JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v7i1.203

A novel extraction method for the preparation of heparinized chicken (*Gallus gallus domesticus*) and horse (*Equus caballus*) whole blood for ^{1}H -NMR metabolomics using Drabkin's reagent

Jennifer N. Niemuth*[1-3]; Michael K. Stoskopf.[1-3]

[1]Department of Forestry and Environmental Resources, College of Natural Resources, North Carolina State University, 2820 Faucette Dr., Raleigh, NC 27695, USA. [2]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, USA. [3]Environmental Medicine Consortium, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, USA.

Received: 19 October 2016 **Accepted:** 2 April 2017 **Available Online**: 12 April 2017

ABSTRACT

Despite the ease of collection, heparinized whole blood is underutilized in proton nuclear magnetic resonance spectroscopy-based metabolomics particularly because of the lack of sample homogeneity. Drabkin's reagent, an aqueous solution of potassium ferricyanide, potassium cyanide, and sodium bicarbonate, causes hemolysis and has been used for quantification of hemoglobin. The objectives of this study were to determine if the use of Drabkin's reagent with heparinized whole blood for metabolomics samples would result in consistent hemolysis, while being invisible to proton nuclear magnetic resonance spectroscopy and quenching metabolic activity. Heparinized whole blood from a chicken (*Gallus gallus domesticus*) and a horse (*Equus caballus*) was used. All heparinized whole blood samples were mixed 1:10 (volume:volume) heparinized whole blood:Drabkin's reagent. Lyophilized Drabkin's reagent rehydrated with a 100% deuterium oxide solution was invisible to proton nuclear magnetic resonance spectroscopy. Standard (10 min incubation, 20 min centrifugal filtration) and delayed (120 min incubation, 20 min centrifugal filtration) samples were prepared for both animal species. The only differences in spectra noted were minor differences in the amplitude of the major peaks of the metabolites 3-methylhistidine and betaine in the chicken samples. Comparison of standard and delayed samples via two-sample Kolmogorov-Smirnov tests found no significant differences between spectra for either animal species (chicken $p = 1$, horse $p = 0.9887$). Use of Drabkin's reagent resulted in consistent, complete hemolysis, while being invisible to proton nuclear magnetic resonance spectroscopy and quenching metabolic activity for at least 140 min at room temperature. This protocol should be considered when the investigator is interested in questions specific to erythrocyte metabolism and/or when heparinized whole blood is the only sample type available.

Keywords: *Equus caballus*; *Gallus gallus domesticus*; heparinized whole blood; metabolomic quenching; metabolomic sample handling; proton nuclear magnetic resonance spectroscopy.

Abbreviations: 1D: one-dimensional; ^{1}H: proton; D_2O: deuterium oxide; DR: Drabkin's reagent; Hb: hemoglobin; HWB: heparinized whole blood; K-S: Kolmogorov-Smirnov; NMR: nuclear magnetic resonance; TSP: trimethylsilyl propanoic acid; v:v: volume:volume.

1. Introduction

Biofluids, such as plasma, used in ^{1}H-NMR-based metabolomics studies provide a snapshot of the metabolic state of an organism. Advantages of biofluids, over most other tissues, include the relative ease of sample collection and low impact on patients or research animals, particularly when repetitive sampling is necessary. Biofluids also can often be analyzed via NMR with minimal to no sample preparation.

The application of metabolomics techniques to ecological questions where sampling wildlife may be necessary presents

***Corresponding author:** Jennifer N. Niemuth, North Carolina State University, College of Veterinary Medicine, Department of Clinical Science, 1060 William Moore Drive, Campus Box 8401, Raleigh, NC 27607, USA. Phone: +1-919-513-6230, Fax: +1-919-515-4237, E-mail Address: jennifer_niemuth@ncsu.edu

some special sample handling challenges over more routine clinical or laboratory settings. Particularly in the field, where electrical power and standard centrifuge use is often not practical, timely collection of plasma may not be possible. HWB is then a preferred sample. Measuring each HWB sample's hematocrit using battery powered minifuges or gravity methods allows the final spectral data to be normalized to the percentage of erythrocytes present in the blood. Unfortunately, the key first step of quenching the metabolism of metabolomics samples [1], commonly achieved in the field by immediate freezing with dry ice or liquid nitrogen, introduces variability to HWB samples because the amount of hemolysis induced in the freeze and/or thaw may not be consistent across samples.

Additionally, sedimentation and movement of intact erythrocytes during NMR analysis can complicate quality control [2,3]. A potential solution to these issues is to create homogeneous samples by completely hemolysing the sample, releasing all erythrocyte intracellular contents into the plasma.

Complete hemolysis of HWB samples for metabolomics also offers the opportunity to address research questions specifically about erythrocyte metabolism. For example, various metabolites, such as glutathione, have been shown to have much greater intraerythrocytic concentrations compared to plasma [4].

In a pilot study designed to evaluate possible methods for achieving reliable complete hemolysis of HWB, established solvents used in sample extractions for NMR metabolomics, such as chloroform and methanol, as well as other solvents, including deionized water, acetone, and ammonia, either did not produce complete hemolysis or physically changed the samples by greatly increasing viscosity, thereby inhibiting further manipulation (Niemuth, unpublished dataset). Chloroform, methanol, deionized water, and acetone were tested at 1:1, 2:1, 5:1, and 10:1 solvent:HWB sample (v:v). Ammonia was tested in a range of concentrations from 1 M to 14.8 M. Deionized water and ammonia less than 3 M did not produce complete hemolysis. Chloroform or ammonia greater than 3 M resulted in a gelatinous sample, while samples treated with methanol or acetone caused clumping. Physical disruption methods using various tissue homogenizers, lyophilization, and sonicators were inconsistent, required extensive time per sample, and increased sample temperature, which without a permanent quenching method could perturb the resulting metabolomes.

DR is a commercially available aqueous solution of potassium ferricyanide, potassium cyanide, and sodium bicarbonate developed for the cyanhemoglobin spectrophotometric method of quantitative Hb determination [5]. DR causes hemolysis and conversion of Hb to cyanmethemoglobin [6-8].

The objectives of this study were to determine if the use of DR with HWB for metabolomics samples would result in consistent hemolysis, while being invisible to ^{1}H-NMR and quenching metabolic activity. Heparinized chicken (Gallus *gallus domesticus*) and horse (*Equus caballus*) whole blood was employed to allow comparison of results with nucleated and non-nucleated erythrocytes, respectively.

2. Material and Methods

2.1. Biological samples and chemical reagent

HWB for this study was obtained from a horse and a chicken during routine clinical diagnostics and euthanasia, respectively. HWB was frozen after collection, stored at -80 °C, and thawed at room temperature (20-21 °C) before use. Commercially available DR (Ricca Chemical Company, Arlington, TX, USA) was used and the pH measured (accumet Basic AB15 Plus, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.2. Determination of sample and reagent incubation time

To determine necessary incubation time, a HWB sample from chicken and horse was individually mixed 1:10 (v:v) HWB:DR. The absorbance of each sample was measured with a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 540 nm approximately every 5 min for a total of approximately 45 min. Plain DR was used as the blank for the spectrophotometer. Change in absorbance over time was used to determine the incubation time for the remainder of the study.

2.3. Determination of Hb extraction efficacy

To evaluate efficacy of Hb extraction, another HWB sample from each animal species was individually mixed 1:10 (v:v) HWB:DR. Both samples were incubated at room temperature for 10 min then centrifuged (3000 g; 5 min; room temperature). The supernatant was collected and saved (stored away from light at room temperature) until analysis. A new aliquot of DR was added to the HWB pellet, vortexed, and the incubation and centrifugation steps were performed as before. These steps were repeated until a total of 5 supernatants were collected from both samples. The absorbance of each supernatant was measured with a spectrophotometer at 540 nm. Change in absorbance over time was used to determine the efficacy of DR Hb extraction and the necessity of multiple washes per sample for the remainder of the study. A HWB chicken sample was also mixed 1:10 (v:v) HWD:DR, incubated (10 min; room temperature) and examined via light microscopy as air-dried, Wright's stained smears.

2.4. ^{1}H-NMR sample preparation

Amicon Ultra 10K 0.5 mL centrifugal filters (Millipore, Carrigtwohill, County Cork, Ireland) were prepared before

use by soaking in ultrapure water for a minimum of 8 h, washed 4 times with 0.5 mL ultrapure water (14,000 g; 20 min; room temperature), and stored in fresh ultrapure water if not used immediately (room temperature; used within 1 day). All samples were filtered prior to NMR analysis (14,000 g; 20 min; room temperature), including an aliquot of plain DR.

For chicken and horse samples, 40 µL HWB and 400 µL DR were mixed, incubated for 10 min at room temperature, and centrifuged (as above). The resulting filtrate was promptly frozen at -80 °C until lyophilization. Hereafter, these are referred to as standard samples. A second set of samples was prepared in the same manner, but was held at room temperature for 120 min prior to centrifugation. Hereafter, these are referred to as delayed samples. Frozen filtrates were lyophilized (-50 °C; FreeZone 2.5, Labconco, Kansas City, MO, USA) until dry (approximately 6 h), sealed with laboratory wax film (Parafilm, Beemis NA, Neenah WI, USA), and returned to -80 °C until NMR analysis.

Sealed lyophilized samples were thawed at room temperature. All samples were rehydrated with 70 µL D_2O with 20 mM phosphate buffer, 0.1 mM TSP, and 1 mM formate. Formate was used as second reference standard in case of interaction between TSP and plasma proteins [9]. Rehydrated samples were filtered (Fisherbrand SureOne 10 µL, extended, filter, low retention, universal fit pipet tips, Thermo Fisher Scientific, Waltham, MA, USA) via centrifugation (3,000 g; 2 min; room temperature) into vials and capped. For quality assurance, blank samples of the D_2O solution were run before and after each animal species' samples.

2.5. ^{1}H-NMR data collection, processing, and analysis

Samples were analyzed with a Varian Inova 600 MHz multinuclear INOVA NMR spectrometer (Varian Medical Systems, Palo Alto, CA, USA) equipped with a Protasis microcoil NMR probe (Protasis Corporation, Marlboro, MA, USA) to obtain 1D, ^{1}H-NMR spectra at 25 °C with a 1.1 sec acquisition time. The sweep width of 7,193 Hz acquired 8,189 complex points and 4,096 transients. Prior to insertion into the spectrometer, sample pH was measured and no practical differences in pH that would require adjustment of technique were noted.

NMR spectra were processed using ACD labs 12.0 1D NMR Processor (Advanced Chemistry Development, Toronto, Ontario, Canada). Spectra were zero-filled to 16,000 points and Fourier transformed. Spectral phasing and baselines were corrected automatically and adjusted, if necessary. All spectra were referenced to the internal standard TSP peak at 0 ppm. Peak identification was performed with Chenomx NMR suite 8.1 (Chenomx, Edmonton, Alberta, Canada) and the Human Metabolome Database [10].

Prior to statistical analysis, dark regions for each animal species were set to eliminate upstream/downstream areas without metabolites (including TSP peaks) and the water peak. Spectra for each animal species were grouped and intelligent bucketing was performed with a bin width of 0.04 ppm and 50% width looseness. This resulted in 214 and 204 bins for the chicken and horse samples, respectively. Integrals were normalized for each sample by dividing each bin value (i.e. integral) by the sum of the bins for that particular sample. Spectra for each animal species were compared statistically via a two-sample K-S test (two-sided, $\alpha = 0.05$) with R version 3.2.3[11].

3. Results

Measured pH of DR was 8.610. The alkaline pH of the samples did not appear to interfere with peak identification using reference spectra made with standard solvents.

Spectrophotometric evaluation of incubated DR treated samples revealed little change in absorbance after approximately 10-15 min. All samples thereafter were incubated for 10 min after DR addition before further processing, except where noted.

Absorbance of the second through fifth collected supernatants was decreased by >95% versus the initial supernatant. All samples thereafter were subjected to a single 1:10 (v:v) HWB:DR treatment to achieve extraction. The aliquot of the chicken sample used in this portion of the study developed a small gelatinous mass after DR treatment similar to what has been described in trout blood by Larsen and Snieszko [7]. The mass lightened in color after each subsequent DR aliquot. Examination via light microscopy of air-dried, Wright's stained smears resulted in subjectively uniform hemolysis.

DR was invisible to ^{1}H-NMR (Figure 1). Blank samples run prior to and after samples from each animal species did not demonstrate any contamination that carried over into the test samples. Standard and delayed samples for both animal species did not appear considerably different, either in regards to metabolite presence/absence or relative metabolite concentrations. Subjectively, the greatest difference appeared to be with the major peaks of the

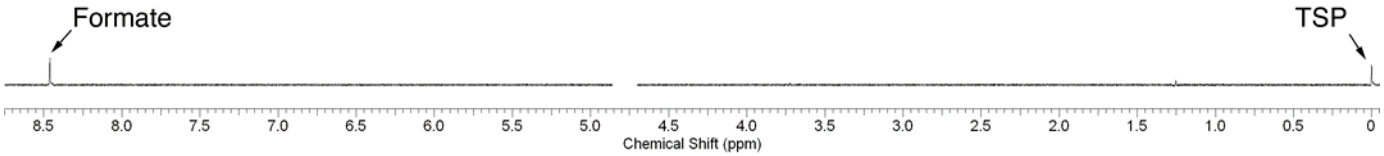

Figure 1. ^{1}H-NMR spectrum of filtered DR. The water peak has been deleted. TSP and formate were added to all samples as reference standards.

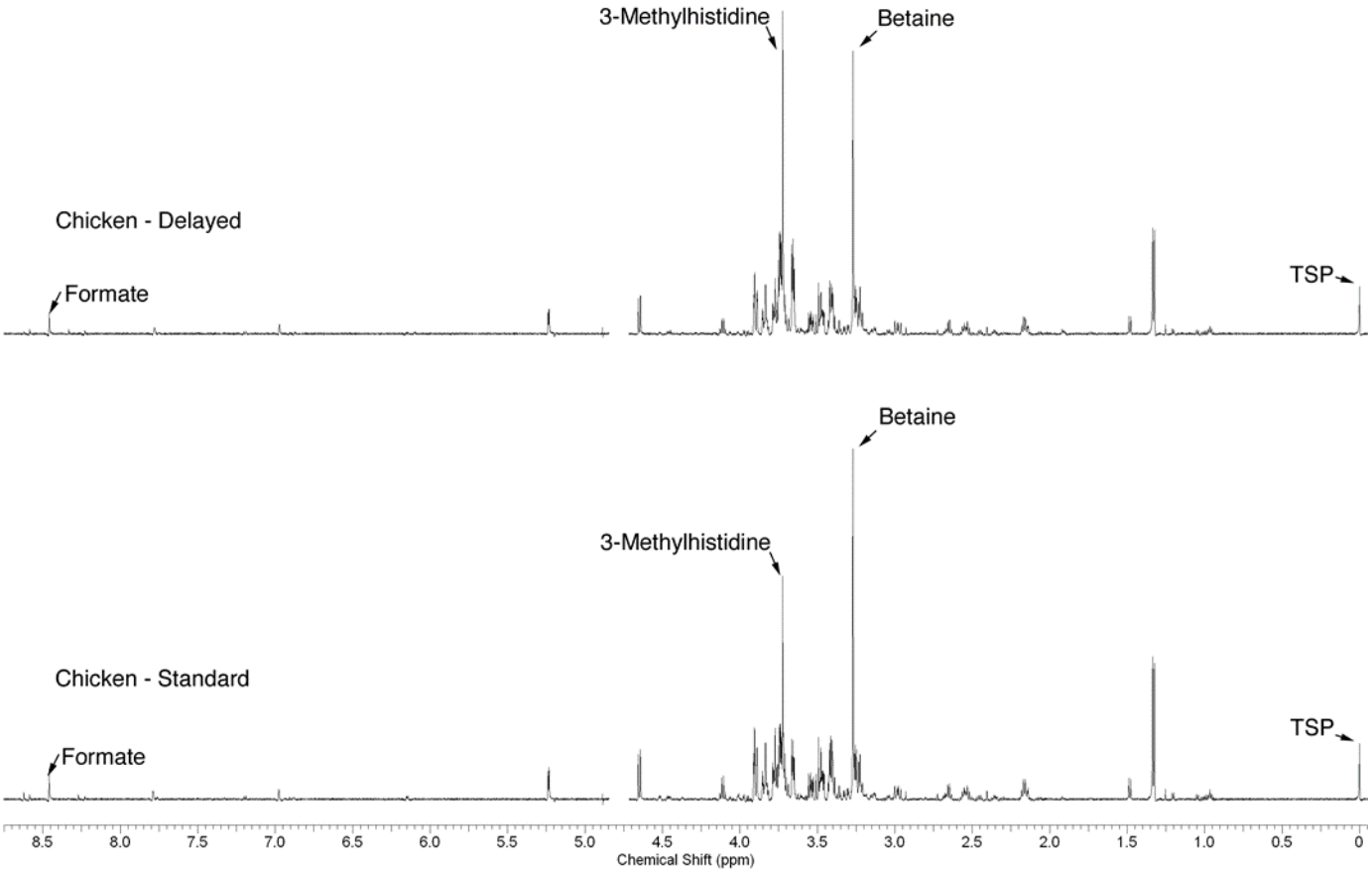

Figure 2. [1]H-NMR spectra of DR treated chicken HWB. The water peak has been deleted. The standard sample was incubated at room temperature for 10 min with DR prior to 20 min of centrifugal filtration. The delayed sample was incubated at room temperature for 120 min with DR prior to 20 min of centrifugal filtration. Subjectively, the greatest difference appeared to be with the major peaks of the metabolites betaine (singlet at approximately 3.3 ppm, methyl group protons) and 3-methylhistidine (singlet at approximately 3.7 ppm, methyl group protons with minor overlap contribution from components of a multiplet and doublet representing single protons on carbons 7 and 8, respectively).

metabolites betaine (approximately 3.3 ppm) and 3-methylhistidine (approximately 3.7 ppm) in the chicken samples (Figure 2). Both horse spectra were very similar (Figure 3). Provisional metabolite identifications and reference chemical shifts based on database comparisons are included as Table 1. Comparison of standard and delayed samples via two-sided, two-sample K-S tests found no significant differences with either animal species (chicken $p = 1$, horse $p = 0.9887$; both are approximate due to the presence of ties).

4. Discussion

The results of this study demonstrate that DR can be used as a component of an effective and simple extraction method for chicken and horse HWB samples for [1]H-NMR metabolomics. DR is commercially available, inexpensive, and has minimal storage requirements (protection from light, freezing, and contact with acids). The amount of potassium cyanide and potassium ferricyanide in 1 L of Drabkin's reagent is much lower than the lowest-observed-adverse-effect level for an average 70 kg human [12].

Depending on the investigator's metabolites of interest, the native pH of DR and the resultant HWB filtrate may require the development of a specific database for DR samples. With our spectra, we were able to identify metabolites using standard reference spectra with minimal to no chemical shift changes. However, the chemical shift of a particular metabolite could be influenced by pH as the molecule is protonated or deprotonated [13]. Titration to a more neutral intracellular pH could be a potential approach to allow peak identification for such metabolites using existing databases. For unknown metabolites, investigators may have success identifying chemical shifts through the calculation of titration curves [13]. However, caution should be exercised as the addition of acid to DR can release hydrogen cyanide [14]. The potential for pH differences between samples should also be considered, especially when studying any physiologic or disease condition resulting in acidosis or alkalosis of the patient [15]. Lastly, one may need to consider the body temperature of the animal species being studied and the temperature at which pH measurements are made. For example, in poikilothermic and ectothermic animal species, such as reptiles, it is necessary to apply a

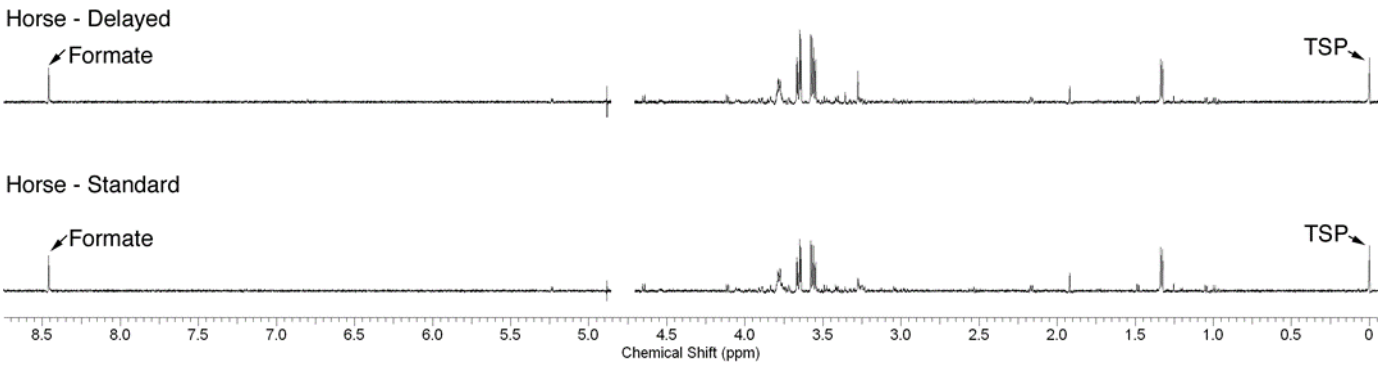

Figure 3. [1]H-NMR spectra of DR treated horse HWB. The water peak has been deleted. The standard sample was incubated at room temperature for 10 min with DR prior to 20 min of centrifugal filtration. The delayed sample was incubated at room temperature for 120 min with DR prior to 20 min of centrifugal filtration. Subjectively, both spectra appear very similar.

correction for the animal's body or environmental temperature to accurately evaluate many clinical measurements, including blood pH [16].

The incubation time of 10 min used in this paper is in agreement with a 5 to 10 min minimum reported in other published protocols for use of DR for hemolysis [5-7]. Hb is a large molecule with a diameter of approximately 5 nm and a total molecular weight of 64,000 Daltons [17]. Our metabolites of interest generally have molecular weights less than 10,000 Daltons. Therefore, we would expect that extraction of Hb from the erythrocyte would also allow for the release of intracellular metabolites. The results of evaluation of blood smears made from DR treated HWB suggested complete hemolysis. This was supported by the near complete extraction of Hb after a single application of DR.

The small gelatinous mass observed in one of the chicken samples during extraction testing was consistent with what has been described in trout [7]. The mass did not interfere with supernatant collection and while no masses were observed in the samples used for NMR analysis, they would have been removed by the filtration step. The formation of these masses has been noted when using DR for hemoglobin quantification and it has been recommended that they be removed (e.g. if within a spectrophotometer cuvette) with a wooden applicator stick [18].

Table 1. Provisional metabolite identifications and reference chemical shifts [10]. Absolute metabolite confirmation would require additional NMR experiments. (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, dd: doublet of doublets, tt: triplet of triplets).

Metabolite	Chemical Shift (ppm) & Peak Shape	Chicken	Equine
3-Aminoisobutyrate	1.16-1.21 (d), 2.57-2.64 (m), 3.00-3.05 (dd), 3.06-3.16 (dd)	•	•
3-Hydroxyisovalerate	1.26 (s), 2.35 (s)	•	•
3-Methylhistidine	3.24 (m), 3.70 (s), 3.93 (dd), 7.05 (s), 7.92 (s)	•	
Acetate	1.91 (s)	•	•
Alanine	1.47 (d), 3.77 (q)	•	•
AMP	4.01 (dd), 4.36 (dd), 4.50 (dd), 6.12 (d), 8.23 (s), 8.58 (s)	•	
Betaine	3.25 (s), 3.89 (s)	•	•
Creatinine	3.03 (s), 4.05 (s)	•	•
Formate*	8.44 (s)	•	•
Glucose	3.233 (dd), 3.398 (m), 3.458 (m), 3.524 (dd), 3.728 (m), 3.824 (m), 3.889 (dd), 4.634 (d), 5.223 (d)	•	•
Glutathione	2.15 (m), 2.54 (m), 2.97 (dd), 3.78 (m), 4.20 (q)	•	•
Glycerol	3.551 (m), 3.644 (m), 3.775 (tt)		•
Inosine	3.832 (dd), 3.902 (dd), 4.257 (dd), 4.420 (dd), 4.798 (s), 6.055 (d), 8.187 (s), 8.305 (s)	•	
Lactate	1.32 (d), 4.10 (q)	•	•
Leucine	0.948 (t), 1.700 (m), 3.722 (m)	•	•
Methanol	3.34 (s)	•	•
Sarcosine	2.73 (s), 3.60 (s)	•	
Succinate	2.39 (s)	•	
Valine	0.976 (d), 1.029 (d), 2.261 (m), 3.601 (d)	•	•

One of the main reasons for selecting DR versus other commercially available hemolysis agents was that we hypothesized DR would be NMR-friendly, expecting lyophilization to remove the only protons in the solution. ^{1}H-NMR analysis of filtered and lyophilized DR confirmed that DR is invisible (Figure 1).

Visually there were only subtle differences in singlet peak amplitudes between the standard and delayed chicken sample spectra. 3-Methylhistidine is an aromatic heteromonocyclic related to muscle breakdown [10], while betaine is involved in glycine, serine, and threonine metabolism [19]. We are not able to determine if the differences in 3-methylhistidine and betaine are due to true sample change or are the result of some other unidentified variability. The Kolmogorov-Smirnov test is used to detect differences in location and shape and did not indicate that either the chicken or horse standard or delayed samples were from different distributions. If it is anticipated that a delay greater than the 20 min necessary for centrifugal filtration will be a required part of a study protocol, it would be prudent to run test samples to determine what, if any, metabolites may be impacted.

5. Concluding Remarks

Use of DR with chicken and horse HWB for metabolomics resulted in consistent, complete hemolysis, while being invisible to ^{1}H-NMR and quenching metabolic activity for at least 140 min at room temperature. This protocol was equally effective with nucleated and non-nucleated erythrocytes of the chicken and horse, respectively, and should be considered when HWB is the only sample type available and/or if the investigator is interested in questions specific to erythrocyte metabolism.

Acknowledgments

This study was funded by a grant from the Bernice Barbour Foundation, Inc. The authors thank Drs. Scott Bailey and Luke Borst for providing blood samples, the Macdonald laboratory and the University of North Carolina Metabolomics Facility at The Hamner Institutes for Health Sciences for use of their magnet, and Dr. Jennifer Neel for her advice on hemolysis determination.

References

1] M.R. Viant, C. Ludwig, U.L. Günther, in: W.J. Griffiths (Ed.), Metabolomics, Metabonomics and Metabolite Profiling, The Royal Society of Chemistry, Cambridge, UK, 2008, pp. 44-70.

2] F.F. Brown, I.D. Campbell, P.W. Kuchel, FEBS Lett, 82 (1977) 12 -16. DOI: 10.1016/0014-5793(77)80875-2

3] J.K. Nicholson, M.J. Buckingham, P.J. Sadler, Biochem J, 211 (1983) 605-615. DOI: 10.1042/bj2110605

4] M.D. Rosenthal, R.H. Glew, Medical Biochemistry: Human Metabolism in Health and Disease, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2009.

5] D.L. Drabkin, J.H. Austin, J Biol Chem, 98 (1932) 719-733. http://www.jbc.org/content/98/2/719.short

6] S.M. Lewis, S. Kumari, Guidelines on Standard Operating Procedures for Haematology, http://apps.searo.who.int/PDS_DOCS/B0215.pdf, Accessed 2016.

7] H.N. Larsen, S.F. Snieszko, Fish culture, 23 (1961) 8-17. DOI: 10.1577/1548-8659(1961)23[8:COVMOD]2.0.CO;2

8] J. Samour, in: G.J. Harrison, T.L. Lightfoot (Eds.), Clinical avian medicine, first ed., Spix Publishing, Palm Beach, FL, 2006, pp. 587-609.

9] M. Kriat, S. Confort-Gouny, J. Vion-Dury, M. Sciaky, P. Viout, P.J. Cozzone, NMR Biomed, 5 (1992) 179-184. DOI: 10.1002/nbm.1940050404

10] D.S. Wishart, C. Knox, A.C. Guo, R. Eisner, N. Young, B. Gautam, D.D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J.A. Cruz, E. Lim, C.A. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H.J. Vogel, I. Forsythe, Nucleic Acids Res, 37 (2009) D603-D610. DOI: 10.1093/nar/gkn810

11] R Core Team, R: A language and environment for statistical computing, http://www.R-project.org/, Accessed 2016.

12] Agency for Toxic Substances and Disease Registry (ATSDR), Toxicological profile for Cyanide, U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia, USA, 2006.

13] G.P. Kreishman, E. Brooks, in: R.S. Macomber (Ed.), A complete introduction to modern NMR spectroscopy, First ed., John Wiley & Sons, Inc., New York, New York, USA, 1998, pp. 252-282.

14] Ricca Chemical Company, Safety Data Sheet - Drabkin's Reagent, http://www.riccachemical.com/Technical-Support/MSDS/2660, Accessed 2016.

15] O. Beckonert, H.C. Keun, T.M.D. Ebbels, J. Bundy, E. Holmes, J.C. Lindon, J.K. Nicholson, Nat Protocols, 2 (2007) 2692-2703. DOI: 10.1038/nprot.2007.376

16] C.A. Harms, K.M. Mallo, P.M. Ross, A. Segars, J Wildl Dis, 39 (2003) 366-374. DOI: 10.7589/0090-3558-39.2.366

17] H.P. Erickson, Biol Proc Online, 11 (2009) 32-51. DOI: 10.1007/s12575-009-9008-x

18] P. Blaxhall, K. Daisley, J Fish Biol, 5 (1973) 771-781. http://dx.doi.org/10.1111/j.1095-8649.1973.tb04510.x

19] M. Kanehisa, Y. Sato, M. Kawashima, M. Furumichi, M. Tanabe, Nucleic Acids Res, 44 (2016) D457-D462. DOI: 10.1093/nar/gkv1070