



**The Robustness of a Simple UV-vis Spectrophotometric  
Method to Determine the Concentration of Eugenol in Water**

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Fisheries professionals need a safe and effective compound to sedate fish for procedures such as collection of tissue samples or morphometric data, surgical implantation of tags or tracking devices, transport, and commercial harvest. During such procedures, sedated fish are less likely to suffer physical injury or be negatively affected by the physiological consequences of handling stress. Ideally, a fish sedative is safe, effective, easy to use, has rapid induction and recovery times, offers some analgesia, is inexpensive, and can be used under a variety of environmental conditions. In many resource management situations, it is also desirable to have access to a sedative where fish can be released or stocked immediately after sedation.

In the U.S., tricaine methanesulfonate (commonly referred to as MS-222) is the only compound approved by the U.S. Food and Drug Administration (FDA) for the temporary immobilization of fish and other aquatic, cold-blooded animals. MS-222 products currently available in the U.S. are Finquel<sup>®</sup> (Argent Chemical Laboratories, Inc., Redmond, WA) and Tricaine-S (Western Chemical Inc. Ferndale, WA). Both products are generally considered to be safe and effective and are often used successfully by fisheries professionals. However, legal use of MS-222 is restricted to four families of fish (Ictaluridae, Salmonidae, Esocidae, and Percidae) and water temperatures above 10°C. Also, a 21-day withdrawal period is required before fish may be released/stocked. For many field-use applications, holding fish is not practical. To avoid such complications, an FDA-approved immediate-release sedative is desperately needed.

Carbon dioxide (CO<sub>2</sub>), which is not approved by FDA but is classified as a drug of low regulatory priority (LRP), can be used as an immediate-release fish sedative. Although some consider CO<sub>2</sub> gas an effective sedative for freshwater fish, many find it difficult to apply uniformly and unpredictable in its effect. To sedate fish with CO<sub>2</sub>, hypercapnia must be induced, which affects all major organ systems and can induce a generalized stress response. Depending on the exposure conditions, full recovery from these disturbances can take hours or days, and in some instances morbidity and mortality are observed. Clearly, better alternatives are needed for fisheries professionals needing to sedate and immediately release fish.

The U. S. Fish and Wildlife Service's Aquatic Animal Drug Approval Partnership (AADAP) Program is working with a variety of public data-generating partners and drug sponsors to identify and obtain approval of a sedative(s) for immediate-release use in fish. Two candidate products, AQUI-S<sup>®</sup> E (50% eugenol) and AQUI-S<sup>®</sup> 20E (10% eugenol), have been developed by AQUI-S New Zealand, Ltd. (Lower Hutt, NZ), and preliminary data and research protocols are being developed to evaluate their efficacy and safety. Such studies conducted in support of FDA approval must also include an FDA-accepted analytical method to verify actual sedative concentrations tested during efficacy and safety studies. Typically, FDA requires that drug concentrations be measured by 'high tech' instrumentation, such as high pressure liquid chromatography coupled with mass spectrometry (HPLC-MS). However, more readily available instrumentation (e.g., UV-vis spectrophotometry) may be used if data generated are comparable to HPLC-MS or otherwise accepted by FDA after it has been demonstrated that the method is consistent across a broad range of environmental parameters. Development of HPLC-MS methodology is costly, time consuming, requires considerable expertise, and is not suited to field applications. Moreover, subsequent analytical verification of individual samples are costly. A simple UV-vis method has been developed that measures the concentration of eugenol in water, and the method appears to be precise and accurate. However, its precision and accuracy have not yet been verified for water from a variety of sources (i.e., robustness). Accordingly, we conducted a trial to evaluate the robustness of this eugenol UV-vis method by preparing and analyzing eugenol standards made from six water sources of varying water quality parameters (e.g., water hardness, alkalinity, and pH).

**Methods**

Water samples were collected from three sites where AADAP researchers plan to conduct effectiveness testing of a eugenol-based compound on representative cold-, cool-, and warmwater fish species: Fish Breeders of Idaho (FBI; Hagerman, ID); Montana Fish, Wildlife and Parks Miles City State Fish Hatchery (MCSFH, Miles City, MT); and FWS Bozeman Fish Technology Center (BFTC, Bozeman, MT). At each site, samples were collected from two different water sources (Table 1). A 500 ug/L eugenol stock standard was prepared from each water sample collected by dissolving 0.5 g eugenol (≥99.5%, PT Indesso Aroma, Jakarta, Indonesia) in 50 mL 100% ethyl alcohol (Fisher

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Scientific, Pittsburgh, PA) and diluting to 1,000 mL in a volumetric flask. A series of five eugenol working standards (5, 10, 25, 40, and 60 mg/L) was prepared by transferring the appropriate volume of stock standard into 100-mL volumetric flasks and diluting with the same water as that used to prepare the stock standard. Four or five aliquots of each working standard were transferred into individual disposable cuvettes (Fisherbrand, plastic, 4.5 mL capacity, 10 mm lightpath, Fisher Scientific) and measured by UV-vis spectrophotometry (Genesys 2, Thermo Electron Corp, Madison, WI) at 279.0 nm. Before a set of working standard samples were measured, the spectrophotometer absorbance reading was set to zero by using an empty cuvette. Mean absorbances were plotted against eugenol concentrations, and linear standard curves were fit in SigmaPlot 11 (SYSTAT 2008). The R<sup>2</sup> value, slope, and y-intercept values were determined from a standard curve generated for each set of working standards. These values were used to make an overall assessment of the robustness of the method.

### Results and Discussion

The R<sup>2</sup> value for each standard curve was  $\geq 0.9999$ ; the slope ranged from 0.0085 to 0.0093; and the y-intercept was near 0.0 for all sets of working standards (Table 1; Figure 1). The values were considered comparable considering that pH, hardness, and

alkalinity ranged from 7.7 to 8.9, 12 to 332 mg/L (as CaCO<sub>3</sub>), and 98 to 459 mg/L (as CaCO<sub>3</sub>). Slight differences in the R<sup>2</sup> and slope values were likely caused by quantitative transfer error. Differences in the y-intercept values were likely caused by slight differences in water clarity not visible to the naked eye (all water appeared clear). As such, the UV-vis spectrophotometric method for determining eugenol concentrations in water appears to be accurate and precise in a variety of different waters. Consequently, we concluded that the method is robust and adequate for determining the concentration of eugenol in solutions prepared to evaluate the safety and effectiveness of AQUIS-E or AQUIS-S 20E.

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### References

SYSTAT. 2008. SigmaPlot 11. SYSTAT Software, Inc., San Jose, California.

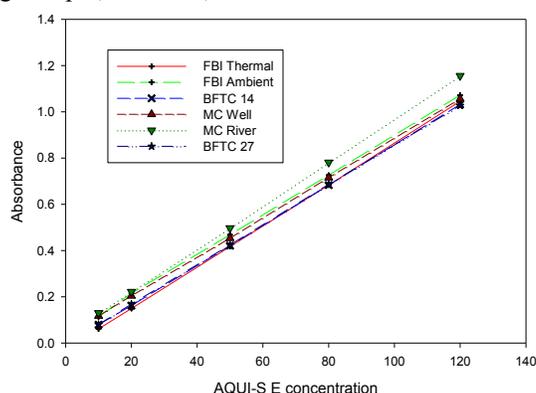


Figure 1. Linear regression lines of six eugenol working standard curves prepared with water from six different sources.

**Table 1.** Linear regression coefficients and selected water chemistry parameters for each water type used to evaluate the accuracy and precision (i.e., robustness) of the UV-vis spectrophotometric method to determine concentration of eugenol in water. Hardness and alkalinity concentrations are reported as CaCO<sub>3</sub>.

Site	Water type	Water temp (°C)	Hardness (mg/L)	Alkalinity (mg/L)	pH	R <sup>2</sup>	Slope	y-Intercept
FBI	Geothermal well	32	24	98	8.6	1.0000	0.0089	-0.0287
FBI	Ambient spring	13	332	256	8.4	0.9999	0.0085	0.0444
BFTC	Cold/warm	14	232	167	7.7	1.0000	0.0087	-0.0104
BFTC	Warm spring	27	246	146	8.2	1.000	0.0086	-0.0040
MCSFH	Yellowstone	2	124	186	8.6	1.000	0.0093	0.0346
MCSFH	Well	11	12	459	8.9	1.000	0.0085	0.0291