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## Use and Advantages of Ethanol Solution of Alizarin Red S Dye for Staining Bone in Fishes

VICTOR G. SPRINGER AND G. DAVID JOHNSON

**A solution of 75% ethyl alcohol (ETOH) and alizarin red S can be substituted for KOH-alizarin red S to stain bone. ETOH-alizarin, unlike KOH-alizarin, does not damage muscle or cartilage. ETOH-alizarin and acidified alcian blue (a cartilage stain) can be used in any order and as often as required to stain bone and cartilage in a single preparation that has not been exposed to KOH-alizarin or trypsin, remembering that the acidic effects of the alcian blue solution should be neutralized to prevent decalcification of weakly ossified elements.**

**F**OR many years, ichthyologists have been using, perhaps exclusively, a potassium hydroxide (KOH)-alizarin red S solution to stain bone red in fishes. It has also been known for many years that KOH macerates muscle—early methods of staining bone for osteological purposes involved clarifying associated muscle in KOH solutions. During our ongoing studies of gill-arch muscle-bone anatomy, we initially used KOH-alizarin to stain bone. We found, however, that KOH weakened and frayed the muscles, making it difficult or impossible to obtain satisfactory preparations. Although ethyl alcohol (ETOH)-alizarin as a bone stain probably has been known for hundreds of years (Hollister, 1934), we were unaware of the possibility and tried ETOH-alizarin as a “shot-in-the-dark” attempt to solve our problem. The results were excellent. We have used the solution successfully on over 200 gill-arch muscle-bone preparations involving a wide variety of specimens preserved in ETOH but fixed initially in alcohol or formalin and collected from recently to about 100 years ago. All of these preparations were also stained for cartilage using acidified ETOH-alcian blue (Potthoff, 1984). Because the utility of using ETOH-alizarin to stain bone was unknown to several colleagues with whom we discussed the method, we report it and some of its advantages, recognizing that we may have only “rediscovered the wheel.”

### MATERIALS AND METHODS

The method is simple and inexact. We use a solution containing 75% ETOH and 25% H<sub>2</sub>O in which we dissolve enough alizarin dye crystals to give the liquid a urine-yellow color (percentage of alcohol and amount of dye are not critical). The structures to be stained are placed in the solution until we are satisfied the bone is adequately stained, as determined by periodic monitoring. For limited structural features,

such as gill arches, overstaining is not a problem, and we have left gill arches in ETOH-alizarin unmonitored for several days without damage.

ETOH-alizarin also may be used to stain bone in specimens to be used for clearing and staining before or after muscle has been digested with trypsin (Taylor and Van Dyke, 1985) and/or cartilage has been stained with acidified alcian blue (Potthoff, 1984). In such cases, specimens should be monitored to prevent overstaining the bone, the same as required when using KOH-alizarin.

The advantages of ETOH-alizarin for staining bone are many. First, ETOH is relatively harmless; KOH is caustic and requires care in handling and storage. ETOH also preserves muscle; KOH macerates and damages muscle. Furthermore, ETOH-alizarin is stable; even after the solution has been reused many times over many months, the stain is effective and never precipitates. KOH-alizarin degrades after use over relatively short periods of time, with the stain often precipitating.

Fourth, muscle-bone-cartilage preparations not previously exposed to KOH (or trypsin) may be stained first with either ETOH-alizarin or alcian blue (Potthoff, 1984) and restained with either solution in any order as often as desired (note: alcian blue solution is acidic, and if its effects are not neutralized after staining, it could decalcify weakly ossified elements). This facility is important: cartilage covered by thick muscle and/or connective tissue frequently does not stain. Dissection in stages after initial staining permits exposing unstained cartilage, which then can be stained. If KOH-alizarin is used to stain bone of muscle-bone-cartilage preparations, the cartilage should be stained first (with alcian blue) for best results. Staining of cartilage after exposure to KOH is usually not possible.

Additionally, KOH is particularly destructive

of larval-fish tissue (P. Mabee, unpubl.), and using ETOH-alizarin for staining bone in larvae should be advantageous. Finally, fish muscles are frequently whitish, and it is often difficult to distinguish the limits of various muscles without staining. ETOH-alizarin often imparts a permanent pink tinge to muscle, increasing its contrast, which we find desirable.

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