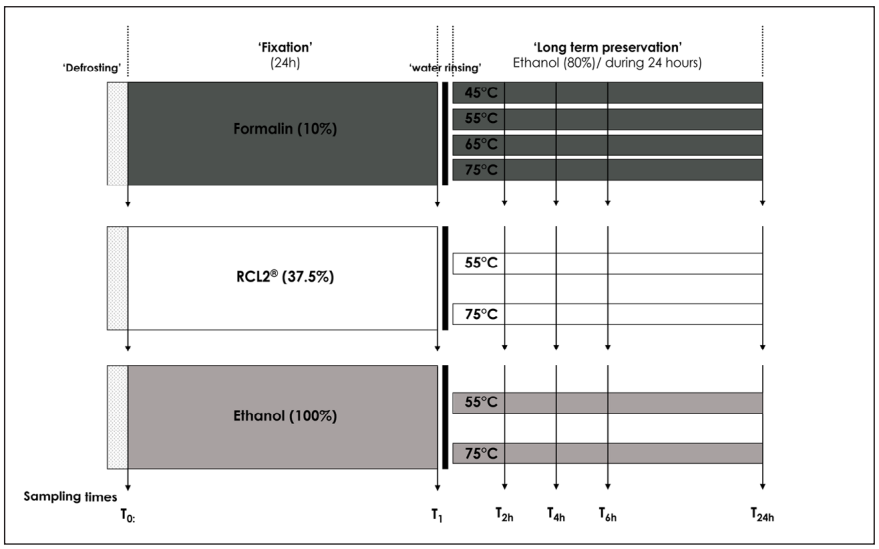

Figure 1
Protocol design of preparation and sampling on study collections

Differential Scanning Calorimetry (DSC)

Stability of the protein structure in the skin was studied by following the thermally induced denaturation of collagen to gelatin using DSC. Collagen denaturation, an endothermic reaction, can be followed during a linear increase of temperature, producing a curve on a thermogram from which both the heat required to cause collagen-gelatin phase transition (change in enthalpy, ΔH) and the onset temperature (of collagen denaturation) are observed. The values are used to investigate the role of non-covalent and covalent crosslinks by treatments in the stabilization of collagen triple helix. The evaluation of the degradation state of the collagen by DSC was applied to cultural heritage (Chahine 2000), but in the specific case of natural history collections no study has been done on spirit specimens.

DSC measurements were performed using a Perkin–Elmer DSC 7 calorimeter calibrated for temperature and heat flow with indium. For each time sampling, dehaired skin samples (n = 5 per rat) were weighed (2–3 mg) and immersed in pure water for 30 minutes, then hermetically sealed into aluminium crucibles (Chahine 2000). The two pans (reference and sample) are heated from 20 to 150°C at the constant rate of 10°C min^{-1}. Thermal changes such as peak denaturation, T_{peak} (°C), extrapolated onset temperature, T_{onset} (°C), and enthalpy, ΔH (J g^{-1}), were measured. Peak temperature is defined as the temperature at which the maximum rate of heat flow (dH/dT) passes into the sample. The extrapolated onset temperature is defined as the temperature corresponding to the intersection of the extrapolation of the tangent to the low temperature side of the melting peak and the baseline (Loke 1995).

Fourier Transform Infrared - Attenuated Total Reflectance (FTIR-ATR)

FTIR spectroscopy is a measurement of wavelength and intensity of the absorption of IR radiation by a sample and IR spectral data are usually interpreted in terms of vibrations of a structural repeat. In the specific case of protein, amide I (~1650 cm^{-1}) and II (~1550 cm^{-1}) bands are the most prominent vibrational bands of the protein backbone attributed respectively to the (ν(C=O)) and the (ν(C-N)) vibrations (Barth 2007). FTIR is commonly used to study changes in the secondary structure of collagen by decomposing the amide I and II bands for archaeological parchment (Odlyha 2009) or bones (Chadefaux 2009). For natural history specimens and especially those preserved in fluid, it appears that only Gentner (1999) applied FTIR to observe the effects of formalin and ethanol storage (on nematodes cuticule).

In this study, dehaired skin samples (0.5 cm^2) were placed in contact with a ZnSe crystal in the ATR cell and analyzed using a Thermo Nicolet FTIR-ATR spectrophotometer. All spectra are the sum of 128 scans collected at a resolution of 4 cm^{-1} over the range 4000-700 cm^{-1}. As skin is a complex and non-homogenous media, spectra could vary from area to area within a piece: for each sampling time, three skin samples per rat were analyzed

(n = 9). Fresh skin spectra were highly reproducible and their uniformity was indicative of a consistent depth penetration of the infrared radiation into the material. Spectra presented are an 'average spectrum' of nine spectra (with baseline correction). The ratio of the amide I and amide II bands was calculated. By using the relative ratio of the two bands, any intensity differences between the spectra were normalized. The separation ($\Delta\nu$) of the amide I and II bands was calculated; its increase signifying the denaturation of the collagen to gelatin (Doyle 1976).

DNA extraction yield

The molecular studies were done on 1 cm² skin samples. After tissue grinding, DNA was extracted with a Gentra® Puregene® Tissue kit (Qiagen). Quantities of nucleic acids were defined by dosage with a spectrophotometer NanoDrop 1000 (ThermoFisher Scientific). DNA extraction yield is measured as the square of the weight (µg DNA / mg tissue) of nucleic acids extracted. Results gave information on the capacity of liberation of nucleic acids from the tissue after its fixation treatment and along its long term preservation in ethanol.

DNA contamination ($A_{260/280}$ and $A_{260/230}$ ratios)

The quality of DNA extracted was evaluated by measuring the absorbance at 230 nm (organic compounds and other contaminants), 260 nm (nucleic acids) and 280 nm (proteins) with spectrophotometer NanoDrop 1000 (ThermoFischer Scientific). For minimal contamination, the absorbance ratios $A_{260/280}$ and $A_{260/230}$ should be between 1.7–1.9 and around 1.8 respectively. They were determined using software associated with the equipment (ND-1000 3.3, NanoDrop Technologies). Good ratios indicate that the subsequent use of DNA is useful.

RESULTS AND DISCUSSION

Collagen thermal stability (DSC)

Fresh (T_0) and formalin treated skin (T_1) thermograms showed an increase of T_{onset} after formalin fixation from (57.50 ± 1.40)°C for untreated skin to (84.90 ± 1.76)°C with enthalpy values of shrinkage from (2.6 ± 0.38) J g^{-1} to (4.7 ± 0.73) J g^{-1} (Figure 2). Formalin fixation enhanced hydrothermal stability of collagen around 30°C was observed by Gustavson (1947) due to new crosslink formation (methylene bridge). For ethanol solution aged material, T_{onset} slowly decreased with ageing. This is more pronounced at higher ageing temperatures: from 85.30 ($T_{2h\text{-}45°}$) to 85 ($T_{6h\text{-}45°}$), 84.10 ($T_{2h\text{-}55°}$) to 80.40 ($T_{6h\text{-}55°}$) and 81.00 ($T_{2h\text{-}65°}$) to 75.40 ($T_{6h\text{-}65°}$) (Figure 3). However, the thermal stability of the skin was still high, despite the drastic conditions, thanks to crosslinking made by the aldehyde (Figure 2). Skin fixed in ethanol (T_1) had the same T_{onset} when compared with fresh skin (T_0) (Figure 3). Despite ageing at 55°C, the skin preserved in ethanol retained its hydrothermal stability from T_{2h} to T_{24h}. Nevertheless, treatment at 75°C

dramatically affected the collagen, with the lack of peaks on thermograms revealing that the collagen was completely dissolved under the ageing conditions. Skins fixed with in RCL2 gave no peak on thermograms, indicating an alteration of the collagen by the fixative.

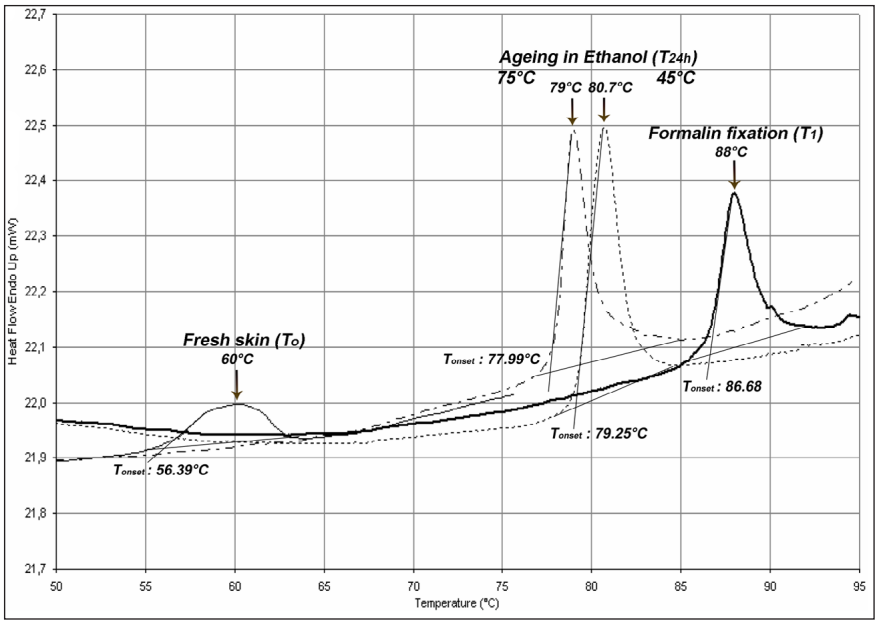

Figure 2

Thermograms of fresh skin T_0 (solid), formalin skin T_1 (solid bold) then ageing in ethanol $T_{24h\text{-}45°C}$ (dash) and $T_{24h\text{-}75°C}$ (dash point)

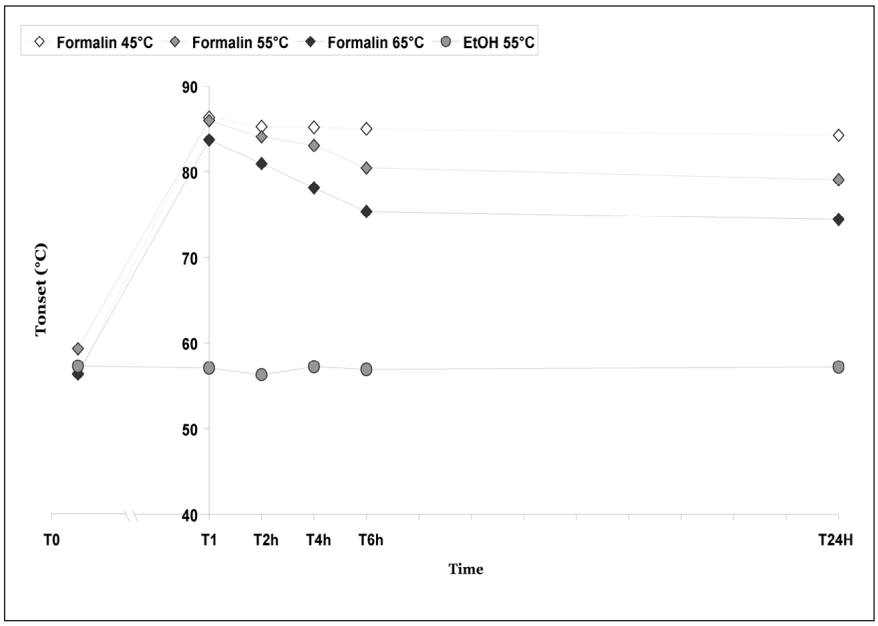

Figure 3

Results of T_{onset} obtained by DSC for formalin and ethanol series

The triple-helical structure of the collagen molecule is held together by hydrogen bonds. Formalin, being an aqueous solution, contains water. It creates a network by covalent crosslink, trapping water molecules within. In the case of pure ethanol, water present in the tissue is evacuated, thereby 'drying' the tissue and creating a spatial conformation which reduces inter- and intramolecular spaces within the collagen, and easily making

new molecular interactions which stabilize the structure. The hydroxyl group of amino acids (hydroxyproline) cannot form hydrogen bonds, but stabilizes the triple helix by the formation of hydrogen bond 'water bridges'. However, it is possible for the hydroxyl group to form hydrogen bonds with a backbone group of other molecule when adjacent molecules are close enough. Reduced hydration causes increased temperature stability (Miles 1995). Nevertheless, this arrangement and its 'solidity' reached its limits in drastic conditions such as high temperature, while formalin crosslinking was more resistant.

RCL2, as a mixture of acetic acid and ethanol, utilizes polarity and ionic interactions of polypeptide chains. Apparently, the acid pH (ca. 3) of the solution partly solubilizes the collagen and its structural network. Even after transferring specimens from RCL2 to ethanol, all thermograms still presented no signal, indicating that an irreversible degradation of collagen had occurred.

Structure of collagen (FTIR-ATR)

FTIR spectra from fresh and formalin-treated skins are presented (Figure 4). Peak picking was performed on the 1800–900 cm^{-1} fingerprint region. Absorption features at 1454, 1400, 1338, 1280, 1240 and 1205 cm^{-1} may be attributed to the $\delta(CH_2)$ and $\delta(CH_3)$ wagging and deformation, and ν(C-N) and δ(N-H) stretching absorptions of collagen (Belbachir 2009). Amide I and II absorptions were revealed at 1632 cm^{-1} and 1548 cm^{-1} respectively for untreated skin. Spectra exhibited absorptions at 1035 cm^{-1} and 1080 cm^{-1} which arise from the ν(C-O) and ν(C-O-C) absorptions of the carbohydrate moieties. Skin fixed in formalin presented similar spectra which overlaid the fresh skin one with no significant shift in the position of

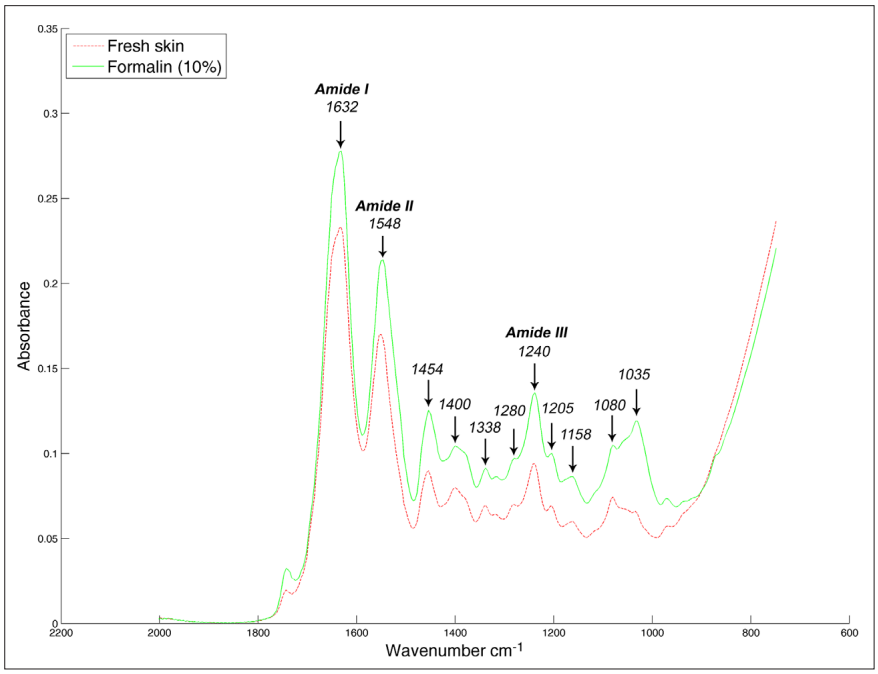

Figure 4
Fourier-Transform Infrared spectra of fresh T_0 and formalin skins T_1 (2000-800 cm^{-1})

the amide I (1633 cm^{-1}) and amide II (1547 cm^{-1}) absorptions. An increase in the peak intensity of amides I and II was noticed, passing respectively from 0.261 (T_0) to 0.275 (T_1) and 0.201 (T_0) to 0.210 (T_1). This is related to formalin crosslinks that appeared as a true fixative since it prevented the cell lysis (Péquignot 2006) and 'locked in' the secondary structure of the protein. During ageing in ethanol, the intensity of amide I and II bands decreased. The shift in the amide I band position to lower wavenumbers and the loss of its sharpness is a result of ethanol-aged induced protein denaturation (Odlyha 2009). Nevertheless, the uniformity of the spectrum indicates that formalin preserved the collagen structure even under drastic treatments ($T_{24h\text{-}75°C}$) (Figure 6). Ethanol fixation increased the absorbance of amide I and II bands in comparison with fresh skin respectively +0.043 (I) and +0.053 (II), but lower than formalin (Figure 5). A shift in lower wavenumbers was observed for amide I and, more significantly, for amide II (-3.9 cm^{-1}). The presence of ethanol inside the tissue was characterized by carbohydrate absorbance bands around 1080–1050 cm^{-1}. For RCL2 samples, the intensity of the peaks of amides I and II decreased from 0.283 (To) to 0.260 (T_1) and from 0.225 to 0.194 respectively (Figure 6). Furthermore, an important shift appeared for both peaks: amide I (4 cm^{-1}) and amide II (5.6 cm^{-1}). In addition, the $\Delta\nu$ increased from 84.4 (T_0) to 92.30 (T_1), revealing a transformation of collagen into gelatin (Table 1) (Derrick 1991). This denaturation induced by RCL2 was noticed on the whole spectra, especially the amide III band. Transferring in ethanol redefines the FTIR spectra, in particular the amide III band. Nevertheless, the stability of the spectra yields proportionally with thermal ageing. This phenomenon intensified for the 75°C series. This degradation could be linked to the oxidation of the polypeptide chain, resulting in the formation of carbonyl

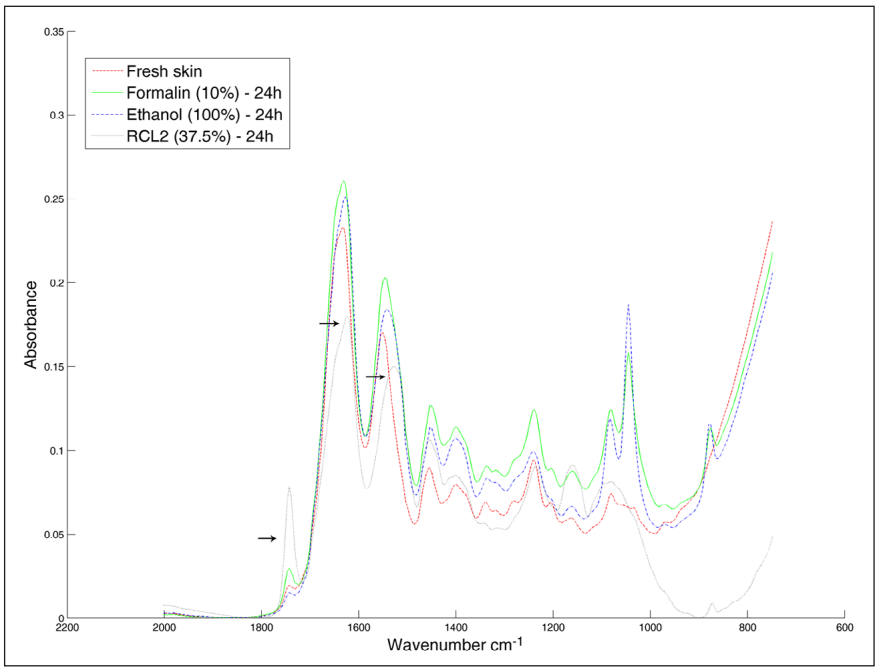

Figure 5

Fourier-Transform Infrared spectra of fresh skin T_0 and skins fixed T_1 with formalin, ethanol and RCL2 (2000-800 cm^{-1})

Table 1

Amide I and II band ratio (Ratio I/II) and change in position between the two amide bands (Δv) for the three fluids series and along the artificial ageing treatment

	45 °C		55 °C		65 °C		75 °C	
	Ratio I/II	ΔV	Ratio I/II	ΔV	Ratio I/II	ΔV	Ratio I/II	ΔV
Formalin								
To	1.299	81.7	1.371	80,0	1.321	80.9	1.320	83.2
T1	1.310	86.9	1.308	87.2	1.317	86.4	1.299	82.6
2h	1.337	83.7	1.276	83.3	1.273	83.9	1.289	83.6
4h	1.306	81.6	1.260	87.8	1.303	83.7	1.306	83.8
6h	1.302	86.1	1.249	85.4	1.307	83.5	1.276	86.8
24h	1.264	85.9	1.262	82.6	1.291	86.2	1.286	85.7
Ethanol								
To			1.387	80.9			1.350	83.2
T1			1.218	86.4			1.228	82.6
2h			1.244	83.9			1.335	83.6
4h			1.209	83.7			1.355	83.8
6h			1.237	83.5			1.338	86.8
24h			1.253	86.2			1.364	85.7
RCL2								
To			1.258	83.6			1.344	84.4
T1			1.340	84.4			1.541	92.3
2h			1.304	82.6			1.231	87.2
4h			1.297	81.1			1.205	90.6
6h			1.370	82.1			1.187	86.6
24h			1.363	83.5			1.200	96.6

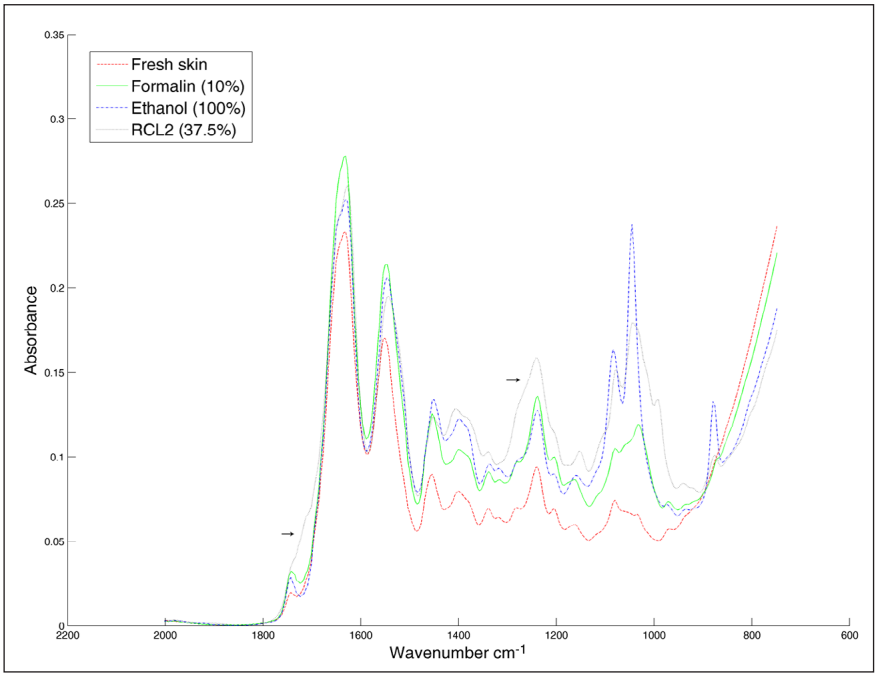

Figure 6

Fourier-Transform Infrared spectra of fresh skin T_0 and skins fixed with formalin, ethanol and RCL2, artificially ageing $T_{24h\text{-}75°C}$ (2000-800 cm^{-1})

compounds observed at 1700–1750 cm^{-1}, or as a slight shoulder appeared on the amide I band (Figures 5, 6) (Badea 2008).

DNA extraction yield and purity

Ability to extract DNA depends on the nature of the fixative used. Formalin induces a drastic and significant loss of the extraction yield along the ageing (Figure 7). Alternatively, alcohol-based fixatives (ethanol and RCL2) do not affect DNA liberation; similar levels of extraction yields are

obtained along the preservation treatment. Ratios of purity indicate that no contamination can be detected after alcohol-based fixative treatments; only a clear diminution of the $A_{230/260}$ ratio is obtained after formalin fixation (data not shown). Nevertheless, due to the loss of DNA liberation, these values cannot be associated with definite organic contamination. The effects described here can be associated with the chemical properties of the tested fluids. Unlike alcohol-based fixatives, formalin creates cross-linking and chemical modifications which impede DNA liberation (Sririnavasan 2002, Tang 2006). Of the evaluated criteria, alcohol-based fixatives are better for DNA extraction from fluid-preserved collections.

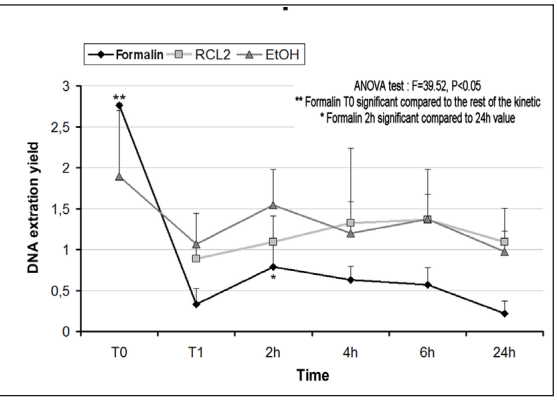

Figure 7
Average DNA extraction yield from fresh skin T_0, skin fixed with the three fixatives T_1 and artificially ageing $T_{2\text{-}24h}$ at 55 and 75°C

CONCLUSIONS

This study on natural history collections preserved in fluid is the first characterization of effects by fixatives at various levels. Among the results, it is possible to note various answers to the process of fixation: formalin is better than ethanol and RCL2 for fixation and preservation of collagen, but it is less successful for DNA availability for subsequent studies.

Finding a formalin substitute can be difficult, since each fluid by its chemical nature has positive and negative effects. A balance needs to be found to optimize the preservation of all potential information contained in a specimen preserved in fluid. Future research needs to be done on other substitutes or on the adaptation of formalin practices.

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MATERIALS LIST

RCL2® CS 100 (Alphelys)
Formalin '37% Formaldehyde /10-15 % Methanol' (Sigma–Aldrich)

Absolute ethanol GPR Rectapur (VWR)
Rat 'Long Evans, RjOrl:LE' / AFAQ Iso 9001:2000 (Janvier S.A.)