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Quantification of Ethanol Levels in Zebrafish Embryos Using Head Space Gas Chromatography

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Cover Letter

UNIVERSITY OF LOUISVILLE

Department of Biochemistry and Molecular Genetics

Dear Dr. Jewhurst,

We would like to resubmit our manuscript "Quantification of ethanol levels in zebrafish embryos using head space gas chromatography," for consideration to be published in *JoVE.* We have addressed the concerns of the editorial reviews and feel that their suggestions have made for a stronger and more rigorous manuscript. We utilize headspace gas chromatography to determine ethanol tissue concentration in 24 hours post fertilization zebrafish embryos in two independent groups over 2 days. We feel that this manuscript describes the process of quantifying tissue ethanol concentrations in zebrafish alcohol research clearly and succinctly. Our protocol is straight forward and produces results the can be easily replicated and are consistent with other methods for quantifying ethanol tissue concentrations in zebrafish embryos. This manuscript will enable researchers to standardize ethanol treatment regimens for zebrafish and gain greater understanding in how ethanol timing and dosage contributes to alcohol induced developmental defects by being able to directly quantify ethanol in zebrafish embryos. None of the original material contained in the manuscript has been previously published nor is currently under review for publication elsewhere. The authors have no conflict of interests.

Thank you for your consideration.
Sincerely,

C. Ben Lovely, Ph.D.
Biochemistry and Molecular Genetics; Alcohol Research Center
University of Louisville

TITLE:

Quantification of Ethanol Levels in Zebrafish Embryos Using Head Space Gas Chromatography

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KEYWORDS:

zebrafish, development, ethanol, fetal alcohol spectrum disorders, head space gas chromatography, embryonic ethanol concentration, animal model

SUMMARY:

This work describes a protocol to quantify ethanol levels in a zebrafish embryo using head space gas chromatography from proper exposure methods to embryo processing and ethanol analysis.

ABSTRACT:

Fetal Alcohol Spectrum Disorders (FASD) describe a highly variable continuum of ethanol-induced developmental defects, including facial dysmorphologies and neurological impairments. With a complex pathology, FASD affects approximately 1 in 100 children born in the United States each year. Due to the highly variable nature of FASD, animal models have proven critical in our current mechanistic understanding of ethanol-induced development defects. An increasing number of laboratories has focused on using zebrafish to examine ethanol-induced developmental defects. Zebrafish produce large numbers of externally fertilized, genetically tractable, translucent embryos. This allows researchers to precisely control timing and dosage of ethanol exposure in multiple genetic contexts and quantify the impact of embryonic ethanol exposure through live imaging techniques. This, combined with the high degree of conservation of both genetics and development with humans, has proven zebrafish to be a powerful model in which to study the mechanistic basis of ethanol teratogenicity. However, ethanol exposure regimens have varied between different zebrafish studies, which has confounded the interpretation of zebrafish data across these studies. Here is a protocol to quantify ethanol concentrations in zebrafish embryos using head space gas chromatography.

INTRODUCTION:

Fetal Alcohol Spectrum Disorders (FASD) describes a wide array of neurological impairments and craniofacial dysmorphologies associated with embryonic ethanol exposure[1]. Multiple factors, including timing and dosage of ethanol exposure and genetic background, contribute to the variation of FASD[2,3]. In humans, the complex relationship of these variables makes studying and understanding the etiology of FASD challenging. Animal models have proven crucial in developing our understanding of the mechanistic basis of ethanol teratogenicity. A wide variety of animal

model systems has been used to study multiple aspects of FASD and results have been remarkably consistent with what is found in exposure in humans[4]. Rodent model systems are used to examine many aspects of FASD, with mice being the most common[5–7]. The majority of this work has focused on developmental defects to early ethanol exposure[8], though later exposure to ethanol has been shown to cause developmental anomalies as well[9]. Moreover, the genetic capabilities of mice have greatly aided in our ability to probe the genetic underpinnings of FASD[10,11]. These studies in mice strongly suggest that there are gene-ethanol interactions with the sonic hedgehog pathway, retinoic acid signaling, *Superoxide dismutase, Nitric oxide synthase I, Aldh2* and *Fancd2*[8,10–21]. These studies show that animal models are critical to advancing our understanding of FASD and its underlying mechanisms.

The zebrafish has emerged as a powerful model system to examine many aspects of ethanol teratogenesis[22,23]. Due to their external fertilization, high fecundity, genetic tractability, and live imaging capabilities, zebrafish are ideally suited to study factors such as timing, dosage, and genetics of ethanol teratogenesis. Ethanol can be administered to precisely staged embryos and the embryos can then be imaged to examine the direct impact of ethanol during developmental processes. This work can be related directly to humans, because the genetic programs of development are highly conserved between zebrafish and humans and can therefore help guide FASD human studies[24]. While zebrafish have been used to examine ethanol teratogenesis, a lack of consensus in reporting embryonic ethanol concentrations makes comparison to humans difficult[25]. In mammalian systems, blood-alcohol levels correlate directly to tissue ethanol levels[26]. Many of the zebrafish studies treat embryos before complete formation of their circulatory system. With no maternal sample to examine, a process to assess ethanol concentrations is required to quantify ethanol levels within the embryo. Here we describe a process to quantify ethanol concentrations in a developing zebrafish embryo using head space gas chromatography.

PROTOCOL:

All zebrafish embryos used in this procedure were raised and bred following established IACUC protocols[27]. These protocols were approved by the University of Texas at Austin and the University of Louisville.

NOTE: The zebrafish line *Tg(fli1:EGFP)*^{y1} was used in this study[28]. All water used in this procedure is sterile reverse osmosis water. All statistical analyses were performed using Graphpad Prism v8.2.1.

1. Making embryo media

1.1. To make a 20x stock of embryo media, dissolve 17.5 g of NaCl, 0.75 g of KCl, 2.9 g of $CaCl_2$, 0.41 g of K_2HPO_4, 0.142 g of NA_2HPO_4, and 4.9 g of $MgSO_4 \cdot 7H_2O$ in 1 L of water. Ignore the white precipitate that forms; this will not impact the media. Filter sterilize the stock solution and store at 4 °C.

1.2. To create the working embryo media solution, dissolve 1.2 g of $NaHCO_3$ in 1 L of 20x embryo media stock and add 19 L of water. Maintain the working embryo media solution at 28 °C.

2. Measuring the embryonic volume using water displacement

NOTE: In this protocol, 24 h postfertilization (hpf) embryos (**Figure 1**) are used. The embryos used in the volume measurements are not used in the ethanol analysis.

2.1. Place 10 embryos and extraembryonic fluid in 1.5 mL microcentrifuge tube marked at a volume of 250 µL (**Figure 2A**). Add water to the 250 µL fill line (see sample with dyed water in **Figure 2B**).

2.2. Repeat step 2.1 to set up receptacles for embryos that have had their chorions removed.

2.2.1. To remove the chorion, place the embryos in their chorions in a 100 mm Petri dish with 2 mg/mL of protease cocktail in embryo media at room temperature (RT) for 10 min. Every few minutes, gently swirl the embryos to break the chorion.

2.2.2. Once all the embryos are free of their chorion, remove the protease cocktail/embryo media and replace with fresh embryo media to wash the embryos. Repeat the wash step 2x. Transfer the embryos to a fresh 100 mm Petri dish.

2.3. Using the smallest tip possible and without damaging the embryos, carefully remove all liquid from around the embryos using a p200 micropipettor (**Figure 2C**) and weigh the water with a scale with <0.1 mg precision. To determine the volume of the sample of 10 embryos, subtract the weight/volume of the water (1 mL of water = 1 g of water.) removed from 250 µL. To determine the volume of a single embryo, divide the difference between 250 µL and the weight of the water removed by 10.

$$Volume\ of\ the\ sample\ of\ 10\ embryos = 250\ \mu L - weight\ of\ water\ removed$$

$$Volume\ of\ an\ individual\ embryo = volume\ of\ the\ sample\ of\ 10\ embryos \div 10$$

3. Treating embryos with ethanol

3.1. Gather embryos from mating tanks and place in a standard 100 mm Petri dish. Count out no more than 100 embryos per single Petri dish and incubate at 28.5 °C.

NOTE: If the chorion is to be removed (step 2.2), it needs to be removed before adding ethanol.

3.2. At 6 hpf, add up to 100 embryos to a new standard 100 mm Petri dish either with embryo media or embryo media + 1% ethanol (v/v). Cover, but DO NOT seal the Petri dish. Place the embryos in a low temp incubator set at 28.5 °C for 18 h, or until the embryos reach the developmental time point of 24 hpf.

4. Preparing workflow before processing the embryos for head space gas chromatography

4.1. Make a solution of 5 M NaCl in water (450 µL will be needed per sample tube) to denature all proteins and prevent ethanol metabolism. Make the protease cocktail (**Table of Materials**) at a concentration of 2 mg/mL in embryo media.

4.2. Label a 1.5 mL microcentrifuge tube and a 2 mL gas chromatograph vial for each sample. Label additional 2 mL gas chromatograph vials for the ethanol standards described below as well as air, water, and the 5 M NaCl/protease cocktail blanks.

4.3. Set up three p200 micropipettors two set to 50 µL, and the third set to 200 µL; a p1000 micropipettor set to 450 µL and a p2 micropipettor set to 2 µL. For the glass pipettes used to transfer embryos from the Petri dishes to the 1.5 mL microcentrifuge tubes, quickly pass the pipette tip through a flame to smooth the edges to not damage the embryos when drawing them into the pipette.

5. Processing embryos for head space gas chromatography

NOTE: Both embryos in their chorions and those previously removed from their chorions are treated the same for consistency in the calculation of dilution factors.

5.1. Using the two p200 micropipettors set to 50 µL, draw 50 µL of the protease cocktail solution into one and draw 50 µL of water into the second.

5.2. Using the glass pipette and micropipettor, quickly place 10 embryos (from step 3.2) in a 1.5 mL microcentrifuge tube and close the cap (as described in **Figure 2A**). Repeat for all samples to be tested, controls, and ethanol treated embryos.

5.3. Using the p200 micropipettor set to 200 µL, quickly open the cap and remove all residual embryo media (as described in **Figure 2C**). Quickly place the pipette containing 50 µL of water in the tube and rapidly but gently (to not damage the embryos) add, then remove the water. Quickly add 50 µL of the protease cocktail solution from the waiting pipettor and close the tube cap (**Figure 2D**).

NOTE: Perform this process one sample at a time.

5.4. Let the sample sit at RT for 10 min to allow the protease cocktail to degrade the chorion. Then, quickly add 450 µL of 5 M NaCl and close the tube cap (**Figure 2E**). Vortex the samples for 10 min. To speed up the process, set up the mixer to vortex multiple samples at the same time.

NOTE: Add a small amount (~100 µL) of a silica bead mixture (2 sizes, 0.5 mm and 1 mm bead) to any tube with embryos older than 24 hpf. In older embryos, the notochord will remain intact regardless of how long they are homogenized.

5.5. After homogenizing for 10 min, quickly remove 2 µL of homogenized embryo supernatant and add to a gas chromatograph vial. Quickly seal the vial with the polytetrafluoroethylene cap.

6. Preparing media and ethanol standards

6.1. To prepare the media standards, dilute the media by a factor of 10 with a 5 M NaCl/protease cocktail solution. Add 2 µL of each sample to a gas chromatograph vial and seal with a polytetrafluoroethylene cap.

6.2. To prepare the ethanol standards, create a serial dilution of 100% ethanol in 5 M NaCl/protease cocktail solution to the following concentrations: 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, and 40 mM. Add 2 µL of each standard to a gas chromatograph vial and seal with a polytetrafluoroethylene cap.

7. Preparing the head space gas chromatograph

NOTE: This setup and protocol may need to be changed depending on the gas chromatograph used. Head space gas chromatography is used to quantify ethanol levels, not for separation.

7.1. Set the heater for the autosampler to 58 °C and turn on. Allow the heater to reach 58 °C, and turn on the air and hydrogen gas lines feeding the gas chromatograph (for flame ionization used to quantify the ethanol).

NOTE: The psi should be set to properly operate the chromatograph according to the manufacturer's specifications. Make sure the helium line is on and set to the proper psi.

7.2. For the septum, make sure the number of samples injected is not >100. For the injection fiber, make sure the number of samples injected is not >500.

NOTE: If either is over the amount of injections, they will need to be changed before running the test samples.

7.3. Turn on the analysis software and make sure the workstation is set up properly. Store all data according to lab/department standards. Create a new sample list for the samples to be run.

7.4. Initiate the startup method and wait for the flame ionization detector to stabilize before running the samples. Once stable, run the software startup method to clean the fiber.

8. Sample measurements using head space gas chromatography

8.1. Once the startup method is complete, activate **Run Dialysates** from the methods menu in the analysis software.

8.1.1. Fill out the sample list, starting with the air, water, 5 M NaCl/protease cocktail blanks. Then enter in the standards in order, from 0.3125 to 40 mM. Follow the standards with a second round of the air, water, and 5 M NaCl/protease cocktail blanks.

8.1.2. Enter all homogenized embryo supernatant samples to be tested, from lowest to highest predicted ethanol concentration. End by entering a third and final round of the air, water, and 5 M NaCl/protease cocktail blanks.

8.2. Add the gas chromatograph vials to the autosampler in the order in which the samples were entered. Allow samples to warm for 10–15 min. Start the sample runs in the software.

8.3. After all the samples and the final blanks have run, activate the shutdown method in the software. Back up all data acquired during the sample runs. Turn off the equipment in the following order: autosampler heater, hydrogen tank and, only after the chromatograph temperature reaches 30 °C, the air tank. Leave the helium tank on to preserve the wax column.

9. Sample ethanol peak integration and sample concentration analysis

9.1. Once the shutdown method is complete, click on **View/Edit** chromatogram. Open the folder containing the results and select the samples to be integrated.

9.2. In the results, click on **Reintegrate Now**. Make sure the correct peaks have been integrated (ethanol peaks between 2 and 2.5). Once all samples have been integrated, print or export the results.

9.3. Plot the peak height of the ethanol standards on a graph. Calculate the slope, Y intercept and R^2 values for the ethanol standards (R^2 should be >0.99).

NOTE: These values will be used to determine the ethanol concentration from the sample peak height.

9.4. For each sample, subtract the Y intercept of the ethanol standards from the peak height for the sample. Divide this value by the slope of the ethanol standards to obtain the ethanol value for each sample in the GC vials.

$$Sample\ ethanol\ value = (Y\ intercept - sample\ peak\ height)/slope$$

9.5. To calculate the ethanol concentration in the embryos, first calculate the dilution factor for each sample. Take the volume measure calculated in step 2.3 of this protocol and divide it by the volume measure plus 500 µL. This represents the 5 M NaCl/protease cocktail solution added to each sample during embryo processing (steps 5.3 and 5.4).

$$Sample\ dilution\ factor = embryonic\ volume/(embryonic\ volume + 500\ \mu L).$$

9.6. Using this sample dilution factor, multiply by the sample ethanol value for each sample. The results will be in mM concentration. Calculate media reference samples by multiplying the media ethanol value by the dilution factor of 10.

$$Embryonic\ ethanol\ concentrations = \ sample\ ethanol\ value \times sample\ dilution\ factor.$$

$$Media\ concentration = media\ ethanol\ value \times 10$$

REPRESENTATIVE RESULTS:

Blood ethanol levels cannot be determined in early embryonic zebrafish, because they lack a fully formed circulatory system. To determine the level of ethanol concentration in the zebrafish embryos, the ethanol levels are measured directly from homogenized embryonic tissue. To properly measure the embryonic ethanol concentrations, the embryonic volume has to be taken into account. The embryo (yolk attached) sits inside the chorion (eggshell) surrounded by extraembryonic fluid (**Figure 1**). Any volume measure of the embryo has to be made on embryos at the time of exposure, because embryonic volume will change over developmental time as the embryo increases in size. In addition, how the embryo is processed has to be taken into account as well as the embryonic volume, because both of these elements create dilution factors used to calculate embryonic ethanol levels.

Because the embryo is not a perfect sphere, especially after 10 hpf, water displacement was used to assess embryonic volume. This study used embryos at 24 hpf. The volume of embryos both in their chorion and those removed from their chorion were measured. These volumes were 1.97 (± 0.4 SD) µL for the embryo with extraembryonic fluid, and 1.1 (± 0.22 SD) µL for the embryo alone (**Table 1**). This difference between the embryo with extraembryonic fluid and the embryo alone is the volume of the extraembryonic fluid, which surrounds the embryo inside the chorion (**Figure 1**). This difference is critical in determining the ethanol concentration of the embryo alone in the samples. From the analysis, the embryo comprised 56% of the total of the volume of the embryo with extraembryonic fluid inside the chorion (**Table 1**). As the embryo grew, the embryonic volume increased over time, resulting in a decrease in the volume of the extraembryonic fluid[25]. When measuring ethanol concentrations from embryos still in their chorion, the water content in both the embryo and the extraembryonic fluid must be taken into account.

Previously published work has shown that the water fraction in the embryo alone is 73.6%[29]. This result was obtained using a combination of volume measures, electron spin resonance spectroscopy, and magnetic resonance microscopy. To confirm this result, embryos were weighed alone before and after baking at 70 °C, as previously described[25]. This process removes all water from the embryo. The change in weight represents the amount of water in the embryo. From this analysis, a 24 hpf embryo contained ~72% water while a 48 hpf embryo contained ~76%. Both of these values are extremely similar to the 73.6% embryonic water volume previously calculated[29]. This suggests that the water volume of the embryo changes little throughout early development.

Based on the published work using multiple approaches to calculate water volume in the embryo, 73.6% was used as the embryonic water content for all of the presented analyses. This resulted in water comprising 0.82 µL of the 1.1 µL of embryonic volume (**Table 1**). From this, the volume of the extraembryonic fluid was calculated as 0.86 µL. Of the total water volume of the embryo with extraembryonic fluid, 49% is contained in the embryo and 51% is in the extraembryonic fluid (**Table 1**).

For the gas chromatography analysis, only 24 hpf embryos still in their chorion were used (**Figure 1**). In this ethanol regimen, embryos were treated with 1% (171 mM) ethanol media concentration from 6–24 hpf, though different ethanol concentrations and exposure time windows can be used depending on experimental design. Two groups of 15 samples (10 embryos per sample) and the media with which they were treated over 2 different experimental days (**Table 2**) were analyzed. Media levels can vary from experiment to experiment. To account for this, media ethanol levels were measured 5x per experimental group. In this analysis, media levels were 143.6 (± 2.3 SD) mM for group 1 and 133.6 (± 2.7 SD) mM for group 2. These values are significantly different using an unpaired *t* test ($p = 0.0002$). The embryos with extraembryonic fluid averaged 63.5 (± 17.1 SD) mM and 53.1 (± 12.6 SD) mM for groups 1 and 2, respectively. However, the concentration was not significantly different between the 2 groups of embryos with extraembryonic fluid (unpaired *t* test, $p = 0.07$). Untreated control embryos were measured at 0 mM. Due to the variation in media levels, a ratio of embryonic ethanol concentration/media levels of ethanol for each sample was calculated. Group 1 had 44% of the media ethanol levels, while group 2 had 40% of the media ethanol levels (**Figure 3** and **Table 2**).

FIGURE AND TABLE LEGENDS:

Figure 1. An image of an embryo at 24 h postfertilization (hpf) inside the chorion. The embryo and the yolk are surrounded by the extraembryonic fluid, all located inside the chorion.

Figure 2. Protocol to measure embryonic volume and process the embryos for analysis. (**A**) Ten 24 hpf embryos transferred to a 1.5 mL microcentrifuge tube marked at a 250 µL volume. (**B**) The microcentrifuge tube with embryos filled with water (water is dyed so it is easier to see at the 250 µL mark). (**C**) All of the water is removed from the tube and weighed. (**D**) Ten embryos transferred to a 1.5 mL microcentrifuge tube. The water was removed as in (**C**) and replaced with 50 µL of protease cocktail. (**E**) After 10 min, 450 µL of 5 M NaCl was added to the tube from (**D**).

Figure 3. Box and whisker plot of the ratio of ethanol concentration of samples to media. Ethanol concentration for each group was divided by the average of the media samples of that group. Group 1 contained 44% of media levels, while group 2 contained 40%. In both groups, $n = 15$; 10 embryos per sample. Groups were compared using a one-way ANOVA with a Tukey's post hoc analysis (****$p < 0.0001$). The whiskers represent minimum to maximum.

Table 1. Calculation of water volume for the embryos with extraembryonic fluid. The volume was calculated from 13 samples (10 embryos per sample) from 24 hpf embryos with extraembryonic fluid still in the chorion and embryos removed from their chorion. Values are reported as the mean ± SD.

Table 2. Calculations of ethanol concentration in embryos with extraembryonic fluid and media (mM). Media samples were direct measures from the media (n = 5 per group). Embryonic ethanol concentration was calculated from 15 samples (10 embryos per sample) from 24 hpf embryos with extraembryonic fluid. The ratio of the ethanol concentrations of the embryos with extraembryonic fluid to media were calculated. Values are reported as the mean ± SD.

Table 3. Calculation of ethanol in the embryo. Embryonic ethanol concentration was calculated as 56% of the total ethanol concentration of the embryo with extraembryonic fluid. This was then reported as a ratio of embryo to media. Values are reported as the mean ± SD.

DISCUSSION:

As a developmental model system, zebrafish are ideally suited to study the impact of environmental factors on development. They produce large numbers of externally fertilized embryos, which allows for precise timing and dosage paradigms in ethanol studies. This, combined with the live imaging capabilities and the genetic and developmental conservation with humans, make zebrafish a powerful model system for teratology studies. Described is a protocol for measuring embryonic ethanol concentrations in developing zebrafish embryos using head space gas chromatography.

Determining embryonic ethanol concentration is critical to understanding the impact of ethanol to the developing embryo. In rodent models, blood ethanol concentrations correlate to tissue concentrations[26]. However, it is not possible to assess blood ethanol levels in early embryonic development in a zebrafish. Head space gas chromatography has been used to measure ethanol levels in various experimental paradigms, including zebrafish[25,30–35]. However, this protocol can be used to measure many different environmental factors, even though the volatility of these various factors needs to be high enough to measure quantitatively and a gas chromatograph column needs to be chosen based on the polarity of the compounds in question. In addition, zebrafish are best suited to study the impact of water-soluble factors, because exposure to hydrophobic factors is extremely difficult to analyze in fish.

This protocol will allow researchers to directly assess ethanol concentrations in zebrafish embryos. However, several factors have to be addressed to ensure accurate measurements of embryonic ethanol concentrations. Because ethanol levels in total embryonic tissues were measured, embryonic volume had to be considered. After 10 hpf the embryo begins somitogenesis (somite formation) and is no longer a sphere (**Figure 1**). To assess embryonic volume, water displacement was used on 10 pooled embryos per sample. Each sample consisted of 10 embryos for two reasons: first, to reduce error from pipetting small volumes, and second, to average out small fluctuations in embryo volumes. When measuring embryonic volume, it is important to consider the precision of pipetting and weighing. Even the use of 10 embryos per sample is at the lower limit of precision for both the pipettes and the scale used to weigh the embryos. With the equipment available, using fewer than 10 embryos would have impacted the analyses. This does not limit researchers from using fewer embryos per sample as long as equipment precision is considered.

Embryonic volume is one factor impacting sample analysis. A second key factor is embryonic processing speed, because ethanol is volatile. In addition, ethanol quickly equilibrates in multiple animal studies[25,36–37]. Our wash protocol is designed to quickly remove any ethanol adhering to the exterior surface of the chorion. In this study this wash protocol did not impact the embryonic ethanol levels, though longer or multiple washes could impact embryonic ethanol concentrations[25,38]. Yet a third factor to take into account is the dilution of the embryonic volume during processing. This protocol uses a protease cocktail to degrade the chorion as well as 5 M NaCl to denature all proteins, including alcohol processing enzymes.

Finally, a range of analyses and reporting methods have been described in a wide variety of zebrafish studies. Due to variation within different samples of a study or comparing different studies, proper reporting of ethanol concentration is critical[25,38]. Methods using indirect (usually enzymatic measures) rely on analysis of a secondary metabolite. In that case, the rate of enzymatic activity can vary and impede precise analysis of ethanol concentration. This method directly measures ethanol levels, and ethanol concentrations are reported as a ratio of embryo to media concentrations. Our results are in line with a growing consensus of a 24 hpf embryo containing ~30% of media concentrations (**Table** 3)[25,38–40]. Surprisingly, this 30% embryonic ethanol concentration is independent of media concentration, and it is not well understood what creates this equilibrium[25,38]. Embryos older than 24 hpf show decreases in ethanol concentrations, with 48 hpf embryos containing nearly half of the ethanol content of a 24 hpf embryo[25]. However, our results do not determine the ethanol-water equilibrium. Given the speed at which ethanol equilibrates, as well as the volatility of ethanol, it is incredibly difficult to assess water volume changes in the embryo itself due to ethanol exposure. Trying to measure the volume of an ethanol treated embryo sample using water displacement will result in loss of ethanol from the embryo during the measurement. Using more precise microscopy and spectroscopy methods than those described above, it may be possible to determine the ethanol-water equilibrium in an embryo by directly measuring both at the same time.

Overall, the protocol described here is a powerful tool with which to assess embryonic ethanol concentrations in developing zebrafish embryos. This information is critical in standardizing FASD studies using zebrafish. The ability to precisely control ethanol treatment paradigms and directly quantify embryonic ethanol concentrations strengthens the functionality of the zebrafish model for human FASD studies. Ultimately, this work will greatly improve our understanding of FASD.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

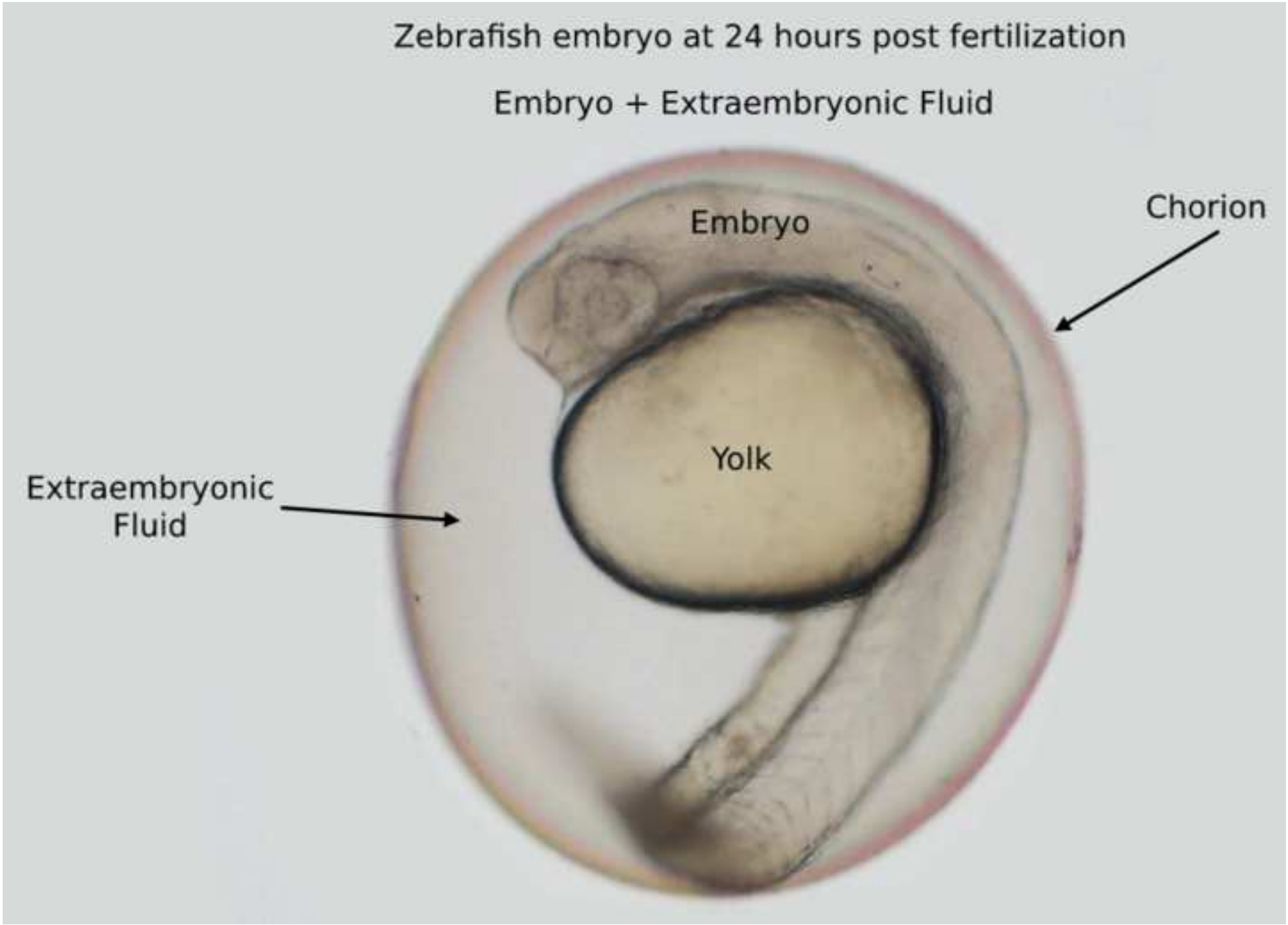


Figure 2

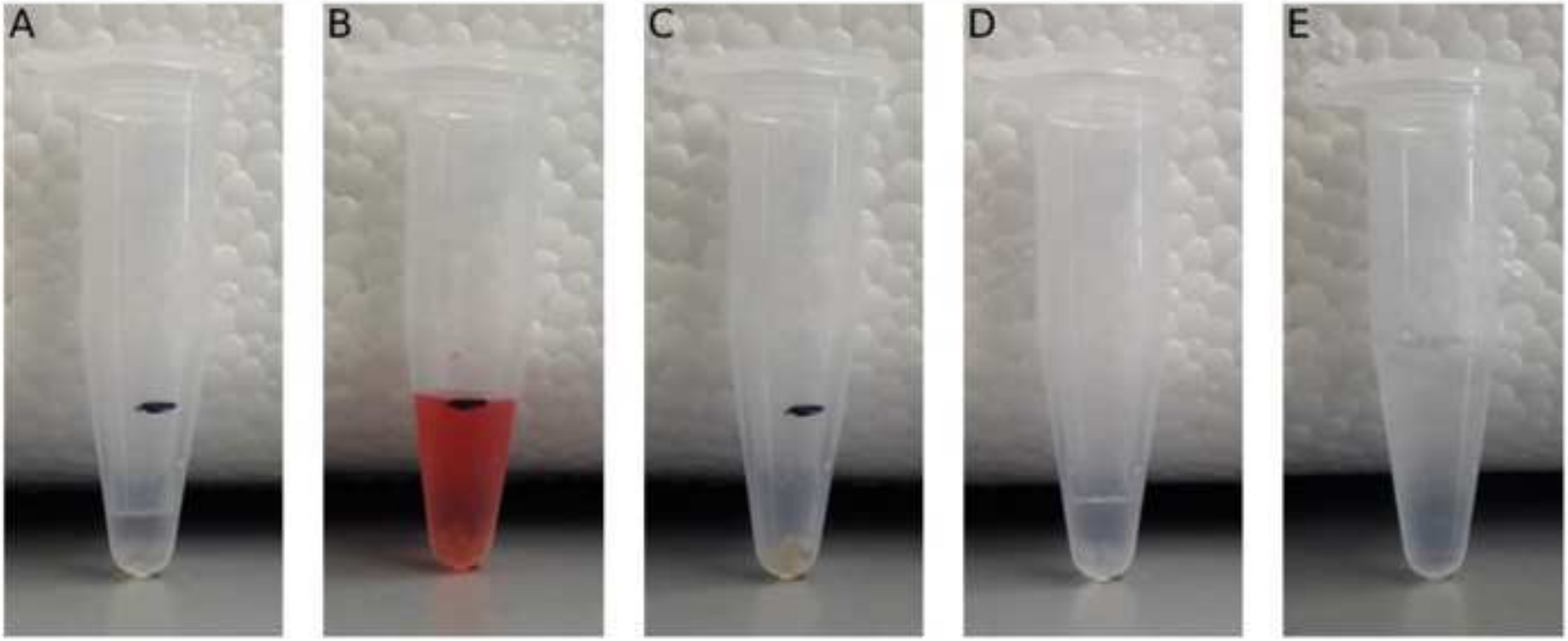
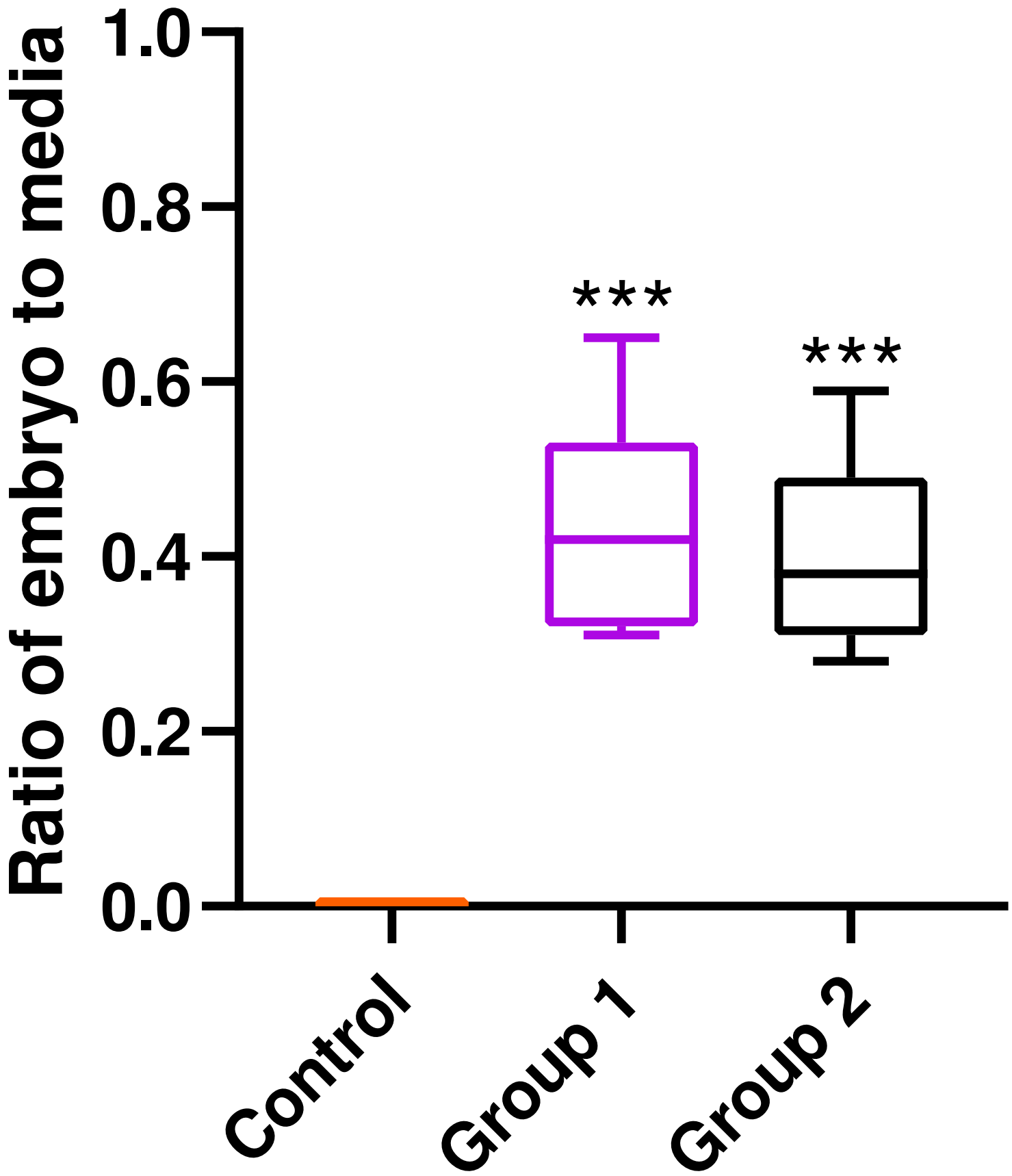


Figure 3

Click here to access/download;Figure;Figure 3.eps



	Embryo and Embryo + Extraembryo Water Volumes (µL)				
			Water[a]		
	Total Volume[b]	% of Total Volume (V)	Embryonic Volume (W)	Extraembryonic Volume (X)	% Water in Embryo (Y)
Embryo + Extraembryonic (EE)[c]	1.97 ± 0.4				
Embryo 24 hpf (E)[c]	1.11 ± 0.22	56%	0.82	0.86	49%
	Calculations				
[V] $Embryo\ \%\ of\ Total\ volume = Total\ Voume\ of\ E \div Total\ Volume\ of\ EE$					
[W] $Embryonic\ Water\ Volume = V \times 73.6\%$					
[X] $Extraembryonic\ Water\ Volume = W - Total\ Volume\ of\ EE$					
[Y] $\%\ Water\ in\ Embryo = (W + X)/W \times 100$					
[Z] $\%\ Water\ in\ Extraembryonic = 100 - Y$					

[a]Embryonic water volume was calculated from Hagedorn et al.[29].

[b]Values are reported as ± standard deviation (SD).

[c]n = 13; 10 embryos per sample

% Water in Extraembryonic (Z)
51%

Ethanol Concentration of embryos and media (mM)[a]			
	Group 1	*Group 2*	*Combined*[d]
Media (M)[b]	143.6 ± 2.3	133.6 ± 2.7	138.6 ± 5.8
Embryo + Extraembryonic (EE)[c]	63.5 ± 17.1	53.1 ± 12.6	58.3 ± 15.7
Ratio of ethanol - embryo to media (1)	0.44 ± 0.12	0.40 ± 0.09	0.42 ± 0.11

Calculations
[1] $Ratio\ of\ ethanol = EE \div M$

[a]Values are reported as ± SD.

[b]n = 5

[c]n = 15; 10 embryos per sample

[d]Values are the average of groups 1 and 2.

Calculation of ethanol in the embryo[a,b]	
Embryo (Y)	*Ratio to media (Z)*
32.7 ± 8.8 mM	0.24 ± 0.06
Calculations	
[Y] $Embryonic\ Ethanol = 58.3\ mM\ (Table\ 2) \times 56\%\ (Table\ 1)$	
[Z] $Ratio\ of\ ethanol = Y \div 138.6\ mM\ (Table\ 2)$	

[a]Values are the average of groups 1 and 2.

[b]Values are reported as ± SD.

Materials

Name	Company	Catalog Number	Comments/Description
Air			Provided by contract to the university
Analytical Balance	VWR	10204-962	
AutoSampler, CP-8400	Varian		Gas Chromatopgraph Autosampler
Calcium Chloride	VWR	97062-590	
Ethanol	Decon Labs	2701	
Gas chromatograph vial with polytetrafluoroethylene/silicone septum and plastic cap 2 mL	Agilent	8010-0198	Can reuse the vials after cleaning, but not the caps/septa
Gas Chromatograph, CP-3800	Varian		Gas Chromatopgraph
Helium			Provided by contract to the university
HP Innowax capillary column	Agilent	19095N-123I	30 m x 0.53 mm x 1.0 µm film thick
Hyrdogen			Provided by contract to the university
Magnesium Sulfate (Heptahydrate)	Fisher Scientific	M63-500	
Microcentrifuge tube 1.5 mL	Fisher Scientific	2682002	
Micropipette tips 10 µL	Fisher Scientific	13611106	
Micropipette tips 1000 µL	Fisher Scientific	13611127	
Micropipette tips 200 µL	Fisher Scientific	13611112	
Petri dishes 100 mm	Fisher Scientific	FB012924	
Pipetman L p1000L Micropipette	Gilson	FA10006M	
Pipetman L p200L Micropipette	Gilson	FA10005M	
Pipetman L p2L Micropipette	Gilson	FA10001M	
Polytetrafluoroethylene/silicone septum and plastic cap	Agilent	5190-7021	Replacement caps/septa for gas chromatograph vials
Potassium Chloride	Fisher Scientific	P217-500	
Potassium Phosphate (Dibasic)	VWR	BDH9266-500G	
Pronase	VWR	97062-916	
Silica Beads .5 mm	Biospec Products	11079105z	
Silica Beads 1.0 mm	Biospec Products	11079110z	
Sodium Bicarbonate	VWR	BDH9280-500G	
Sodium Chloride	Fisher Scientific	S271-500	
Sodium Phosphate (Dibasic)	Fisher Scientific	S374-500	

Solid-phase microextraction fiber assembly Carboxen/Polydimethylsiloxane	Millipore Sigma	57343-U	Replacement fibers
Star Chromatography Workstation	Varian		chromatography software
Thermogreen Low Bleed (LB-2) Septa	Millipore Sigma	23154	Replacement inlet septa

We would like to thank the editorial reviewers of our manuscript “Quantification of ethanol levels in zebrafish embryos using head space gas chromatography” for their insightful and constructive criticism. We are glad that the reviewers felt that the manuscript was describing an important protocol and wish to thank the reviewers for the suggestions to strengthen the manuscript. We feel that we have addressed all the concerns raised by the reviewers, which has made for a stronger, more rigorous manuscript. All changes recommended by the reviewers are highlighted by track changes.

Editorial Comments:

1. Are these embryos used later in the experiment, e.g., in section 3?

We have added text in the Note from step 2 to state “The embryos used in this step (determination of embryonic volume) are not used in later sections.”

2. How to remove chorions? According to step 5.4?

We have added text explaining how to remove the chorion using the protease cocktail. This is not the exact same procedure as described in step 5.4. The added text states “To remove the chorion place the embryos in their chorions in a 100 mL Petri dish with 2 mg / mL of protease cocktail in embryo media at room temperature for 10 minutes. Every few minutes, gently swirl the embryos to break the chorion. Once all the embryos are free of their chorion, remove the protease cocktail / embryo media and replace with fresh embryo media to wash the embryos. Repeat the wash step two more times. Transfer the embryos to a fresh 100 mL Petri dish.”

3. Why do you need two different types of embryos (i.e., with or without chorions removed)? Can you explain this a bit?

To explain the reason for using both embryos with and without the chorions removed we modified text in the results section (lines 271-278). “We measured the volume of both embryos in their chorion and those removed from their chorion. We measured these volumes at 1.97 (+/- 0.4 S.D.) µL for the embryo + extraembryonic fluid and 1.1 (+/- 0.22 S.D.) µL for just the embryo (Table 1). This difference between the embryo + extraembryonic fluid and the embryo alone is the volume of the extraembryonic fluid, which surrounds the embryo inside the chorion (Figure 1). This difference is critical in determining the ethanol concentration of the embryo alone in our samples. From our analysis, this results in the embryo comprising 56% of the total of the volume of the embryo + extraembryonic fluid inside the chorion (Table 1).”

4. Please specify the time points tested in this protocol.

We have corrected the text in step 3.2 to state that the at 6 hpf the embryos were treated with ethanol.

5. Please give some examples (of ethanol concentration).

We also state in step 3.2 that the embryos were treated with 1% ethanol v/v (in embryo media).

6. For how long are the embryos treated with ethanol?

In step 3.2 we state that the embryos were treated for “…18 hours or until the embryos reach the developmental time point of 24 hpf.”

7. From which step? Are they treated with ethanol?

In step 5.2 we now state that “…ten embryos (from step 3.2)…” and that this step should be repeated “…for all samples to be tested, both control and ethanol treated embryos.”

8. I suggest un-highlight this section because the steps are routine procedures when using the gas chromatography instrument.

As suggested by the reviewer, we have un-highlighted step 7.

9. Is this a menu to be selected?

For clarification in step 8.1, we have now stated “Once the startup method is complete, activate “run dialysates” from the methods menu in the analysis software”

10. Do you mean homogenized embryo supernatant?

We have clarified the text in step 8.1.2 to state “all homogenized embryo supernatant samples.”

11. Correct?

In step 9.5 the reviewer added the text …(steps 5.3 and 5.4 of this protocol)... Yes this is correct and properly clarifies step 9.5. We thank the reviewer for adding this text.