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Tissue extraction methods for metabolic profiling of a freshwater bivalve, *Elliptio complanata* (Lightfoot, 1786)

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Abstract: Much is still unknown about why freshwater mussels (Unionidae) are particularly sensitive to environmental change. A better understanding of freshwater mussel metabolism is needed, and the field of environmental metabolomics holds the promise to inform these questions. A number of protocols exist for the extraction of metabolites for identification from animal tissues. As a first step in the application of environmental metabolomics to the study of freshwater mussels, we compared extraction protocols using an inorganic oxidizing acid (perchloric acid), an organic nitrile (acetonitrile), and a salt/water solution (Ringer's solution) to establish an uncomplicated, robust, repeatable and inexpensive tissue extraction protocol for freshwater mussel tissue. Perchloric acid resulted in notable extraction of energy-related nucleotides (AMP/ADP/ATP), yet had the lowest peak count of the three extraction methods and showed poor repeatability. Acetonitrile and Ringer's solution yielded metabolite extraction results similar to each other with Ringer's solution having the greatest number of peaks particularly in the 3.0–4.5 ppm sugar/amino acid range. Ringer's solution is simple to use, safe and consistent and bears consideration when selecting an extraction protocol for ^{1}H nuclear magnetic resonance experiments.

Key words: freshwater bivalve, metabolomics, NMR, nuclear magnetic resonance, Ringer's solution

The imperiled status of freshwater mussel populations worldwide is due to a suite of physical and chemical environmental changes including pollution, habitat change and loss of food resources (Bogan 1993). One of the challenges in understanding the effects of these impacts is determining which single or combination of factors are affecting a given population or individual. A growing body of knowledge has aided in the diagnostic capability of investigators, yet there is still much we do not understand about freshwater mussel metabolism, the effects of noxious stimuli, and how to detect signs of illness, especially in the early stages.

Environmental metabolomics, defined as the measurement of metabolic response to external stimuli, characterizes the interaction between the organism and its environment by measuring physiological changes at a molecular level (Morrison *et al.* 2007, Bundy *et al.* 2009). Environmental metabolomics techniques have been used to evaluate a wide range of taxa. Among other contributions, metabolomics has been used to: 1) detect withering disease in abalone (*Haliotis rufescens* (Swainson, 1822)) (Viant *et al.* 2003), 2) characterize heat stress and recovery in fruit flies (*Drosophila melanogaster* (Meigen, 1830)) (Malmendal *et al.* 2006), 3) distinguish between earthworms (*Lumbricus rubellus* (Hoffmeister, 1843), *Lumbricus terrestris* (Linnaeus, 1758) and *Eisenia andrei* (Bouché, 1972)) from a metal contaminated and non-contaminated site (Bundy *et al.* 2004) and 4) suggest potential biomarkers for disease in whale sharks (*Rhincodon typus* (Smith, 1829)) (Dove *et al.* 2012). Recently, metabolomic profiling has been used to identify factors that may be contributing to the global decline in freshwater mussels (Unionidae) (Leonard *et al.* 2014, Roznere *et al*. 2014). Data from environmental metabolomic studies can inform a wide range of ecological and environmental studies (Miller 2007, Bundy *et al.* 2009), and elucidate important life history questions (Tikunov *et al.* 2010, Dove *et al.* 2012). Metabolomic and metabonomic techniques evaluate tens to thousands of endogenous, low molecular weight

(< 1000 Da) organic metabolites as they occur within a cell, tissue, whole organism or biofluid, providing a snapshot of physiologic status for an organism (Nicholson *et al.* 1999, Viant 2007, Bundy *et al.* 2009). These measurements can then be mechanistically related to the organism's phenotype by considering established metabolic pathways to generate a robust multi-dimensional image of health status, in contrast to the limited information obtained when measuring a single biomarker. The value of the data yielded in this process is directly dependent on the use of reliable and repeatable sampling and processing techniques.

A metabolome is comprised of fatty and amino acids, vitamins, lipids, carbohydrates and other organic intermediate and end products of metabolic processes; each with distinct chemical characteristics that affect their stability and behavior in solution (Dunn and Ellis 2005). A number of protocols exist to extract these metabolites from animal tissues (Table 1); however, to date there is no universally accepted technique (Lin *et al.* 2007, Viant 2007, Liebeke and Bundy 2011). For example, some extraction solvents selectively solubilize polar metabolites, while others are more efficient at extracting non-polar compounds (Lin *et al.* 2007). Still others precipitate large macromolecules that may interfere with identification of smaller metabolites resulting in improved spectral resolution (Lin *et al.* 2007). The ideal protocol for environmental metabolomic studies would be one that reproducibly and efficiently extracts a broad range of metabolites classes, does not destroy or modify the metabolites during processing, and is safe for the user and the environment (Maharjan and Ferenci 2003, Lin *et al.* 2007, Barton *et al.* 2008, Liebeke and Bundy 2011).

As a preliminary step in the evaluation of metabolomics as a tool to evaluate freshwater mussel health and further our knowledge about freshwater mussel metabolism, we present a comparison of tissue extraction protocols for obtaining the

Table 1. Comparative studies of extraction methods. Asterisk indicates method(s) recommended by article authors.

Species	Extraction Method	Reference
Mammal cells	Acetonitrile*	Dietmair *et al.* 2012
	Methanol (freeze)*	
	Cold 50% methanol*	
	Methanol/chloroform*	
	Hot 80% methanol	
	Cold 100% methanol	
	Hot ethanol	
	Hot ethanol with HEPES	
	Cold ethanol	
	Hot water	
	Potassium hydroxide	
	Perchloric acid	
Fish muscle and liver	Cold perchloric acid	Lin *et. al.* 2007
	Acetonitrile/water (1:1)*	
	Acetonitrile/water (2:1)*	
	Methanol/water (1:1)	
	Methanol/water (2:1)*	
	Methanol/chloroform/water*	
	Methanol/chloroform/water with KCl	
Fish liver	Step-wise methanol/chloroform/water	Wu *et. al.* 2008
	Two-step methanol/chloroform/water*	
	All-in-one methanol/chloroform/water	
Earthworms	Chloroform/methanol	Liebeke and Bundy 2011
	Aqueous acetonitrile/methanol*	
	Aqueous isopropanol/methanol	
	Hot aqueous ethanol	
Earthworms	D2O buffer**	Brown *et al.* 2008
	Acetonitrile-d3	
	Benzene-d6	
	Chloroform-d	
	Methanol-d4*	
	DMSO-d6*	

^{1}H -Nuclear Magnetic Resonance (NMR) metabolic profile of *Elliptio complanata* (Lightfoot, 1786), a freshwater mussel common in the Eastern United States. To establish an uncomplicated, robust, repeatable and inexpensive tissue extraction protocol for freshwater mussel tissue, we evaluated two solvents previously used to extract metabolites from other bivalve species, perchloric acid (Tikunov *et al.* 2010) and acetonitrile (Tuffnail *et al.* 2009), and a less toxic extraction protocol using Ringer's solution (Gibb 1997a,b Bundy *et al.* 2002). We compared the extraction protocols by: 1) analyzing the supernatants immediately after homogenization and after an additional 30 minute incubation time; 2) comparing the sum of the results of multiple serial extractions with the first extraction fraction of each sample and; 3) measuring the variability in the metabolic profiles observed between and within individual animals.

MATERIALS AND METHODS

All chemicals were purchased through Fisher Scientific (Waltham, MA, U.S.A.).

Three adult Eastern Elliptio (*Elliptio complanata*) were collected from the Eno River (Neuse River Basin), near Hillsborough (Orange County), NC and transported in river water to the Aquatic Epidemiology and Conservation Laboratory at North Carolina State University College of Veterinary Medicine, Raleigh, NC. An oyster shucking knife was used to open the valves of the mussel and excise the mantle and adductor muscles from one valve. The anterior and posterior adductor muscles were sharply excised and placed into a 15 ml polyethylene tube and snap frozen in liquid nitrogen within 10 seconds of opening the shell.

The frozen muscle tissues from each animal were divided into eight portions and weighed to the nearest milligram. Sectioning and weighing were done at room temperature, with each sample out of liquid nitrogen for approximately 45 seconds. Each sample was placed individually into a 1.5 ml polyethylene microcentrifuge tube and assigned a treatment by extraction solvent and time protocol (Table 2) and replaced into liquid nitrogen.

For metabolite extraction, 500 µL of the extraction solvent (Ringer's solution, Acetonitrile/deionized water 40%, Perchloric Acid/deionized water 70%) was added to each sample. All solvents were maintained at room temperature, and samples were allowed to thaw during homogenization. Homogenization beads (200 µL 0.9–1.6 mm stainless steel beads, Next Advance) were added and the tubes were placed in a Bullet Blender homogenizer (Next Advance, Averill Park, NY, U.S.A.) and homogenized for 7 minutes. Additional beads (200 µL 2 mm zirconium oxide, Next Advance) were added to any samples that contained identifiable tissue pieces and an additional 3 minutes of homogenization was performed. Samples were considered completely homogenized once the tissue sample was reduced to a liquid consistency. Samples were retained on ice between and after homogenization steps.

Samples designated for Time Protocol A were centrifuged at room temperature (AccuSpin Micro 17, Fisher Scientific, Waltham, MA, U.S.A.) within 5 minutes after homogenization at 13,500 rpm for 20 minutes to separate the tissue from the supernatant. The supernatant was collected and placed in a 4 °C refrigerator. An additional 500 µL of extraction solvent was added to the remaining tissue pellet. The samples were then vortexed for approximately 30 seconds until the pellet was resuspended and incubated in a 4 °C refrigerator for 30 minutes. Centrifugation was repeated, the supernatant collected and a third volume of solvent added. The vortexed samples were incubated for a second thirty minutes, centrifuged and supernatant collected.

Samples designated for Time Protocol B were vortexed and placed in a 4 °C refrigerator to allow the tissue to incubate

Table 2. Designation of treatment of each mussel by Solvent, Time Protocol, and Replicate. Includes tissue sample weight. * indicates a sample not included in final analysis due to poor spectral quality from contaminant or other signal interference.

	Mussel W		Mussel X		Mussel Y	
Sample	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)	Solvent, Time Protocol, (Replicate)	Tissue weight (mg)
1	Ringer's, A (a)	119	Ringer's, A	47	Ringer's, A	84
2	Ringer's, A (b)	92	Ringer's, B	56	Ringer's, B	89
3	Ringer's, A (c)	108	Acetonitrile, A (a)	49	Acetonitrile, A	83
4	Ringer's, B	79	Acetonitrile, A (b)	65	Acetonitrile, B	155
5	Acetonitrile, A	85	Acetonitrile, A (c)	60	Perchloric Acid, A (a)	101
6	Acetonitrile, B	104	Acetonitrile, B	55	Perchloric Acid, A (b)	81
7	Perchloric Acid, A	81	Perchloric Acid, A	44	Perchloric Acid, A (c)	90
8	Perchloric Acid, B	97	Perchloric Acid, B	36	Perchloric Acid, B	130*

in the extraction solvent for 30 minutes. After incubation, the samples were centrifuged at 13,500 rpm for 20 minutes at room temperature. The supernatant was collected, a second volume of solvent was added and the sample was incubated a second 30 minutes. After a second centrifugation and collection, the process was repeated a third time.

All collected Ringer's and acetonitrile extractions were frozen at -80 °C. Perchloric acid samples were buffered with 0.5 M potassium hydroxide to achieve a final pH of 7–7.4 then centrifuged at 13,500 rpm for 20 minutes to remove any precipitate. The resulting supernatant was removed and frozen.

Frozen samples were lyophilized overnight (Lyoph-Lock 18 Freeze Dry System, Laboconco, Kansas City, MO, U.S.A.), reconstituted with 700 µl 10% deuterium oxide (D_2O) solution containing 0.1 mM deuterated trimethylsilyl propionate-D4 (TSP) as an internal standard, then placed in microcentrifuge tubes. Samples were centrifuged at 13,500 rpm for 20 minutes to separate any remaining solid material. Supernatant solutions were pipetted into 5 mm borosilicate NMR tubes (Wilmad Labglass, Vineland, NJ, U.S.A.).

The pulsed field NMR experiments were performed on a Bruker AVANCE 500 MHz Spectrometer with Oxford Narrow Bore Magnet, HP XW 4200 Host Workstation, and Topspin 1.3 Software version. The instrument is equipped with three Frequency Channels with Wave Form Memory and Amplitude Shaping Unit, three Channel Gradient Control Unit (GRASP III), variable Temperature Unit, Pre-Cooling and Temperature Stabilization Unit. A 5 mm ID 1H/BB (^{109}Ag-^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.) was used for all 1D ^{1}H presaturation experiments. The NMR probe was tuned to the ^{1}H frequency of 500.128 MHz. All spectra were acquired at temperature 294 K. The instrument parameters for acquisition of the one-dimensional proton data and are listed in Table 3.

Data were analyzed using an ACD/Labs 12.0 1D NMR Processor (ACD/Labs, Toronto, Ontario, Canada). The ^{1}H spectra were zero-filled to 32,000 points, and line broadened using a 1.0 Hz exponential Gaussian function. The resulting spectra phase and baseline were corrected before integration using the ACD Intelligent Bucketing feature with a bin width of 0.04 ppm excluding water and reference peaks (total 186 bins). Spectra were included for integration and additional analysis if a clear spectrum was obtained and the TSP peak was symmetrical with a half-peak width of 0.02 ppm or less. The integral values for the first, second, and third extractions were added together for calculation of summed extraction analysis. Automatic peak counts were performed using a noise factor of 3.5 and minimum signal to noise ratio of 10.

The integral tables were normalized and Pareto scaled using Microsoft Excel 2010 (Microsoft, U.S.A.) to minimize inter-sample differences due to sample mass and to minimize effects of large amplitude differences respectively (Craig *et al.* 2006). The resulting transformed data was imported into SAS JMP v.10 (Cary, NC, U.S.A.) for principal components analysis (PCA). Through examination of a biplot PCA map, the contribution of metabolite peaks to the variation of the principal component line can be determined (Liebeke and Bundy 2011). Peak identification was performed using Chenomx NMR Suite 7.6 (Chenomx, Edmonton, Alberta, Canada) and the Human Metabolome Database (www.hmbd.ca).

Table 3. ^{1}H data collection parameters

Parameter	^{1}H value
Spectrometer frequency (MHz)	500.128 MHz
Spectral width (ppm)	13.2 ppm
Number of data points	32 K
Relaxation Delay (s)	1 s
Acquisition time (s)	2.47 s
Pulse width (µs) and tip angle	9.5 µs at 90 °
Number of transients	128
Number of dummy scans	4
Presaturation delay (s)	2 s
Presaturation power (db)	58 db

RESULTS

In this study, all three extraction solvents yielded metabolite profiles useable for metabolite identification and principal component analysis (see Hurley-Sanders *et al.* 2015 for list of metabolites). One sample (Animal Y, 30 minute incubation) was not included in the analysis due to poor spectral quality characterized by poor detection of metabolites over background noise for all extraction fractions. For each solvent, the first component of a three fraction serial extraction yielded the greatest quantity and number of metabolites with the second and third fraction yielding few measurable metabolites. Solvent type accounted for the greatest variability between samples in both the first fraction and summed extractions (Fig. 1A, B). Ringer's solution yielded the most peaks and the least variable profiles within the peaks obtained for an individual (Fig.1 A, B). No obvious effect of time-to-first-extraction was evident (Fig. 1C).

DISCUSSION

Comparison of solvents

Several studies looking at differences in extraction protocols for NMR analysis of animal tissues have displayed the diversity in techniques available and contrasting

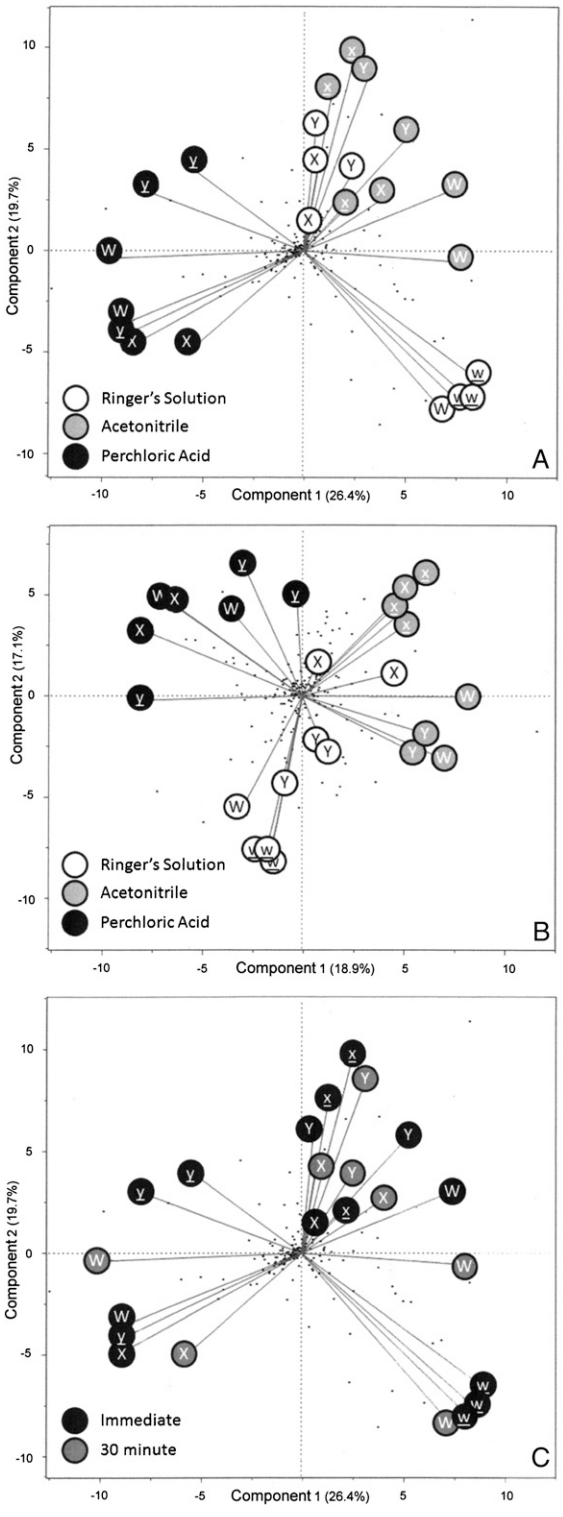

Figure 1. Principal components biplots of 1H-NMR spectra integrals showing clustering of samples with percentage variance. Letters within circles (W, X, Y) indicate the animal sampled lower case underlined letters indicate replicates of the same sample. **A,** Principal components map of solvent type calculated using first extraction of each sample. Perchloric acid samples (black) separate from other samples along PC 1. Ringer's solution (white) and acetonitrile (grey) group similarly however samples from Animal W group separately due to a strong glycogen peak. **B,** Principal components map of solvent type calculated using summed integrals from all three serial extractions of each sample with a particular solvent. With summation of the extraction fractions there is greater separation of the groups due to differences in the metabolite profile extracted by each solvent. **C,** Principal components map of time to first extraction calculated using the first extraction of each sample. There appears to be no correlation between metabolite profile and whether the extraction fluid is collected immediately after centrifugation (black) or incubated for 30 minutes prior to collection (grey).

recommendations (Table 1). Prior NMR studies using molluscan tissue reflect this diversity with use of perchloric acid (Graham and Ellington 1985, Viant 2003, Rosenblum *et al.* 2006) and Ringer's solution (Gibb 1997a) reported for gastropod tissue and perchloric acid (Tikunov *et al.* 2010), acetonitrile (Tuffnail *et al.* 2009), and methanol/chloroform with or without water (Hines *et al.* 2007, Jones *et al.* 2008, Liu *et al.* 2011a, 2011b, 2011c, Zhang *et al.* 2011a, 2011b, Kwon *et al.* 2012, Ji *et al.* 2013, 2014a, 2014b) reported for marine bivalves. Animal tissues contain a large variety of metabolites with greatly differing physical and chemical characteristics, and no single technique currently known is capable of reliably yielding full extraction of all metabolites (Viant 2003, Dunn and Ellis 2005, Lin *et al.* 2007, Fan and Lane 2008, Dietmair *et al.* 2012).

Often, mixtures of solvents are used to meet multiple protocol requirements (*e.g.*, disrupt cell membranes, fractionate metabolite classes, solubilize metabolites, precipitate macromolecules) (Fan and Lane 2008, Liebeke and Bundy 2011, Dietmair *et al.* 2012); however, this can increase the number of protocol steps and introduce variability. With this study we evaluated two commonly used extraction solvents (perchloric acid and acetonitrile) with a reported but less established physiologic solution (Ringer's solution) (Gibb 1997a, 1997b, Bundy *et al.* 2002). Our goal was to identify and characterize an easy to use, inexpensive, non-toxic protocol that gives high metabolite yield for global metabolite evaluation, and has good reproducibility.

Perchloric Acid

Perchloric acid ($HClO_4$) is a commonly used extraction solvent for NMR spectroscopy of biological samples, particularly for the extraction of nucleotides and other water-soluble metabolites including amines (Lowry *et al.* 1971, Shyrock *et al.* 1986, Lin *et al.* 2006, 2007). It is useful for metabolic profiling as it denatures and precipitates proteins and other

macromolecules by itself or via precipitation with potassium hydroxide (KOH), removing the influence of these molecules on resonance spectra (Fan and Lane 2008). However, other metabolites can be lost due to adsorption to perchlorate precipitates (Chen *et al.* 1977, Shyrock *et al.* 1986). As a highly volatile, strong acid, and a strong oxidizer at concentrations > 70%, perchloric acid requires special storage and handling and must be used in special ventilation hoods. Buffering steps are often included with perchloric acid protocols to minimize the effects of the low pH on the shift qualities of hydrogen metabolites in ^{1}H-NMR and to minimize destruction of acid-labile metabolites (Shyrock *et al.* 1986, Maharjan and Ferenci 2003, Fan and Lane 2008). This titration is time-consuming and can be technically difficult with small sample sizes, potentially resulting in wide alkaline and acid pH swings that can destroy metabolites of interest. Even if neutralized appropriately, peak shifting of metabolites due to acid effects is a major source of variation in perchloric acid protocols (Defernez and Colquhoun 2003, Lin *et al.* 2007, Brown *et al.* 2008, Dietmair *et al.* 2012).

Our findings were consistent with these characterizations of perchloric acid extractions. Using principal components analysis to compare the methods, perchloric acid resulted in the most efficient extraction of energy-related nucleotides (AMP/ADP/ATP) along principal component 2 (Fig. 2). Perchloric acid samples had the lowest peak counts of the three extraction methods, suggesting lower numbers of metabolites extracted. However, this may also suggest less degradation of macromolecules due to improved stability of the solution. We also found poor consistency between the replicate samples that did not improve with summation of the three extraction fractions (Fig. 1).

Processing was more laborious for the perchloric acid samples than for the other methods. The perchloric acid samples were more likely to require additional homogenization time to achieve a liquefied sample, increasing the time needed

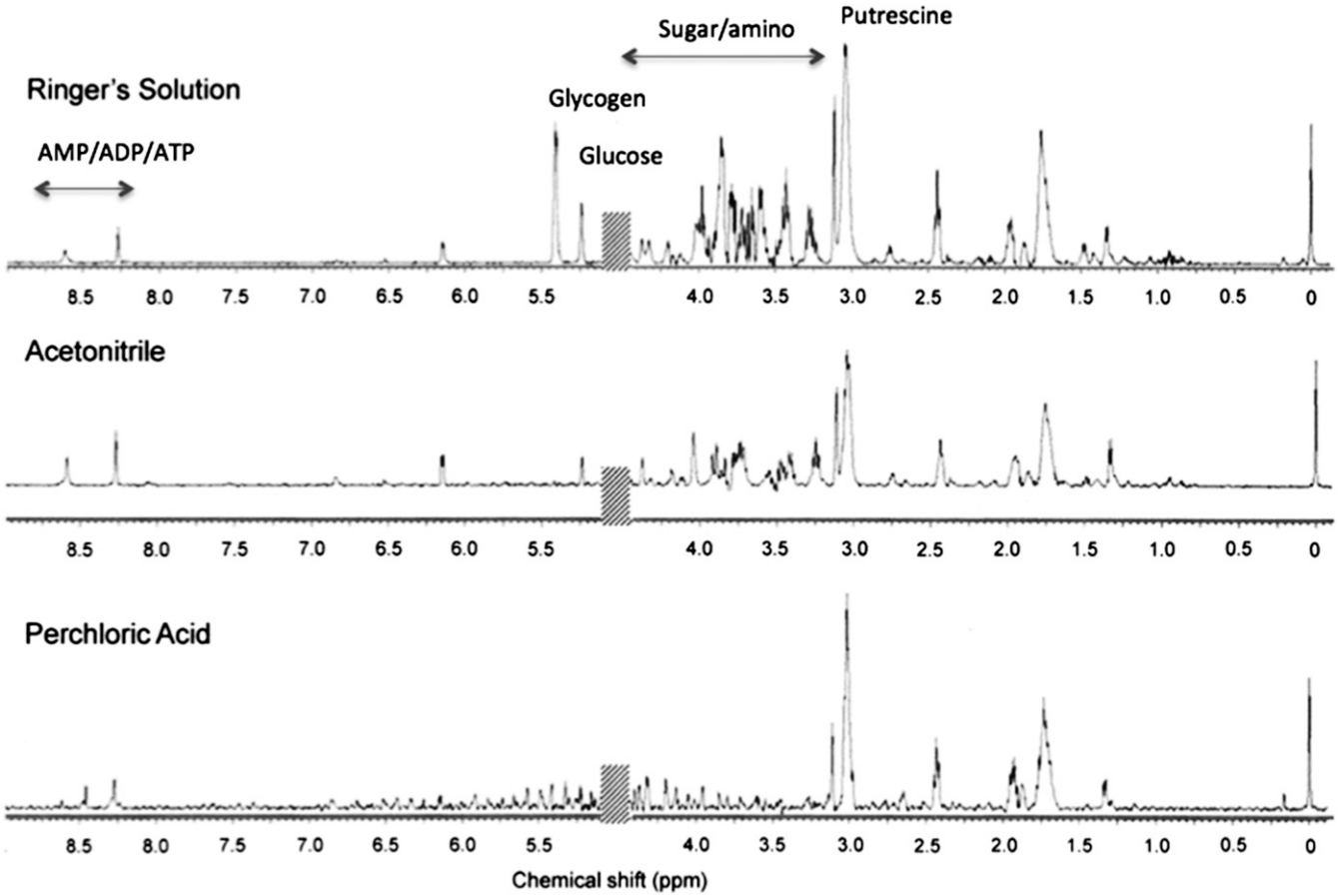

Figure 2. First extraction, immediate collection ^{1}H-NMR spectra from each extraction solvent. All samples represent one animal.

for processing. Perchloric acid processing time was also increased 25–50 minutes per sample due to inclusion of the buffering step and subsequent centrifugation not needed for the acetonitrile or Ringer's solution protocols.

Acetonitrile

Acetonitrile (CH_3CN) is an aqueous, neutral, organic solvent with medium polarity that is usually better for extracting acid-labile metabolites than perchloric acid (Fan and Lane 2008). Reproducibility is also improved for acetonitrile extractions as compared to perchloric acid (Lin *et al.* 2007). Acetonitrile is primarily useful for extraction of hydrophilic metabolites (Coen *et al.* 2003, Stentiford *et al.* 2005, Lin *et al.* 2007); however, it can also recover lipids and other macromolecules that result in superimposition of broad peaks on resonance spectra. These broad peaks complicate metabolite identification and quantification (Lin *et al.* 2007, Fan and Lane 2008). Acetonitrile should be handled carefully, inhalation, ingestion and possibly skin absorption of acetonitrile can result in toxicity from its metabolite, hydrogen cyanide (Greenberg 1999).

In our study, acetonitrile showed good reproducibility only after the three extraction fractions were summed (Fig. 1). The 30 minute incubation showed greater peak numbers than the immediate collection after homogenization. This could be related to improved solubilization of metabolites over time. However, enzymatic activity resulting in the breakdown of macromolecules into smaller compounds (*i.e.*, proteins to amino acids) cannot be ruled out. Principal components analysis showed similarities between acetonitrile and ringer's with overlap primarily due to putrescine and glucose metabolites (Fig. 2).

Ringer's Solution

In the early 1880s, Ringer's solution was developed through investigation of the blood constituents needed to maintain contractility in frog cardiac muscle (Ringer 1882, 1883). As a salt solution isotonic to animal body fluids, this solution has become ubiquitous in physiologic studies and is used in modified form in human and veterinary medicine as an intravenous electrolyte solution. Ringer's solution is primarily a sodium chloride (NaCl), calcium chloride ($CaCl_2$) and potassium chloride (KCl) mixture with or without additives (Ringer's Solution 2007). The composition of mineral salts is adjusted to accommodate individual species and meet their differing physiologic requirements for cell metabolism and osmolarity. This results in a slightly alkaline solution (pH 7.3–7.4), ideal for many NMR experiments. Mammalian and amphibian Ringer's solutions are inexpensive and readily obtainable through scientific supply companies, but can also be easily made in the laboratory (Ringer's Solution 2007). The specific composition of a freshwater mussel Ringer's solution has not yet been determined, therefore, an amphibian Ringer's solution composed of only NaCl, KCl, and $CaCl_2$ in water (Fisher Scientific, Waltham, MA, U.S.A.) was used in this study. The low concentration of paramagnetic elements (Ca^{2+}, Na^{+}) in the Ringer's solution should have minimal effect on the NMR signal. Especially as any residual salt is further diluted through reconstitution of the sample with water (D_2O) during sample processing.

As with the use of phosphate buffer solutions (Brown *et al.* 2008, Bundy *et al.* 2009), detrimental effects on metabolites should be minimal and Ringer's solution should allow solubilization of metabolites at physiologic ratios. As an electrolyte solution that does not contain organic solvents or strong acids, this solution can be handled safely and disposed of without any special protocols. A suspected disadvantage is that Ringer's has no quenching effect on enzymes and, therefore, may not maintain a temporally stable sample for analysis as long as extraction methods that denature proteins. It is possible that in a non-polar solution such as Ringer's solution, hydrophobic components may settle with centrifugation (Brown *et al.* 2008).

In this study, Ringer's solution yielded the largest number of peaks, particularly in the 3.0–4.5 ppm sugar/amino acid range (Fig 2). These metabolites were responsible for the separation of the majority of Ringer's samples from the samples processed with the two other extraction methods by principal component 1 (PC1). This may indicate improved extraction of these groups of metabolites in Ringer's solution indeed acetonitrile has been shown to be less effective for glycogen extraction (Tikunov *et al.* 2013). Alternatively, the greater peak numbers may reflect degradation of macromolecules due to poor quenching of metabolic activity by the protocol. The replicate Ringer's samples had good consistency as shown by tight grouping on the principal components map when compared to all extraction methods and when only Ringer's samples were evaluated (Fig. 1). This was seen in both the first extraction fraction and when the three extraction fractions were summed (Fig. 1). Despite good reproducibility within an individual, PCA did show notable separation of mussel W from the other two individuals. This separation was not as apparent for the other extraction methods. Examination of the PCA biplot suggests that this grouping is due to variation in glycogen, indicating Ringer's may be a particularly good solution for extraction of this metabolite.

Other solvents

Methanol-based extraction protocols were not evaluated even though methanol/chloroform is well established for use in extractions of tissues of marine bivalves (Hines *et al.* 2007, Jones *et al.* 2008, Liu *et al.* 2011a, 2011b, 2011c, Zhang *et al.* 2011a, 2011b, Kwon *et al.* 2012, Ji *et al.* 2013, 2014a, 2014b) and other species (Lin *et al.* 2007, Wu *et al.* 2008, Liebeke and

Bundy 2011, Dietmair *et al.* 2012). The Ringer's method required fewer steps than needed for extraction with methanol/chloroform and a comparison of Ringer's solution with methanol/chloroform may be useful. In addition, Ringer's solution is less toxic and engineering controls needed to prevent exposure to methanol or chloroform are not needed when processing tissues with Ringer's solution.

Comparison of time protocol

Optimal time protocols varied with the extraction protocol. For the perchloric acid extractions, an immediate collection of supernatant following homogenization appeared to result in a greater number of spectral peaks than collection after 30 minutes incubation whereas acetonitrile yielded a greater peak number after incubation. For the Ringer's extraction, both time collections resulted in roughly the same number of peaks. Evaluation for time effect using principal components analysis, however, showed no apparent time effect suggesting that variation between individuals is greater than variation due to incubation time (Fig. 1C).

Comparison of serial extraction fractions

One extraction with 500 μL of solvent per extraction was not adequate for retrieval of all metabolites, although many published extraction protocols extract tissues only once, with lower volume to tissue weight ratios and/or for shorter extraction times (Gibb *et* al 1997a, 1997b, Tuffnail *et al.* 2009, Tikunov *et al.* 2010, Dietmair *et al.* 2012). Although the peak numbers and intensities were notably lower on the second and third extractions than the first extraction, the addition of the three extractions was necessary to improve yield of metabolites for each extraction solvent tested. Extraction replicates had diminished variability when extractions were summed. These findings suggest that a minimum of three extractions are needed to adequately extract metabolites. However, increasing the ratio of solvent to tissue may achieve the same result if the solubilization of metabolites is not related to time of incubation, but rather saturation of the extraction solution.

Variation across individual mussels

An important quality of an extraction protocol for NMR is reproducibility (Dunn and Ellis 2005, Lin *et al.* 2007, Brown *et al.* 2008). The introduction of variability through processing, such as pipetting errors, pH swings during titration, or temperature changes, can mask biological variation or create an artificially increased variation compared to actual biological variation (Dunn and Ellis 2005, Lin *et al.* 2007, Brown *et al.* 2008). In our study, Ringer's solution had the least intra-mussel variation based on principal components analysis. For Ringer's solution, the replicate variability was characterized by tight grouping of the replicate samples on both the first extraction and with all three extraction fractions summed. There was notable variability within the individual for the first extraction of acetonitrile, but reproducibility improved with the addition of the subsequent two extractions. Notable intra-mussel variability was seen with perchloric acid extraction that did not improve with the additional extractions.

Variability related to the solvent utilized was greater than the individual variability between mussels, especially once extraction fractions were summed (Fig. 1A, B). As previously noted, with Ringer's solution extraction there was a difference in the amount of glycogen extracted from the tissues of mussel W.

CONCLUSIONS

Proton nuclear magnetic resonance spectroscopy using each of the three tested extraction solvents yields spectra that can be used for identifying metabolites in freshwater mussel adductor tissue. Determining effective protocols is the first step in the application of this technology to greater questions of freshwater mussel health. The strengths and weaknesses of each extraction procedure should be considered when selecting an extraction protocol for a metabolomics experiment. When specific metabolites are desired, selection of a specific protocol will result in optimal extraction of the markers of interest (Fan and Lane 2008). In the early stages of many environmental metabolomic studies, a non-specific extraction is required as it may not be initially clear which are the metabolites of importance (Viant *et al.* 2003). The Ringer's solution protocol examined in this study is simple to use, safe and consistent, yielding a robust metabolite profile. Ringer's solution bears consideration when selecting an extraction protocol for ^{1}H NMR experiments conducted to assess the health of freshwater mussels.

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