SALTING OUT: A SIMPLE AND RELIABLE METHOD TO DISTINGUISH BETWEEN COMMON FLUID PRESERVATIVES AND ESTIMATE ALCOHOL CONCENTRATION

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Abstract.—This paper details the salting-out method, which uses the salts potassium carbonate and sodium chloride to distinguish between the three most commonly used fluid preservatives: ethanol, isopropanol, and formalin.

A summary of other methods to identify fluid preservative type and a review of the salting-out method published by Mayfield (2013, Distinguishing between ethanol and isopropanol in natural history collection fluid storage, *Society for the Preservation of Natural History Collections*, https://spnhc.org/wp-content/uploads/2018/11/Mayfieldfinalwithtablechanges.pdf) are provided. A new salting-out method is presented, which requires a small fluid sample (2–4 ml). It is simple, quick, and relatively inexpensive to implement, making it a viable method to distinguish between common fluid preservatives. The materials and equipment for the salting-out test cost just over $100 US, and tests take approximately 3 minutes per container.

Results of testing on known concentrations and combinations of ethanol, isopropanol, and formalin (a solution of formaldehyde in water) and on samples of fluid preservatives from specimen containers in the Smithsonian National Museum of Natural History and Bernice Pauahi Bishop Museum collections are presented. The results of salting-out tests have been verified by direct analysis in real time mass spectrometry (DART-MS) (Cody et al., 2005, Versatile new ion source for the analysis of materials in open air under ambient conditions, *Analytical Chemistry* 77(8):2297–302), which confirmed the results of salting-out tests but also highlighted some limitations, particularly when combinations of fluid preservative are encountered.

Key words.—alcohol, DART-MS, fluid preservative, form, salting-out.

Introduction

Fluids have been used as a method for preserving biological specimens since the late 17th century (Moore 1999, Simmons 2014). Many different types of fluid preservatives as well as additives have been used; for details please refer to Simmons (2014) and Moore (1999). Currently, the most commonly used fluid preservatives for preservation of biological specimens are ethanol (undenatured or denatured), isopropanol, and formalin (Simmons 2001). Glycerol is also used but can be easily distinguished by its high viscosity compared with other fluid preservatives.

The salting-out method presented in this article is a simple, quick, and relatively inexpensive test that uses the salts potassium carbonate (K_2CO_3) or sodium chloride (NaCl) to distinguish among commonly used fluid preservatives: ethanol (CH_3CH_2OH) (EtOH), isopropanol [$(CH_3)_2CHOH$] (IPA), and formalin (CH_2O in water). Some other methods to identify fluid preservatives rely on differences in density or odor, but the salting-out method exploits differences in solubility.

The optimized salting-out method builds upon research published by Mayfield (2013). It helps determine whether a fluid sample is ethanol, isopropanol, or an aqueous solution. For the purpose of this test, the aqueous-based solution is considered to be formalin. What is commonly referred to as "10% formalin" is 3.7% weight/weight (w/w) or 4% weight/volume (w/v) formaldehyde gas in water and is an aqueous solution. "10%" refers to the dilution

factor of 1:9—one part 37% w/w or 40% w/v formaldehyde stock solution diluted with nine parts water.

Known concentrations and combinations of ethanol, isopropanol, neutral buffered formalin, and unbuffered formalin were tested with the salting-out method to determine which concentrations and combinations salted out with potassium carbonate or sodium chloride and which did not. Following this, fluid samples from fluid specimen containers from the Smithsonian National Museum of Natural History (USNM) and the Bernice Pauahi Bishop Museum (BPBM) collections were tested. Direct analysis in real time mass spectrometry (DART-MS) (Cody et al. 2005) was used to verify the results of the simpler salting-out method. The results of testing with both the salting-out method and DART-MS are presented and discussed, along with limitations and notes on the salting-out method, costs, and health and safety precautions.

Background

Identification of Fluid Preservatives

While ideally the individual containers and collection records for specimens in natural history collections would contain a label or record of the type of fluid used as the preservative, this is rarely done in practice, particularly in older collections. Simmons (2014) notes that some collections use different types of containers or lids to distinguish fluid preservatives, but using a label is recommended as the most effective way to document the fluid type.

There are several methods to determine an unknown type of fluid preservative from a fluid specimen container. Pure ethanol, isopropanol, and formalin all have distinct odors and can be distinguished by smell, though this method is strongly discouraged due to potential exposure to toxic or carcinogenic compounds (Waller and McAllister 1986, Simmons 2014). Fluid density can be used to determine fluid type as detailed in Carter (1994) and Moore (1999). For example, a hydrometer can be used to determine density, as described in Simmons (2014); however, this requires comparatively large volumes of fluid to float a hydrometer as well as corrections for temperature variations. Fluid specimen containers can be small (<10 ml) and do not always contain the required volume to take these measurements. The use of digital density meters has been recommended for some time to determine alcohol concentration quickly (Carter 1994, Moore 1999, Simmons 2014) because they require a much smaller sample size (2 ml) and are more precise. A digital density meter is, however, a costly piece of equipment that is not accessible for all collecting institutions. Furthermore, it can be challenging to interpret the readings obtained from a digital density meter, particularly when trying to distinguish ethanol from isopropanol or formalin from a low concentration alcohol, as may be found in older fluid collections.

An alternative method based on fluid density differences was proposed by Moore (1999) in which a self-made gravimetric device is used to distinguish between fluid preservatives based on fluid density. This method can only distinguish formalin from alcohol solutions above 55% concentration and requires plastic pin heads that float in alcohol, which are now difficult to source. It is likely that lower concentrations of alcohol may be encountered in unknown fluid preservatives, particularly where there has been alcohol evaporation. The commercial version of this idea, called Alcomon Indicator System (Alcomon Company, www.alcomon.com), consists of floating discs and relies on fluid density to determine ethanol concentration as the indicators sink or float.

Instrumental analysis methods, such as gas chromatography–mass spectrometry (GC-MS), high performance liquid chromotography (HPLC) (MacLeod 2008), or Fourier-transform infrared spectroscopy (FTIR) (Kay and Ivison 2003) can be used to determine fluid preservative type, but these methods are beyond the budget capacity of many institutions, and obtaining results can be time consuming (Simmons 2014). Qualified staff are generally required to interpret the complex results. DART-MS, with its ready ability to thermally desorb volatile materials and insensitivity to matrix effects compared with the other techniques described, is used herein only as a confirmatory analysis for the salting-out test.

There are many published methods to determine if formalin or formaldehyde is present in fluid preservatives, which could be used in conjunction with the salting-out method presented in this article. These include leuco-fuchin indicator test strips to distinguish alcohol solutions from formalin (Waller and McAllister 1986), methods to test if aldehydes are present, such as Schiff reagent (Moore 2009), commercial formaldehyde test strips (van Dam 2009, Simmons 2014, Finkelde and Waller 2019, 2021), and titration methods (Simmons and Waller 1994, Waller and Simmons 2003, Finkelde and Waller 2019, 2021).

Mayfield (2013) identified salting out as a method to distinguish between ethanol and isopropanol and suggested this as a viable method of identification of fluid preservatives. Many institutions do not have resources for some of the methods listed above, such as instrumental analysis or a digital density meter, and there can be challenges in using density alone to determine fluid type. The salting-out method offers an alternative to determine fluid preservative type when it is unknown and can also be used to give an approximation of alcohol concentration.

Salting Out

The process of salting out is the separation of an organic phase from an aqueous phase by the addition of salt (Shakhashiri 1989, Smith 1996). Smith (1996:1) states that "weak intermolecular forces (e.g., hydrogen bonds) between organic molecules or nonelectrolytes and water can be easily disrupted by the hydration of the electrolytes." Shakhashiri (1989:267) elaborates on this, stating that "the phenomenon of salting out is common when salts are added to aqueous solutions of nonelectrolytes. From a molecular standpoint, the strong hydration of the electrolyte ties up the water and makes it unavailable for the relatively weak hydrogen bonding with the nonelectrolyte. Because it is the hydrogen bonding between water and the nonelectrolyte that keeps it in solution, the solubility of the nonelectrolyte decreases when the hydrogen bonding is disturbed." Since alcohol-based fluid preservatives are solutions of alcohol with water, the salting-out method is a viable process to distinguish between them, since salts disrupt the hydrogen bonding. Water and ethanol can be made immiscible by the addition of potassium carbonate (Smith 1996). Water and isopropanol can be made immiscible by the addition of potassium carbonate or sodium chloride (Mayfield 2013). The effects of electrolytes (salts or acids) on formaldehyde solubility can be either a decrease in solubility with potential for salting out or an increase in solubility resulting in salting in (Ma et al. 2018). In the experiments described in this paper, no instances of salting out of formalin were observed with potassium carbonate.

Mayfield's Salting-Out Method

Mayfield (2013) exploited the salting-out property as a way to distinguish between ethanol and isopropanol in natural history fluid collections. Mayfield used sodium chloride (NaCl) and potassium carbonate (K_2CO_3) to salt out 50% isopropanol, and potassium carbonate (K_2CO_3) to salt out 70% ethanol (pure and denatured). A distinction could be

made between the two fluid types, since ethanol did not salt out with sodium chloride. Mayfield used a large amount of fluid (20 ml per 3 g of salt) in her tests. The article noted that 10% buffered formalin did not salt out with either salt. Fortunately, common contaminants to the alcohols, such as formalin or glycerin, did not affect the salting out of the alcohol. Mayfield found that with ethanol and isopropanol combinations, once the concentration of ethanol in total volume of alcohol exceeded 25.9%, the salting out with sodium chloride no longer occurred. However, Mayfield's salting-out method has some issues. First, the sample sizes were quite large: 20 to 40 ml is a large volume to remove from a small fluid specimen container. Second, tests were not conducted on lower concentrations of alcohols, which can sometimes be found in fluid collections, particularly when evaporation has occurred.

Materials and Methods

All salting-out tests were conducted within a fume hood wearing appropriate personal protective equipment, as detailed in Hawks et al. (2010) and Simmons (2014, 2019).

Initial Testing with Mayfield's Fluid-to-Salt Ratio

Initial tests were conducted on various concentrations of ethanol and isopropanol using the method published in Mayfield (2013), but with a smaller fluid sample. Instead of using a fluid sample of 20 ml with 3 g of each salt, a 2-ml fluid sample was tested with 0.30 g of each salt. For all tests, the salt was measured using a Mettler PC 220 analytical scale, capable of measuring to three decimal places.

Salting-Out Method Optimization

The procedure has been adapted from those published in Mayfield (2013), North Carolina State University Department of Chemistry (n.d.), and Smith (1996) to use a smaller sample size of 2 ml instead of 20 ml. The fluid-to-salt ratio has also been altered to a 2-ml fluid sample and 0.60 g or 0.90 g of salt, depending on the alcohol concentration. The method is broken down into three tests: Test A, Test B, and Test C. The materials, suppliers, and cost are detailed in Appendix 1. Refer to Appendix 2 for a ready-to-use methodology and flow chart diagram. Initial preparations of test vials containing 0.60 g of K_2CO_3 or 0.60 g of NaCl were made following the method in Appendix 2.

- Test A: A 2 ml fluid sample was removed with a syringe and deposited in a vial containing 0.60 g K_2CO_3. One drop of bromothymol blue indicator was added to the sample solution, and the lid was secured. The vial was shaken for 30 seconds, then allowed to stand for 30 seconds.
- Test B: A 2 ml fluid sample was removed with a syringe and deposited in a vial containing 0.60 g NaCl. One drop of bromothymol blue indicator was added to the sample solution, and the lid was secured. The vial was shaken for 30 seconds, then allowed to stand for 30 seconds.
- Test C: If the sample solution did not salt out in Test A, the following steps were undertaken. A scale, weighing paper, and spatula were used to weigh out 0.30 g K_2CO_3. Working under a fume hood, the lid was removed from the Test A sample vial, and the 0.30 g K_2CO_3 was carefully poured into the vial (for a total of 0.90 g K_2CO_3 in the sample solution). The lid was secured, and the vial was shaken for 30 seconds, then allowed to stand for 30 seconds.

Further notes on the salting-out method provided by the author, including notes on the color variations due to the bromothymol blue indicator, are available in Appendices 2 and 3.

Estimating Alcohol Concentration

An estimate of the alcohol concentration can be made following Test A by measuring (in millimeters) the relative height of the alcohol layer (the colored layer on top) to the total height of the liquid volume, by using the following equation:

$$\text{Alcohol concentration} = \frac{\text{colored layer height}}{\text{liquid height}} \times 100.$$

Testing the Optimized Salting-Out Method

Known samples and dilutions of ethanol (200 Proof ACS/USP Grade, Pharmco; Aaper, Brookfield, CT), isopropanol (70% Walgreens, Deerfield, IL), neutral buffered formalin (NBF), and unbuffered formalin were tested with the methods above. The 10% formalin solutions were prepared by diluting 40% w/v USP grade formaldehyde solution (Fisher Scientific, Fair Lawn, NJ) 1:9 with reverse osmosis water. The 10% NBF was buffered with 4 g/L sodium phosphate monobasic monohydrate (Fisher Chemical, Hampton, NH) and 6.5 g/L dibasic sodium phosphate anhydrous (Sigma Chemical Company, St. Louis, MO), as described in Simmons (2014).

Combinations of NBF with 70% ethanol or 50% isopropanol were tested. The percentage of NBF tested was consistent with residual amounts found in alcohol preservative fluids from initial fixation (Waller and McAllister 1986, Waller and Simmons 2003).

Combinations of ethanol and isopropanol were prepared from 70% alcohol concentrations of both alcohols, ranging from 5% to 95% of each alcohol, then these combinations were tested with the optimized salting-out method.

Following the testing on known concentrations and combinations, samples from the USNM and BPBM collections were tested. The specimens were thought to be preserved in "ethanol," "isopropanol," or "neutral buffered formalin," respectively. Ten samples were taken from "ethanol" and "isopropanol" fluid preservative containers, and twenty from the "neutral buffered formalin" containers, as the first round of testing on NBF samples demonstrated some limitations of the test.

Direct Analysis in Real Time Mass Spectrometry

Direct analysis in real time mass spectrometry (DART-MS) was used to verify the results of the salting-out tests. A DART 100 probe operated with an SVP controller and Vapur interface (IonSense, Saugus, MA) was mounted in transmission mode in front of an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Sealed glass capillaries were dipped into samples of the fluids and mounted in front of the DART probe. The LTQ was operated in low-mass mode with three microscans and 50-millisecond maximum fill time per scan. Pure solvents were purchased for analysis: 200 Proof ACS/USP Grade ethanol (Pharmco – Aaper); 70% isopropanol (Walgreens); "10%" neutral buffered formalin, prepared from 40% w/v USP grade formaldehyde solution (Fisher Scientific), which was diluted and buffered following the procedure listed above, and liquid chromatography–mass spectrometry (LCMS) grade methanol (MeOH, used as a stabilizer in the formalin) (Fisher Chemical). Pure and mixed solvent samples were analyzed to identify the mass spectral peaks for each fluid type and peak abundance

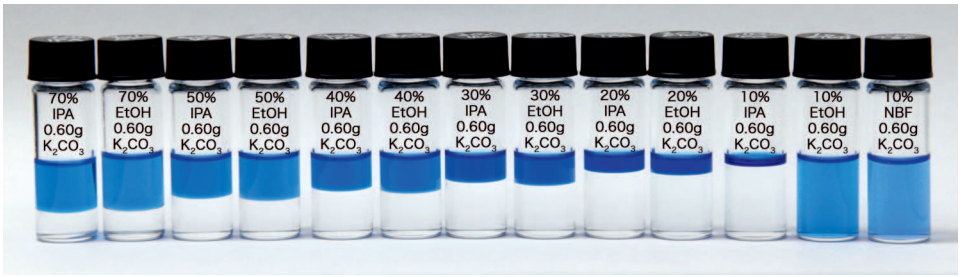

Figure 1. Photograph of salting-out Test A (with 0.60 g K_2CO_3 and one drop bromothymol blue indicator in 2 ml of fluid) (left to right): 70% IPA; 70% EtOH; 50% IPA; 50% EtOH; 40% IPA; 40% EtOH; 30% IPA; 30% EtOH; 20% IPA; 20% EtOH; 10% IPA; 10% EtOH; 10% NBF (© I. Finkelde).

ratios in mixtures. A solution of 37% w/w ACS reagent formaldehyde (Sigma Aldrich), the concentration in 10% NBF, was used to create the EtOH/MeOH peak abundance ratio. Samples from the specimen containers that were thought to contain the fluid preservatives "ethanol," "isopropanol," and "neutral buffered formalin," were then analyzed using the same technique.

Alcohol Concentration

The concentration of the alcohol from the "ethanol" fluid preservative containers was measured with an Anton Paar DMA 35 Digital Density Meter (Anton Paar, GmbH, Graz, Austria).

Results and Discussion

Initial Testing with Mayfield's Fluid-to-Salt Ratio

When conducting initial tests using Mayfield's fluid-to-salt ratio (2 mL fluid sample with 0.30 g salt) with K_2CO_3 on low alcohol concentrations it was found that concentrations of alcohol below 30% for ethanol and below 20% for isopropanol did not salt-out. This is the reason a new fluid-to-salt ratio was developed and tested. It is likely that lower concentrations of alcohol may be found in collections containing older fluid specimens, since alcohol may have evaporated from containers with poor seals.

Testing the Optimized Salting-Out Method

The results of testing the optimized salting-out method outlined above are detailed in Tables 1 and 2.

Distinguishing Alcohols from Aqueous Solutions

When following the procedure outlined in Test A, salting-out was observed for most concentrations of ethanol and isopropanol, as shown in Figure 1, with a clear layer on the bottom and colored alcohol layer on top. The 10% ethanol did not salt-out and would therefore be interpreted as an aqueous solution (formalin). However, Test C will cause the 10% ethanol to salt-out (Fig. 2), and in this manner low concentrations of alcohol can be distinguished from aqueous solutions. This method works for concentrations above 6% ethanol, below which they did not salt-out and may incorrectly be interpreted as formalin. If a fluid preservative is found to have low alcohol content, it is recommended to select the appropriate alcohol for the specimen (ethanol or isopropanol) and step the specimen

Table 1. Results of testing the optimized salting-out method on known concentrations and combinations of ethanol, isopropanol, and formalin.

Known sample (2 ml) and concentration	salted-out with Test A (0.60 g K_2CO_3)	Salted-out with Test B (0.60 g NaCl)	Salted-out with Test C (total 0.90 g K_2CO_3)	Fluid type interpreted from salting-out tests
Ethanol (EtOH)				
70%	Yes	No	—	Ethanol
50%	Yes	No	—	Ethanol
40%	Yes	No	—	Ethanol
30%	Yes	No	—	Ethanol
20%	Yes	No	—	Ethanol
10%	No	No	Yes	Ethanol or low concentration alcohol
9%, 8%, 7%	No	—	Yes	Ethanol or low concentration alcohol
6% and lower	No	—	No	Formalin
Isopropanol (IPA)				
70%	Yes	Yes	—	Isopropanol
50%	Yes	Yes	—	Isopropanol
40%	Yes	Yes	—	Isopropanol
30%	Yes	Yes	—	Isopropanol
20%	Yes	Yes	—	Isopropanol
10%	Yes	No	—	Ethanol or low concentration alcohol
Neutral buffered formalin (NBF) or unbuffered formalin (UF) (tested separately)				
10%	No	—	No	Formalin
5%	No	—	No	Formalin
2%	No	—	No	Formalin
1%	No	—	No	Formalin
Combinations of 70% EtOH or 50% IPA with NBF				
70% EtOH with 2% NBF	Yes	No	—	Ethanol
70% EtOH with 1% NBF	Yes	No	—	Ethanol
70% EtOH with 0.5% NBF	Yes	No	—	Ethanol
70% EtOH with 0.2% NBF	Yes	No	—	Ethanol
70% EtOH with 0.1% NBF	Yes	No	—	Ethanol
50% IPA with 2% NBF	Yes	Yes	—	Isopropanol
50% IPA with 1% NBF	Yes	Yes	—	Isopropanol
50% IPA with 0.5% NBF	Yes	Yes	—	Isopropanol
50% IPA with 0.2% NBF	Yes	Yes	—	Isopropanol
50% IPA with 0.1% NBF	Yes	Yes	—	Isopropanol
Combinations of 70% EtOH and 70% IPA (% EtOH and IPA in total volume of alcohol = 70%)				
5% EtOH:95% IPA	Yes	Yes	—	Isopropanol
10% EtOH:90% IPA	Yes	Yes	—	Isopropanol
15% EtOH:85% IPA	Yes	Yes	—	Isopropanol
20% EtOH:80% IPA	Yes	Yes	—	Isopropanol
25% EtOH:75% IPA	Yes	Yes	—	Isopropanol
30% EtOH:70% IPA	Yes	No	—	Ethanol

Table 2. Results of testing the optimized salting-out method on fluid preservative samples from specimen containers.

Sample no. (2 mL) and specimen container	salted-out with Test A (0.60 g K_2CO_3)	Salted-out with Test B (0.60 g NaCl)	Salted-out with Test C (total 0.90 g K_2CO_3)	Fluid type determined by salting-out tests
Samples from "ethanol" fluid preservatives[a]				
1: USNM 168326	Yes	No	—	Ethanol
2: USNM 102831	Yes	No	—	Ethanol
3: USNM 426199	Yes	No	—	Ethanol
4: USNM 68249	Yes	No	—	Ethanol
5: USNM 396340	Yes	No	—	Ethanol
6: USNM 102366	Yes	No	—	Ethanol
7: USNM 93896	Yes	No	—	Ethanol
8: USNM 245099	Yes	No	—	Ethanol
9: USNM 351717	Yes	No	—	Ethanol
10: USNM 174947	Yes	No	—	Ethanol
Samples from "isopropanol" fluid preservatives				
1: BPBM 19237	Yes	Yes	—	Isopropanol
2: BPBM 15876	Yes	Yes	—	Isopropanol
3: BPBM 35453	Yes	Yes	—	Isopropanol
4: BPBM 19316	Yes	Yes	—	Isopropanol
5: BPBM 27167	Yes	Yes	—	Isopropanol
6: BPBM 21842	Yes	No	—	Ethanol
7: BPBM 27977	Yes	Yes	—	Isopropanol
8: BPBM 27286	Yes	Yes	—	Isopropanol
9: BPBM 16382	Yes	Yes	—	Isopropanol
10: BPBM 9574	Yes	Yes	—	Isopropanol
Samples from "neutral buffered formalin" fluid preservatives[b]				
1: USNM 333111	No	—	No	Formalin
2: USNM 330560	No	—	No[c]	Formalin
3: USNM 330555	No	—	No[c]	Formalin
4: USNM 330953	No	—	No[c]	Formalin
5: USNM 54293	No	—	No	Formalin
6: USNM 167738	No	—	No	Formalin
7: USNM 286277	No	—	No	Formalin
8: USNM 564056	No	—	No	Formalin
9: USNM 313623	No	—	No	Formalin
10: USNM 249397	No	—	No[c]	Formalin
11: USNM 564225	No	—	No	Formalin
12: USNM 564226	No	—	No	Formalin
13: USNM 564227	No	—	No	Formalin
14: USNM 564228	No	—	No	Formalin
15: USNM 564223	No	—	No	Formalin
16: USNM 564224	No	—	No	Formalin
17: USNM 564222	No	—	No	Formalin
18: USNM 564055	No	—	No	Formalin
19: USNM 580244	No	—	No	Formalin
20: USNM 523542	No	—	No	Formalin

USNM = United States National Museum; BPBM = Bernice Pauahi Bishop Museum.

[a] Specimens from Department of Vertebrate Zoology Division of Fishes.

[b] Specimens from Department of Vertebrate Zoology Division of Amphibians and Reptiles.

[c] Samples in which dark toned clumpy layer formed on top when additional K_2CO_3 was added.

Figure 2. Photograph of salting-out tests (with one drop bromothymol blue indicator in 2 ml of fluid) (left to right): 10% IPA with Test A (0.60 g K_2CO_3); 10% EtOH and 10% NBF with Test C (0.90 g K_2CO_3) (© I. Finkelde).

through staged concentrations to bring it to the desired alcohol concentration, using the method detailed in Moore (2001).

Distinguishing between Ethanol and Isopropanol

When following the procedure outlined in Test B, isopropanol concentrations above 20% salted-out with a clear layer on the bottom and a colored layer on the top, and ethanol did not salt-out (Fig. 3). This test could only distinguish isopropanol from ethanol down to 20% isopropanol. Lower concentrations of isopropanol did not salt-out with Test B but could be distinguished as a low concentration of alcohol using Test A (Fig. 4).

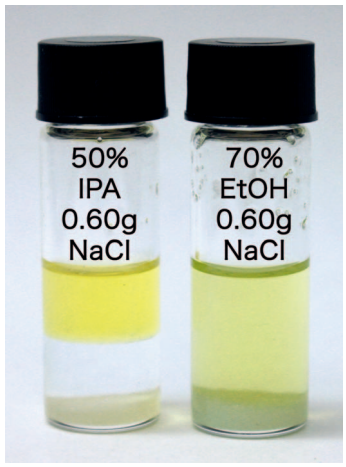

Figure 3. Photograph of salting-out Test B (with 0.60 g NaCl and one drop bromothymol blue indicator in 2 ml of fluid): 50% IPA (left) and 70% EtOH (right) (© I. Finkelde).

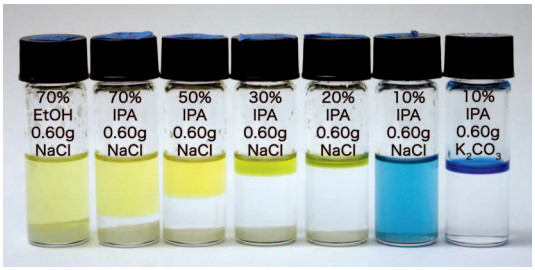

Figure 4. Photograph of salting-out with Test B (0.60 g NaCl and one drop bromothymol blue indicator in 2 ml of fluid): 70% EtOH does not salt-out with Test B; 2 mL IPA concentrations will salt-out down to 20% IPA with Test B; 10% IPA will not salt-out but can be distinguished as a low concentration alcohol with Test A (0.60 g K_2CO_3) (© I. Finkelde).

Combinations of Fluids

Low concentrations of NBF in 70% ethanol or 50% isopropanol were tested. All of the combinations of NBF with ethanol gave a result for ethanol, and all combinations of NBF with isopropanol gave a result for isopropanol. The salting-out test cannot detect the presence of low concentration NBF, but the salting-out test could be conducted in conjunction with methods listed above to determine formalin concentration.

When using Test B on combinations of 70% ethanol and 70% isopropanol, the result is either isopropanol (the sample salted-out) or ethanol (the sample did not salt-out). Once the percentage of ethanol in the total alcohol volume (70%) reached above 25%, the solutions no longer salted-out and would be interpreted as ethanol. This is consistent with the results obtained by Mayfield (2013). All gave a result for alcohol with Test A and could be distinguished from an aqueous solution (formalin).

Tests on Samples of Fluid Preservatives from Specimen Containers

As detailed in Table 2, samples from collection fluid specimen containers were tested using the optimized salting-out method. The specimens were thought to be preserved in "ethanol," "isopropanol," and "neutral buffered formalin," as detailed in their collection records and anecdotally through discussions with staff.

"Ethanol" samples were initially tested with an Anton Paar DMA 35 Digital Density Meter with ranges from 62.5% to 78.7% alcohol. With the salting-out tests (A and B) all ten samples of "ethanol" from the preservative samples returned a result for ethanol.

The "isopropanol" samples were preliminarily identified as having been preserved in 50% isopropanol. Nine out of the ten samples of "isopropanol" from the specimen fluid preservatives returned a result for isopropanol. Test A and B caused the fluid to salt-out. One test sample (Sample 6, BPBM 21842) returned a result for ethanol, with the fluid only salting-out with Test A, and not with Test B.

Owing to limitations highlighted by the first batch of tests, a total of twenty formalin samples were tested with the optimized salting-out method. The specimens were preliminarily identified as having been preserved in 10% neutral buffered formalin.

The first ten samples returned a result for aqueous solutions (formalin), because the fluid did not salt-out with Test A or Test C. However, a darker toned clumpy layer formed on the top of some samples, when additional potassium carbonate was added. It is still unclear why this occurred, but it is speculated that it could be due to lipids in the fluid.

Table 3. Peaks identified with DART-MS on known fluid samples. The peak areas (summed, respective to each sample) from masses in bold were used to compare signals.

Known fluid sample	Main peaks (*m*/*z*)
70% isopropanol	**43**, 39, 41, **61**
100% ethanol (200 proof)	33, **47**
10% neutral buffered formalin	**45**, 33, 65
Methanol (LCMS grade)	**33**, 47, **65**

The second batch of ten samples of formalin from the preservative samples returned results for aqueous solutions (formalin). They did not salt-out with Test A or Test C, and the darker clumpy layer was not noted in these samples.

DART-MS on Collection Specimen Container Fluid Preservatives

Known samples of isopropanol, ethanol, formalin, and methanol were analyzed with DART-MS to identify the peaks for each fluid type, as detailed in Table 3 and Appendix 4, Figures A4.1, A4.2, A4.3 and A4.4. Isopropanol had no other organic components, but methanol was present in 200 proof ACS/USP grade ethanol as signified by the methanol protonated molecule. Molecular and dimer signals from methanol stabilizer were also present in the formalin. Gas-phase reactions between methanol and formalin in the ionization process produced $[C_2H_4O + H]^+$ instead of the expected $[CH_2O + H]^+$ from formaldehyde.

Two calibration curves were constructed from sampling known mixtures of IPA/EtOH (Fig. 5) and EtOH/Formalin (Fig. 6) and comparing peak areas as listed for each solvent in Table 3. The methanol stabilizer signal was compared with trace ethanol signals within each spectrum. Appropriate concentration bounds containing results for unknowns were selected to fit the data to exponential and linear curves, respectively.

The results of DART-MS analysis on the collection specimen fluids are shown in Table 4, presented along with the presumed fluid type from catalog records or anecdotal staff evidence, and the fluid type determined by the salting-out tests. These results confirmed the results of the salting-out tests and indicated that there were a number

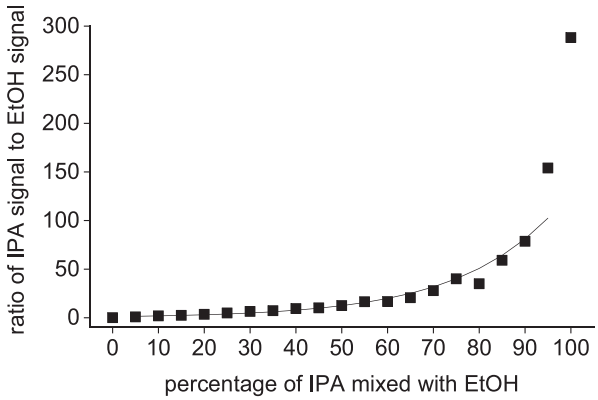

Figure 5. DART-MS calibration curve constructed from mixtures of 70% IPA and 70% EtOH.

Table 4. Results of analysis with DART-MS on fluid samples from specimen containers.

Sample no. and details of specimen container	Fluid type from catalog records or staff	Fluid type determined by salting-out tests	Peaks for EtOH with DART-MS	Peaks for IPA with DART-MS	Peaks for MeOH with DART-MS	Peaks for formalin with DART-MS
Samples from "ethanol" fluid preservatives[a]						
1: USNM 168326	Ethanol	Ethanol	Yes	—	—	—
2: USNM 102831	Ethanol	Ethanol	Yes	—	—	—
3: USNM 426199	Ethanol	Ethanol	Yes	—	—	—
4: USNM 68249	Ethanol	Ethanol	Yes	—	—	—
5: USNM 396340	Ethanol	Ethanol	Yes	—	—	—
6: USNM 102366	Ethanol	Ethanol	Yes	—	—	—
7: USNM 93896	Ethanol	Ethanol	Yes	Yes: 6–7%	—	—
8: USNM 245099	Ethanol	Ethanol	Yes	—	—	Yes
9: USNM 351717	Ethanol	Ethanol	Yes	—	—	Yes
10: USNM 174947	Ethanol	Ethanol	Yes	—	—	—
Samples from "isopropanol" fluid preservatives						
1: BPBM 19237	Isopropanol	Isopropanol	—	Yes	—	—
2: BPBM 15876	Isopropanol	Isopropanol	—	Yes	—	—
3: BPBM 35453	Isopropanol	Isopropanol	—	Yes	—	—
4: BPBM 19316	Isopropanol	Isopropanol	—	Yes	—	—
5: BPBM 27167	Isopropanol	Isopropanol	—	Yes	—	—
6: BPBM 21842	Isopropanol	Ethanol	Yes: 31–33%	Yes	—	—
7: BPBM 27977	Isopropanol	Isopropanol	—	Yes	—	—
8: BPBM 27286	Isopropanol	Isopropanol	—	Yes	—	—
9: BPBM 16382	Isopropanol	Isopropanol	—	Yes	—	—
10: BPBM 9574	Isopropanol	Isopropanol	—	Yes	—	—
Samples from "neutral buffered formalin" fluid preservatives[b]						
1: USNM 333111	Formalin	Formalin	0%–trace	—	Yes	Yes
2: USNM 330560	Formalin	Formalin	0%–trace	—	Yes	Yes
3: USNM 330555	Formalin	Formalin	0%–trace	—	Yes	Yes
4: USNM 330953	Formalin	Formalin	0%–trace	—	Yes	Yes
5: USNM 54293	Formalin, previously in ethanol[c]	Formalin	Yes: 1–2%	—	Yes	Yes
6: USNM 167738	Formalin, previously in ethanol[c]	Formalin	0%–trace	—	Yes	Yes
7: USNM 286277	Formalin	Formalin	0%–trace	—	Yes	Yes
8: USNM 564056	Formalin	Formalin	0%–trace	—	Yes	Yes
9: USNM 313623	Formalin	Formalin	0%–trace	—	Yes	Yes
10: USNM 249397	Formalin	Formalin	0%–trace	—	Yes	Yes
11: USNM 564225	Formalin	Formalin	0%–trace	—	Yes	Yes
12: USNM 564226	Formalin	Formalin	Yes: 1–2%	—	Yes	Yes
13: USNM 564227	Formalin	Formalin	Yes: 1–2%	—	Yes	Yes
14: USNM 564228	Formalin	Formalin	Yes: 1–2%	—	Yes	Yes
15: USNM 564223	Formalin	Formalin	0%–trace	—	Yes	Yes
16: USNM 564224	Formalin	Formalin	0%–trace	—	Yes	Yes
17: USNM 564222	Formalin	Formalin	0%–trace	—	Yes	Yes
18: USNM 564055	Formalin	Formalin	Yes: 1–2%	—	Yes	Yes
19: USNM 580244	Formalin	Formalin	Yes: 1–2%	—	Yes	Yes
20: USNM 523542	Formalin	Formalin	0%–trace	—	Yes	Yes

USNM = United States National Museum; BPBM = Bernice Pauahi Bishop Museum.

[a] Specimens from Department of Vertebrate Zoology Division of Fishes.

[b] Specimens from Department of Vertebrate Zoology Division of Amphibians and Reptiles.

[c] These specimen lots (tadpoles) had previously been fixed in formalin, then stored in ethanol. They were transferred from ethanol back to formalin in 1994 (USNM 54293) and 1985 (USNM 167738).

Note: other peaks were also present in the samples from specimen jars, likely from lipid and fats. Peaks for benzyl butyl phthalate were also detected, likely from plastic liners in sampling vials. Further analysis of the spectra and identification of the molecules is required.

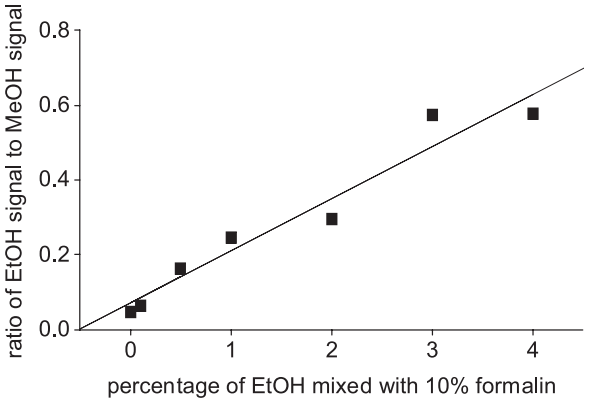

Figure 6. DART-MS calibration curve constructed from mixtures of 97% EtOH and 37% w/w formaldehyde (ACS reagent, Sigma Aldrich), the concentration in 10% NBF.

of jars that had combinations of fluids that the salting-out test could not detect. There were two "ethanol" samples that contained some formalin, and one that contained some isopropanol; one "isopropanol" sample contained ethanol; and six "formalin" samples contained ethanol in the fluid in very low quantities. Other peaks were also present in the samples from specimen containers, likely from lipids and fats. Peaks for benzyl butyl phthalate were also detected, likely from plastic liners in the sampling vials.

The comparison of the curve of known IPA/EtOH combinations (Fig. 5) with the samples indicated that one of the "ethanol" containers (EtOH Sample 7: USNM 93896) had between 6% and 7% isopropanol in the total volume of alcohol. One of the "isopropanol" containers (IPA Sample 6, BPBM 21842) had between 31% and 33% ethanol in the total volume of alcohol. This is the reason it did not salt-out with Test B and gave a result for ethanol with the salting-out tests.

Two "ethanol" samples contained residual formalin, likely from fixation. The comparison with the EtOH/Formalin curve (Fig. 6) indicated that in six "formalin" samples ethanol was present in the fluid in the range of 1–2%. Some specimens had previously been stored in ethanol and were transferred to formalin (NBF Sample 5, USNM 54293, and NBF Sample 6, USNM 167738), but it is unclear why ethanol was present in the other samples.

Limitations of the Salting-Out Test

As with any method, there are limitations to the salting-out test presented in this paper, including:

- Need to open the container to remove the fluid samples. This can be problematic and time consuming in collections with jars that have wax, bladder, bituminous, or gelatin seals.
- Cannot determine the concentration of formalin; can only indicate the fluid is water based.
- Very low concentrations of alcohol (i.e., less than 6% ethanol) may also be interpreted as formalin.
- Cannot determine combinations of fluid types, as highlighted by the results of DART-MS analysis.

- Cannot detect additives in the fluid, such as those listed in Simmons (2014:199–273, 284–288).
- It is unclear why some of the formalin samples had a darker toned clumpy layer on top that was not alcohol. It could be due to the lipids and fats in the fluid, but more research is required.
- When higher concentrations of alcohol (>60%) are tested, it can be difficult to get the salt to dissolve in the fluid, and some settles to the bottom. The separation layer must be observed above the excess salt.
- Tests have not yet been conducted on more exotic fluid preservatives, so it is not known what the results would be.

Further Research Required on Denatured Alcohol

Some collecting institutions use denatured ethanol as a preservative because it is cheaper or more readily available. Denatured ethanol was not tested as part of this study, but it would be useful to determine if it is still a viable method for ethanol containing denaturants. Mayfield (2013) found that 70% denatured ethanol (containing one part methanol (CH_4O), one part ethyl acetate ($C_4H_8O_2$), one part methyl iso-butyl ketone ($C_6H_{12}O$) and one part hydrocarbon solvent per 100 parts ethanol) salted-out with potassium carbonate, but not with sodium chloride. It would be useful to conduct further research to determine whether other denaturants affect the results of the optimized salting-out test method.

Cost and Time

The materials and all equipment required for the salting-out test cost just over $100 US at the time of publication, as detailed in Appendix 1 (Table A1.1). This provides materials for approximately 550 to 830 tests. The glass vials and syringes can be washed and reused. Tests on a single sample take approximately 3 minutes.

Labeling

Once the fluid type has been determined, the container should be labeled with the type of preservative it contains. This will avoid future custodians of the collection from encountering the same issue of unknown preservatives. Refer to Hawks and Williams (2005) and Range et al. (2019) for details on papers and inks to use when labeling fluid-preserved specimens.

Health and Safety

When handling fluid-preserved specimens, one should always use appropriate personal protective equipment (PPE), which includes a lab coat, neoprene or nitrile gloves, and protective eyewear (Hawks et al. 2010; Simmons 2014, 2019). Opening specimen containers needs to be done within a fume hood to avoid breathing the vapor of the fluid preservative (Simmons 2019). It is necessary and important to obtain, review, and document safety data sheets for all chemicals used prior to undertaking testing. A spill kit should be readily available whenever work with fluid preservatives is undertaken.

When a jar containing a fluid-preserved specimen in an unknown fluid is encountered, best practice is to treat it as though it contains a hazardous substance. Formalin is a known carcinogen (Liteplo et al. 2002, Babin et al. 2010, Simmons 2014, IARC 2018), isopropanol is twice as toxic as ethanol (Simmons 2014), and alcohols and formalin are flammable liquids. A number of other hazardous additives have been used in fluid preservatives over the years, as detailed in Simmons (2014:199–273). When undertaking the salting-out method

on unknown fluid types, unknown chemical interactions or reactions could occur due to the presence of these unknown additives, and every precaution should be taken.

Conclusion

Understanding the type of fluid used to preserve a specimen is vital in understanding the way in which a specimen may degrade. It is also important from a health and safety perspective, due to the known hazards associated with fluid preservatives. The type of fluid preservative used is often not documented, and this can make it difficult to identify for collections management and safety purposes. The optimized salting-out test method is a quick, comparatively inexpensive, and reliable method that can be used to distinguish between the concentrations of ethanol, isopropanol, and aqueous-based fluid preservatives commonly encountered in fluid-preserved collections using small sample sizes (2–4 ml). The results of testing on known concentrations and unknown samples from fluid preservative containers indicate that salting-out is a viable method to determine fluid preservative type. DART-MS analysis verified the results of the salting-out tests but also highlighted some limitations of the method, particularly when combinations of fluid are encountered (e.g., traces of formalin fixative in alcohol or residues of previously used alcohol). The results of the salting-out tests and DART-MS analysis highlight the need for a simple, quick test that can distinguish between ethanol, isopropanol, and formalin before topping-up is done. It is likely that the blending of fluid types in some of the samples was due to the incorrect fluid preservative being used. The salting-out tests can be used to give an approximation of alcohol concentration and could be used in conjunction with other methods to determine formalin concentration.

Acknowledgments

The authors would like to thank Catharine Hawks for supervising this research and providing encouragement, valuable comments, and feedback. Without her support, this research would not have been possible. The authors also thank the organizers of the meeting, "Preservation of natural history wet collections: feedbacks and future prospects," held in Paris, France, 5–7 December 2018. This meeting provided a platform for this research to be presented initially, and to discuss fluid preservation issues within the relevant community. This research was also presented at the Society for the Preservation of Natural History Collections Annual Meeting in Chicago, 25–31 May 2019, with the support of the Christine Allen Travel Grant.

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The authors would like to acknowledge the work of Teresa Mayfield for initially researching the salting-out method and presenting it as a viable method to use in the determination of fluid preservatives used in natural history collections. This has provided the basis of the research presented here, and we are very grateful for her contribution to develop methods of fluid preservation identification.

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Résumé

Cet article présente en détail une méthode, basée sur la précipitation saline («salting-out» en anglais) avec des sels de carbonate de potassium et chlorure de sodium, d'identification de trois fluides conservateurs couramment utilisés: l'éthanol, l'isopropanol, et le formol.

Un état de l'art des autres méthodes permettant d'identifier les fluides conservateurs et la méthode de précipitation saline publiée par Mayfield en 2013 sont tout d'abord présentés. La nouvelle méthodologie, qui nécessite un petit échantillon de fluide (2 à 4 ml), est ensuite détaillée. Simple, rapide et relativement peu coûteuse à mettre en œuvre, c'est une méthode viable pour distinguer les fluides courants. Le matériel et l'équipement pour le test coûtent un peu plus de $100 US, et les tests prennent environ trois minutes par bocal.

Les résultats des tests effectués sur des échantillons-modèles de produits purs ou de mélanges, de concentrations connues en éthanol, isopropanol et formol (solution de formaldéhyde dans l'eau) et ainsi que sur des échantillons de fluides provenant des bocaux des collections du Smithsonian National Museum of Natural History et du Bernice Pauahi Bishop Museum, sont montrés. Les résultats de ces tests ont été vérifiés par analyse directe en spectrométrie de masse en temps réel (DART-MS) ce qui a confirmé les résultats, mais a également mis en évidence certaines limites, notamment lorsque des mélanges de fluides conservateurs sont rencontrés.

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Appendix 1. Details of Materials Required for Salting-Out Method

Table A1.1. List of supplies, sources, and costs of materials for salting-out tests.

Item	Source[a]	Cost (US$)
100 × 1 dram (3.7 ml) borosilicate glass vials	J.G. Finneran, 1 dram, 15 × 45 mm clear vial, 13–425 mm black solid top, PTFE/F217 lined, Item No. 84020-1545	$34.90
Sodium chloride	Science Company sodium chloride, 500 g (noniodized) catalog No. NC-0870 (enough for 833 tests)	$8.95
Potassium carbonate	Fisher Scientific potassium carbonate, anhydrous, 500 g, lab grade, catalog No. S25480 (enough for between 555 and 833 tests)	$15.00
Bromothymol blue indicator	Science Company bromothymol blue pH indicator, 1 oz., catalog No. NC-1949	$3.95
Scale that weighs in range of 0.01 g	An analytical balance or jeweler's scale (available from multiple suppliers) can be used	$10.00–$15.00
Weighing paper	Lab exact cellulose weighing paper sheet, nitrogen free, 3 × 3 inches, 500 sheets, UNSPSC Code: 47131900	$15.29
2 × spatulas	Science company micro spatula, stainless steel, catalog No. NC-3062	$5.90
Syringes	Sigma Aldrich syringe PP/PE without needle, 3-ml capacity, 0.1 ml graduated, 100 pack, catalog No. Z116858-100EA	$21.60
Total cost		$115.59–$120.59

[a] Note: Specification of brands or suppliers is not an endorsement; similar products are available from multiple suppliers. Many institutions will already have some of these supplies and equipment available, and this will considerably lower the cost.

Appendix 2. Ready-to-Use Salting-Out Method

This method details the salting-out procedure for those who wish to use it in testing fluid preservatives. A flow chart diagram of the methodology is presented in Figure A2.1.

Materials

Sodium chloride (noniodized) (NaCl); potassium carbonate (K_2CO_3); bromothymol blue indicator; 1 dram (3.7 ml) borosilicate glass vials with lids; weighing scale (capable of measuring 0.01 g range); weighing paper; syringes; spatulas.

Method

All tests should be conducted within a fume hood while wearing appropriate personal protective equipment, as detailed in Hawks et al. (2010) and Simmons (2014, 2019).

This procedure has been adapted from those published in Mayfield (2013), North Carolina State University Department of Chemistry (n.d.), and Smith (1996) to use a smaller sample size of 2 ml instead of 20 ml. The fluid-to-salt ratio has also been altered for a 2 ml fluid sample to 0.60 g or 0.90 g of salt, depending on the alcohol concentration. For all tests, the authors measured the salt using a Mettler PC 220 analytical scale, capable of measuring to three decimal places. The method is broken down into three tests: A, B, and C.

Initial Preparation

- Using a scale, weighing paper, and a spatula, weigh out 0.60 g of K_2CO_3 and place in a glass vial for use in Test A.
- Using a new sheet of weighing paper and a different spatula, weigh out 0.60 g of NaCl and place in another glass vial for use in Test B.
- Repeat for as many test vials as needed.

Note: Fold the weighing paper in half to facilitate pouring into the vials. Weighing paper for each type of salt can be reused for that salt.

Test A: Distinguishing Alcohol (Most Concentrations) from Aqueous Solutions with Potassium Carbonate

- Remove a 2-ml fluid sample with a syringe and deposit in a vial containing 0.60 g of K_2CO_3.
- Add one drop of bromothymol blue indicator to the sample solution and secure the lid on the vial.
- Shake the vial for 30 seconds, then allow to stand for 30 seconds.

If the solution salts-out and separates into two layers—a clear layer of water on bottom and blue or colored layer of alcohol on top—then it is alcohol based. Continue to Test B to determine whether it is ethanol or isopropanol.

If the solution does not salt-out and remains blue or colored throughout, undertake steps in Test C.

Test B: Distinguishing Isopropanol from Ethanol with Sodium Chloride

- Remove another 2 ml fluid sample with a syringe and deposit in a vial containing 0.60 g of NaCl.
- Add one drop of bromothymol blue indicator to the sample solution and secure the lid on the vial.
- Shake the vial for 30 seconds, then allow to stand for 30 seconds.

If the sample solution salts-out (separates into two layers, a clear on bottom and yellow or colored on top) then it is isopropanol. If the solution does not salt-out, it is ethanol or a low concentration of isopropanol. This amount of sodium chloride will salt-out isopropanol concentrations down to 20%. Lower concentrations do not salt-out, but can be distinguished as a low concentration alcohol using Test A or Test C.

Test C: Distinguishing Low Concentration Ethanol from an Aqueous Solution

If the sample solution did not salt-out in Test A, follow the steps below:

- Using the scale, weighing paper, and spatula, weigh out 0.30 g of K_2CO_3.
- Working under a fume hood, remove the lid from the sample vial and carefully pour the 0.30 g of K_2CO_3 into the vial with the sample used in Test A (for a total of 0.90 g K_2CO_3 in the sample solution). Secure the lid on the vial.

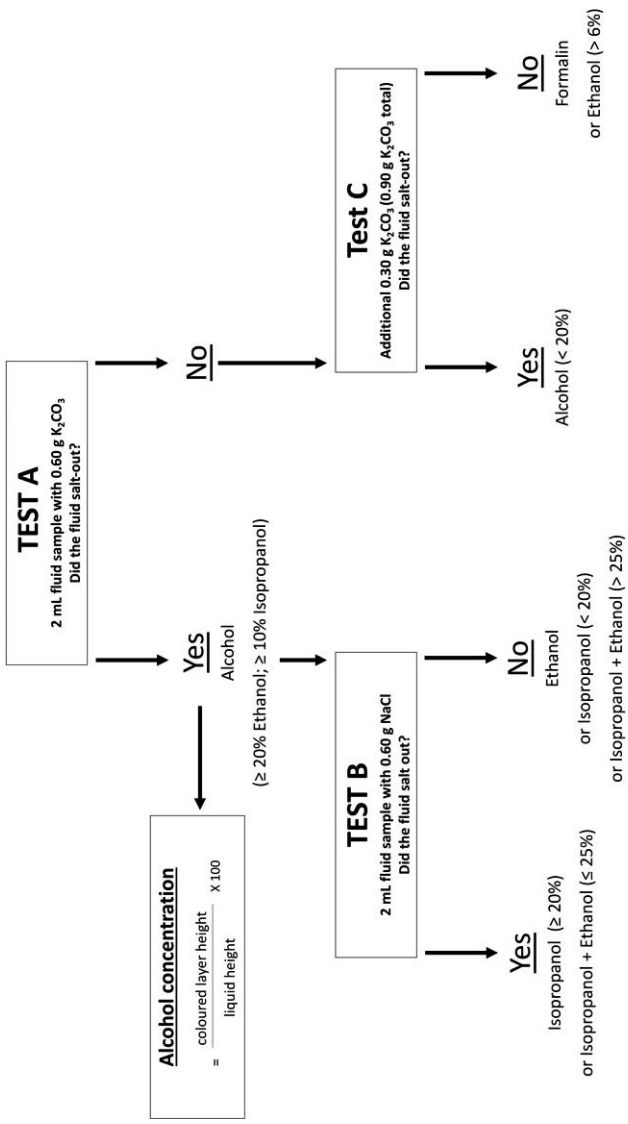

Figure A2.1. Flow chart diagram detailing the salting-out test methodology (© I. Finkelde).

- Shake the vial for 30 seconds, then allow to stand for 30 seconds.

If the sample solution salts-out, it is a low concentration of alcohol. If it does not salt-out, then it is an aqueous solution, probably formalin. This method works down to 6% ethanol, after which very low ethanol concentrations may also be interpreted as formalin.

Disposal of Samples

Following testing, dispose of the samples as hazardous waste, in compliance with applicable regulations. Take into consideration that the top colored layer is pure alcohol, and an alkali solution is formed by the potassium carbonate. The vials can be washed and dried to be used again.

Appendix 3. Notes on the Salting-Out Method

- The color variations that can be seen in the test results are due to the bromothymol blue pH indicator, which binds to the alcohol in the separation from water, and this makes it easier to see the separation layers.
- With this test, the salting-out (the separation of alcohol from water) is what distinguishes between alcohols, not the color variations. The different colors indicate the pH of the solution, but this is affected by the addition of the different salts, which alter the pH. The color results are also affected by the initial color of the fluid, which may have yellowed due to lipid or dye leaching from the specimen.
- When depositing the fluid sample into the vial with the syringe, avoid touching the salt and fluid/salt combination with the syringe tip, since this may lead to contamination. Rinse syringes with water between sampling.
- Sometimes with higher concentrations of ethanol, it is difficult to get the potassium carbonate to dissolve. The authors used a bamboo skewer to break up the spherical clumps of potassium carbonate that form.
- When higher concentrations of alcohol (above 50%) are tested with 0.60 g K_2CO_3, not all the K_2CO_3 will dissolve, and some will settle as a solid in the bottom. Take care to distinguish the salted-out layer above this solid salt.
- In high concentrations of ethanol, the NaCl will settle as a solid in the bottom of the vial; however, it will dissolve in lower concentrations.
- Salting-out causes an exothermic reaction within the fluid, and this can sometimes be felt by warmth in the glass vial.
- When adding the additional K_2CO_3, a highly alkaline solution is formed. With the bromothymol blue indicator, this will show as a blue or purple tone throughout.

Appendix 4. DART-MS Spectra of Known Samples

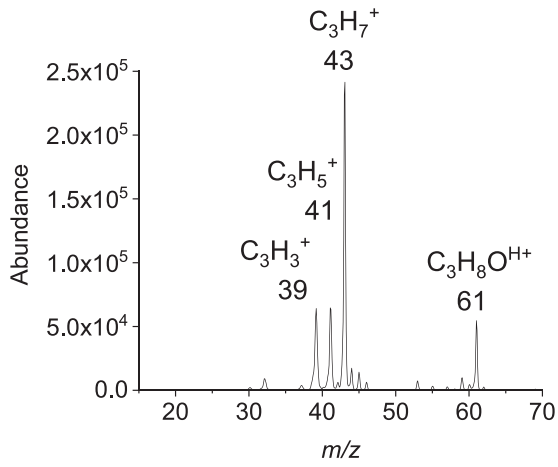

Figure A4.1. Mass spectrum of 70% isopropanol (Walgreens).

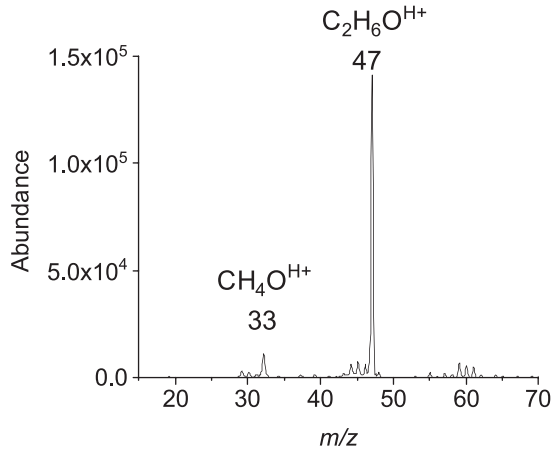

Figure A4.2. Mass spectrum of 200 Proof ACS/USP Grade ethanol (Pharmco – Aaper).

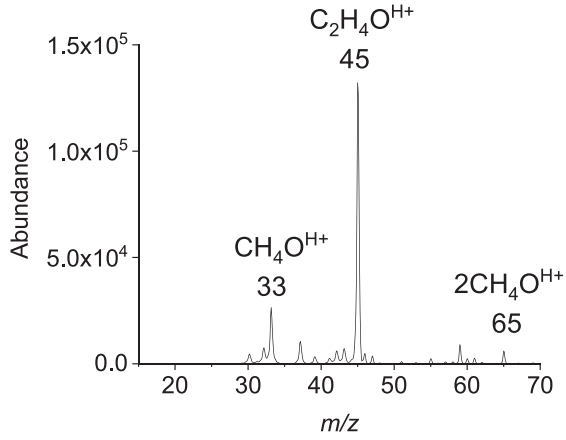

Figure A4.3. Mass spectrum of 10% neutral buffered formalin.

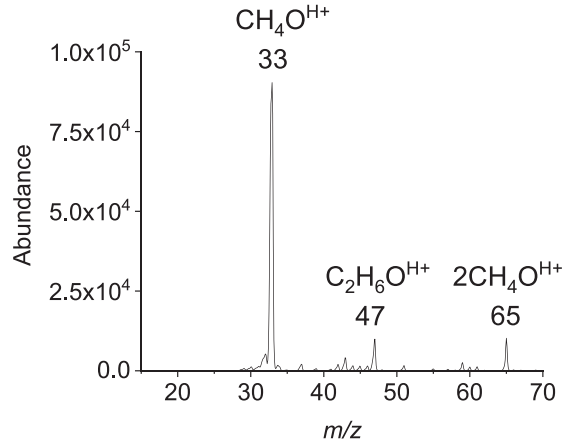

Figure A4.4. Mass spectrum of LCMS grade methanol (Fisher Chemical).