Quantification of tissue creatine content using capillary electrophoresis

Y. Jane Choi[1,2*], Angelo P Bautista[3], Jessica R Terrill[3], J. Jane Pillow[1,2], Peter G Arthur[3].

[1] School of Human Sciences, University of Western Australia, Perth, WA, Australia
[2] Wal-yan Respiratory Research Centre, Telethon Kids Institute, Perth, WA, Australia
[3] School of Molecular Sciences, University of Western Australia, Perth, WA, Australia

*Corresponding author
Email: jane.choi@uwa.edu.au (YJC)

Metadata

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Competing interests
The authors have no conflicts of interest to declare.

Data availability
Data will be made available upon request.

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Abstract

Creatine plays a fundamental role in cellular energy homeostasis. The current protocol describes an alternative method for creatine quantification in biological tissue samples using capillary electrophoresis, with high separation efficiency that enables rapid analysis with low sample volumes. The protocol involves homogenization of snap-frozen tissue in phosphate buffer, followed by electrophoresis through a bare-fused capillary (75 µm internal diameter) and measurement at 200 nm on the Agilent 7100 CE system. Under the optimised conditions, there was excellent linearity in creatine standards between 6.3 – 100 µM. The overall intra-assay variability for concentrations between 6.3 – 100 µM was 1.5 %, and the inter-assay variability was 6.4 %, with a limit of detection at 6 nmol/mg protein. The protocol was further benchmarked against a commercially available enzyme assay kit using lung samples from lambs that received continuous creatine or saline supplementation. There was good agreement between the two methods (mean difference = 0.42 [-0.26-1.1] nmol/mg protein). Importantly, capillary electrophoresis enables reliable detection of creatine in biological samples from just ~1.5 mg of wet-weight lung tissue. Capillary electrophoresis enables rapid (<10 minutes) and highly efficient analysis of tissue samples and avoids challenges faced with traditional enzymatic assays. The current protocol was developed and optimised with ovine lung tissue, but it can be easily adapted to analyse various tissue types. For tissues with higher baseline creatine content, such as the skeletal muscles or brain, <1 mg wet weight tissue would be sufficient to detect creatine using capillary electrophoresis.

Introduction

Creatine is a popular nutritional supplement with a fundamental role in energy homeostasis [1]. The creatine (Cr)/phosphocreatine (PCr) system is intrinsically involved in cellular energy homeostasis by converting PCr to Cr, which yields a high-energy phosphate group used to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). Increasing the tissue creatine content, also called creatine 'loading', increases the intracellular pool of Cr and PCr and provides an alternate pathway to generate ATP rapidly [2]. In skeletal muscles, creatine exerts ergogenic effects in healthy individuals and therapeutic benefits in various myopathies [1,3]. In the brain, the benefit of creatine in neurodegenerative diseases (such as Huntington's and Alzheimer's disease) through improved cerebral bioenergetics is well-described [4]. More recently, there is growing evidence for a role of creatine as a perinatal neuroprotectant via energy preservation and antioxidant protection of the fetal brain during ischemic conditions [5,6].

Several methods are available to measure the creatine content of biological samples. Direct determination of creatine is often used in a clinical context to diagnose inborn errors of creatine metabolism [7]. These quantitative methods involve the analysis of patient serum or urine samples for creatine content via high-performance liquid chromatography [8] or mass-spectrometry-based approaches [9–13]. In a research context, similar HPLC methods are used to assess creatine content in tissue or cell culture samples [14–16] or ^{31}P-magnetic resonance spectroscopy (MRS) to quantify high-energy phosphates in the brain tissue [17]. Alternatively, enzymatic detection of creatine involves fluorometric assay initially described by Passonneau and Lowry, which involves a multi-step enzymatic reaction to infer creatine concentration from the decrease in NADH fluorescence [18]. This method was used to determine creatine content in various tissue types, such as the muscle, brain, heart, liver and kidneys, from murine and ovine models [19–21]. However, enzymatic assays can be affected by the presence of interfering substances in the sample, and often require specific acid extraction during sample preparation that can be time-consuming.

We present an alternative method for creatine quantification in biological tissue samples using capillary electrophoresis. The advantage of capillary electrophoresis includes high separation efficiency that enables cost-effective and rapid analysis with low (nanolitre) sample volumes

[22,23]. These features make capillary electrophoresis amenable to use with human biopsy samples. Capillary electrophoresis provides improved sensitivity at lower sample concentrations and avoids interference in enzymatic assays. While the detection of creatine and creatinine using CE was described previously [24], existing methods are limited to human urinary creatine and creatinine levels. The current protocol describes workflow for determination of creatine content from snap-frozen tissue samples; from sample homogenisation and extraction to optimised conditions for capillary electrophoresis. The current protocol was developed and optimised with ovine lung samples. However, the protocol can be easily adapted to analyse various tissue types.

Materials and Methods

The protocol described in this peer-reviewed article is published on protocols.io (dx.doi.org/10.17504/protocols.io.4r3l22j23l1y/v1) and is included for printing as Supporting Information file 1 with this article.

The creatine peaks on the electropherogram were identified via enzymatic depletion of the signal and enhancement of the endogenous signal after spiking with additional creatine. Creatine concentration was calculated as the area under the peak (AUC) of the electropherogram and expressed as a ratio compared to the reference DMSO signal. Standards were injected in technical duplicates and run at the beginning of each day before sample analysis. The standards were repeated across four days, and a standard curve was generated from each day to assess the inter-assay variability. Samples were standardised to 1 mg/mL protein concentration and injected in technical duplicates to assess the intra-assay variability. Creatine concentration was interpolated from the standard curve and expressed as nmol/mg protein. The results from capillary electrophoresis were subsequently benchmarked against a commercially available creatine assay kit (MAK079, Sigma-Aldrich), which measures creatine without phosphocreatine due to a lack of creatine phosphokinase. Samples were similarly standardised to 1 mg/mL, and the assay was performed according to the manufacturer's guidelines. Creatine concentrations are similarly expressed as nmol/mg protein.

Expected results

Using optimised conditions described in the protocol, creatine peak is confirmed after depletion with creatinase enzyme and increased after spiking with additional creatine (**Fig 1**).

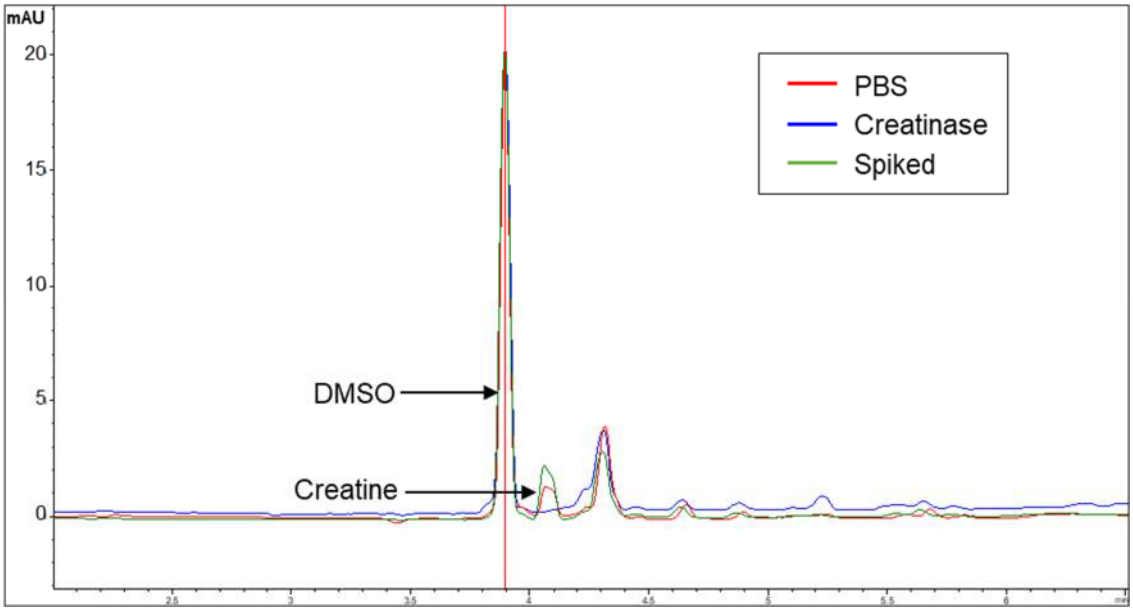

Fig 1. Identification of creatine peak by capillary electrophoresis under optimised conditions with UV detection at 200 nm.
Snap-frozen lung tissues were homogenised in PBS with 0.01 % DMSO (red, baseline), and the creatine peak on the electropherogram was identified via enzymatic depletion with creatinase (blue) and spiking (green) with additional creatine (25 µM). A bare-fused capillary (75 µm internal diameter) at an effective length of 36.5 cm (length to detector 8.5 cm) was used. The background electrolyte contained 75 mM NaH_2PO_4 and 150 mM SDS at pH 6. A voltage of 15 kV was applied at 25 ^{O}C with sample injection at 10 mbar for 10 s.

Linearity and assay variability

We assessed the separation performance via the linearity of the standard curve and the intra- and inter-assay variability. The concentration range for the assessment of linearity was determined based on the expected range of concentration for this sample type. A representative image of the electropherograms generated at each standard concentration is illustrated in **Fig 2**, and the linear regression parameters are described in **Fig 3**. Under the optimised conditions tested, there was excellent linearity in standards between 6.3 – 100 µM. The overall intra-assay variability for concentrations between 6.3 – 100 µM was 1.5 %, and the inter-assay variability was 6.4 %. The limit of detection was 6 nmol/mg protein, but variables such as the

concentration of the sample injected or the capillary diameter could be changed to enhance the lower limit of detection.

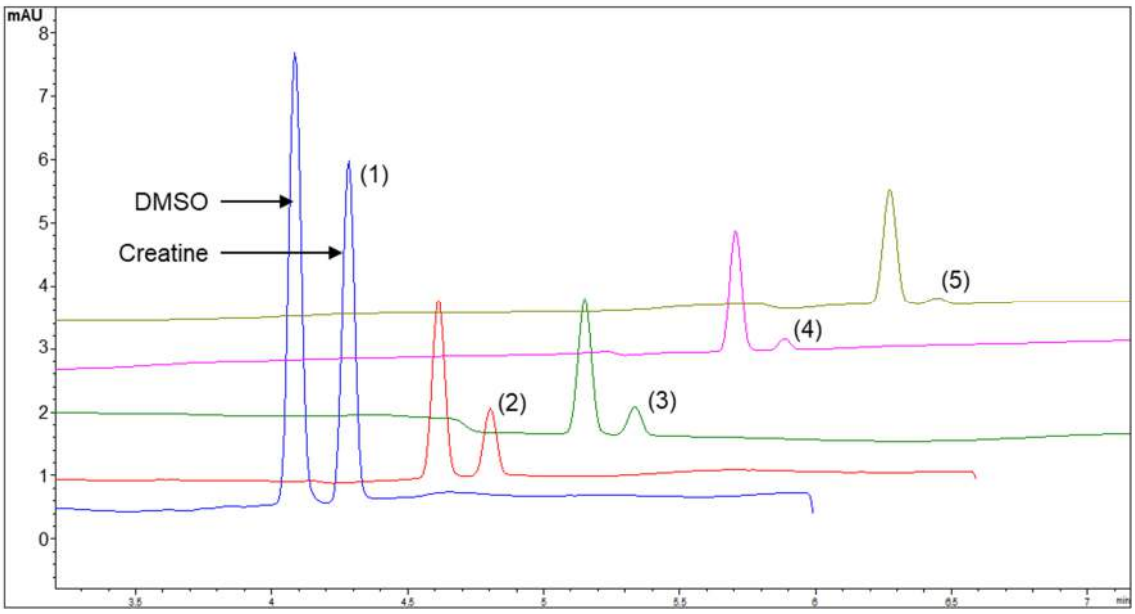

Fig 2. Representative electropherograms of creatine standards under optimised CE conditions with UV detection at 200 nm.

The creatine peaks are identified as: (1) 100 µM, (2) 50 µM, (3) 25 µM, (4) 12.5 µM, (5) 6.3 µM, (6) 0 µM. Creatine standards were prepared in homogenisation buffer, which contains 50 mM NaH_2PO_4, 150 mM SDS and 0.01 % DMSO (as the reference signal) at pH 6. The separation conditions were identical to Fig 1.

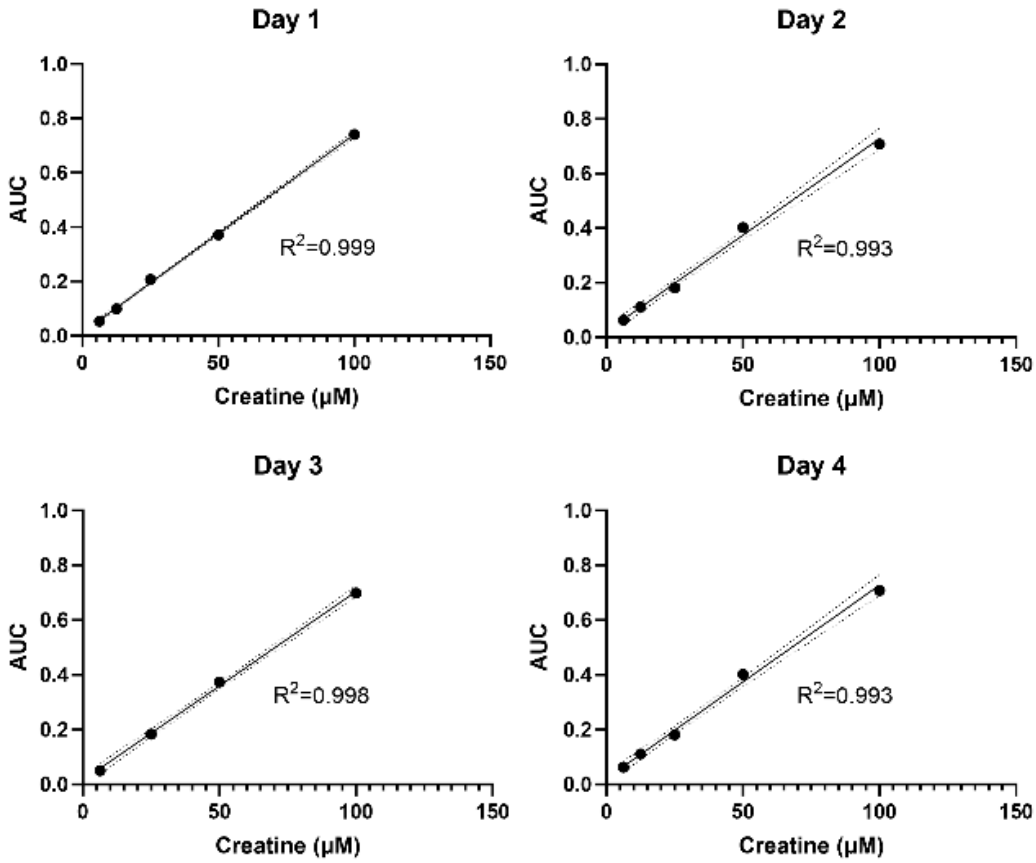

Fig 3. Linearity of creatine standards between 6.3 – 100 µM.
Each panel illustrates the standard curve generated on four separate days. The dotted line represents the 95 % confidence interval of the linear regression. The separation conditions were identical to Fig 1.

Determination of creatine content in fetal lamb lungs

The optimised conditions were used to compare creatine content in fetal lung tissue in groups that received saline or continuous intravenous creatine supplementation for 17 days at 6 mg $kg^{-1}hr^{-1}$. All samples had creatine content within the range of detection. Creatine supplementation significantly increased creatine content in the fetal lungs (mean difference [95 % CI] = 12.5 [9.8, 15.3] nmol/mg protein; $p < 0.0001$) (**Fig 4**).

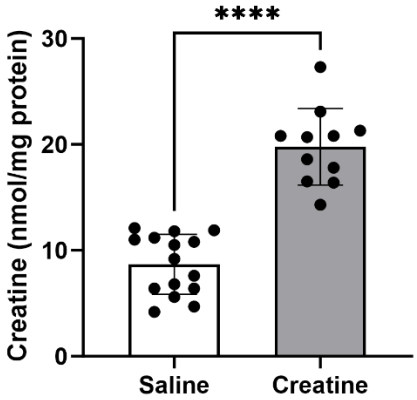

Fig 4. Comparison of lung creatine content with and without creatine supplementation. Intravenous creatine supplementation for 17 days at 6 mg $kg^{-1}hr^{-1}$ increases lung creatine content. Data were analysed using unpaired t-test and presented as mean ± SD. ****$p<0.0001$.

Benchmarking against enzymatic analysis

We tested a random subset of animals from both control and supplemented groups (n = 5/group, based on *a priori* calculation). Fig 5 illustrates a Bland-Altman comparison of the two methods. The two methods showed good agreement, although capillary electrophoresis derived a marginally higher creatine content compared to the enzymatic assay (mean difference [95% CI] = 0.42 [-0.26 – 1.1] nmol/mg protein). While this bias was not statistically significant, it could be explained by potential interference in enzyme assay or lack of completion of the reaction [18]. Regardless, the mean difference was within the pre-determined range of ± 15 %, and both methods showed a similar magnitude of difference between groups that did and did not receive creatine supplementation.

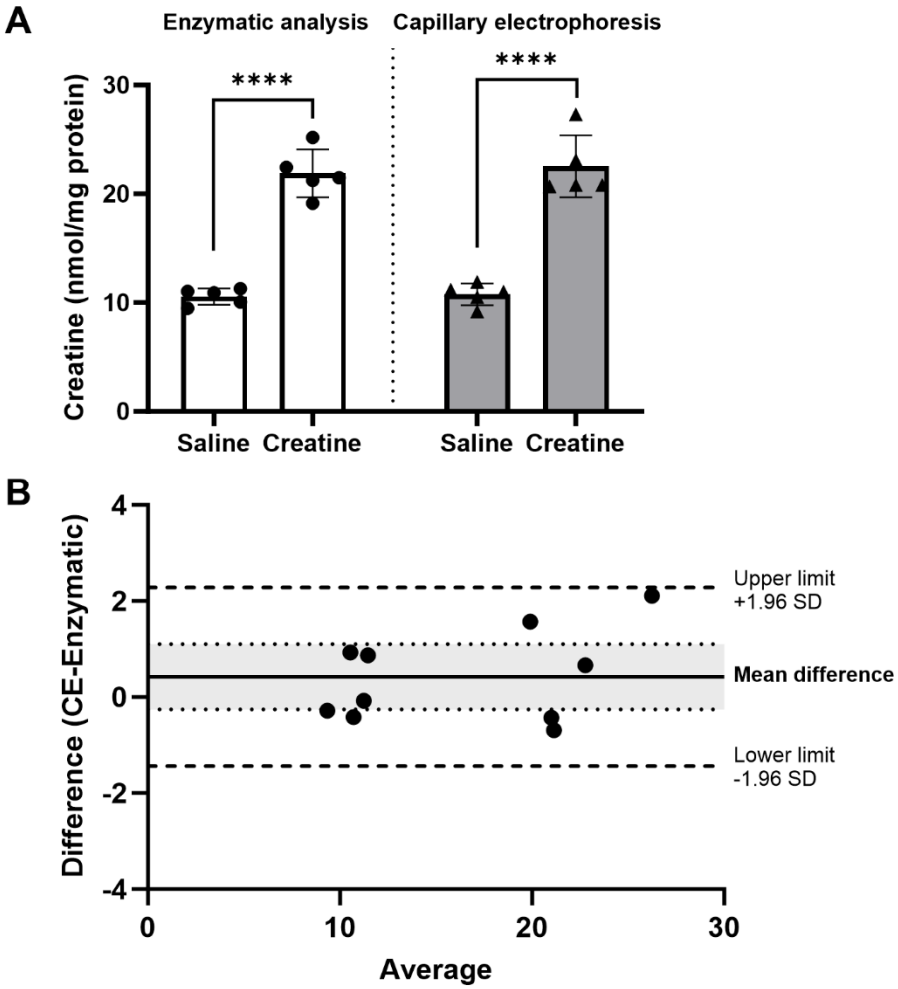

Fig 5. Comparison of capillary electrophoresis (CE) and commercial enzymatic assay kit. (A) Creatine content in the lung tissue was determined using a commercial enzymatic assay kit (MAK079, Sigma-Aldrich) and capillary electrophoresis under separation conditions described in Fig 1. Groups were compared using unpaired t-tests and presented as mean ± SD. ****$p<0.0001$. (B) A Bland-Altman comparison between two methods. The dotted lines on either side of the mean difference denote the 95 % confidence interval of the mean difference.

Comments on the utility of this protocol

Overall, the current protocol provides a streamlined process to analyse creatine content in biological tissue samples. Given the excellent sensitivity of the protocol at low tissue creatine concentrations, we calculated that approximately 1.5 mg tissue (wet weight) would be sufficient to determine creatine content in a lung sample (assuming ~10 nmol/mg protein in control conditions and that each mg of tissue yields ~0.2 mg protein). The current protocol was developed and optimised with ovine lung tissue samples, but it can be easily adapted to analyse various tissue types. We anticipate even less amount of tissue would be sufficient (< 1 mg) to

reliably detect creatine in tissues with higher baseline creatine content, such as the skeletal muscles or brain.

Ethics declarations

Animal ethics approval for the collection of fetal lung tissue was obtained at the University of Western Australia (RA/3/100/1607), and animal research was conducted in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (Eighth Edition).

Supporting information

Supporting Information File 1 – Step-by-step protocol, also available on protocols.io.

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The schematic illustration of the protocol workflow was created with Biorender.com.

Authors contributions

YJC conceptualised, performed the experiments, drafted the protocol and the manuscript. AB conceptualised, performed the experiments and reviewed the protocol. JT interpreted results and reviewed the protocol. JJP obtained funding for the study and reviewed the protocol. PGA conceptualised, interpreted the results, and reviewed the protocol. All authors reviewed and revised the manuscript and approved the final form.

References

1. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80(3):1107–213.
2. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The "phosphocreatine circuit" for cellular energy homeostasis. Vol. 281, Biochemical Journal. Portland Press Ltd; 1992. p. 21–40.
3. Gualano B, Artioli GG, Poortmans JR, Lancha Junior AH. Exploring the therapeutic role of creatine supplementation. Amino Acids. 2010 Jan;38(1):31–44.
4. Persky AM, Brazeau GA. Clinical pharmacology of the dietary supplement creatine monohydrate. Pharmacol Rev. 2001 Jun;53(2):161–76.
5. Dickinson H, Ellery S, Ireland Z, LaRosa D, Snow R, Walker DW. Creatine supplementation during pregnancy: summary of experimental studies suggesting a treatment to improve fetal and neonatal morbidity and reduce mortality in high-risk human pregnancy. BMC Pregnancy Childbirth. 2014 Apr 27;14(1):150.
6. Tran NT, Kelly SB, Snow RJ, Walker DW, Ellery SJ, Galinsky R. Assessing Creatine Supplementation for Neuroprotection against Perinatal Hypoxic-Ischaemic Encephalopathy: A Systematic Review of Perinatal and Adult Pre-Clinical Studies. Cells. 2021 Nov 1;10(11).
7. Schulze A. Creatine deficiency syndromes. Vol. 244, Molecular and Cellular Biochemistry. 2003. p. 143–50.
8. Carducci C, Birarelli M, Leuzzi V, Carducci C, Battini R, Cioni G, Antonozzi I. Guanidinoacetate and creatine plus creatinine assessment in physiologic fluids: An effective diagnostic tool for the biochemical diagnosis of arginine:glycine amidinotransferase and guanidinoacetate methyltransferase deficiencies. Clin Chem. 2002;48(10):1772–8.
9. Cognat S, Cheillan D, Piraud M, Roos B, Jakobs C, Vianey-Saban C. Determination of Guanidinoacetate and Creatine in Urine and Plasma by Liquid Chromatography–Tandem Mass Spectrometry. Clin Chem. 2004 Aug 1;50(8):1459–61.
10. Carducci C, Santagata S, Leuzzi V, Carducci C, Artiola C, Giovanniello T, Battini R, Antonozzi I. Quantitative determination of guanidinoacetate and creatine in dried blood spot by flow injection analysis-electrospray tandem mass spectrometry. Clin Chim Acta. 2006 Feb 1;364(1–2):180–7.
11. Carling RS, Hogg SL, Wood TC, Calvin J. Simultaneous determination of guanidinoacetate, creatine and creatinine in urine and plasma by un-derivatized liquid chromatography-tandem mass spectrometry. Ann Clin Biochem. 2008 Nov;45(6):575–84.
12. Bodamer OA, Bloesch SM, Gregg AR, Stockler-Ipsiroglu S, O'Brien WE. Analysis of guanidinoacetate and creatine by isotope dilution electrospray tandem mass spectrometry. Clin

Chim Acta. 2001;308(1–2):173–8.

13. Fernández-Fernández M, Rodríguez-González P, Añón Álvarez ME, Rodríguez F, Álvarez Menéndez F V., García Alonso JI. Simultaneous determination of creatinine and creatine in human serum by double-spike isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS). Anal Chem. 2015 Apr 7;87(7):3755–63.

14. Sestili P, Martinelli C, Bravi G, Piccoli G, Curci R, Battistelli M, Falcieri E, Agostini D, Gioacchini AM, Stocchi V. Creatine supplementation affords cytoprotection in oxidatively injured cultured mammalian cells via direct antioxidant activity. Free Radic Biol Med. 2006;40(5):837–49.

15. Sestili P, Barbieri E, Martinelli C, Battistelli M, Guescini M, Vallorani L, Casadei L, D'Emilio A, Falcieri E, Piccoli G, Agostini D, Annibalini G, Paolillo M, Gioacchini AM, Stocchi V. Creatine supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts. 2009 Sep 1;53(9):1187–204.

16. Ipsiroglu OS, Stromberger C, Ilas J, Höger H, Mühl A, Stöckler-Ipsiroglu S. Changes of tissue creatine concentrations upon oral supplementation of creatine-monohydrate in various animal species. Life Sci. 2001;69(15):1805–15.

17. Adcock KH, Nedelcu J, Loenneker T, Martin E, Wallimann T, Wagner BP. Neuroprotection of creatine supplementation in neonatal rats with transient cerebral hypoxia-ischemia. Dev Neurosci. 2002;24(5):382–8.

18. Passonneau J, Lowry O. Typical fluorimetric procedures for metabolic assays. In: Passonneau J, Lowry O, editors. A Flexible System for Enzymatic Analysis. New York: Academic Press; 1972. p. 130–1.

19. Watt KKO, Garnham AP, Snow RJ. Skeletal muscle total creatine content and creatine transporter gene expression in vegetarians prior to and following creatine supplementation. Int J Sport Nutr Exerc Metab. 2004;14(5):517–31.

20. Ireland Z, Dickinson H, Snow R, Walker DW. Maternal creatine: does it reach the fetus and improve survival after an acute hypoxic episode in the spiny mouse (Acomys cahirinus)? Am J Obstet Gynecol. 2008;198(4):431.e1-431.e6.

21. Tran NT, Muccini AM, Snow RJ, Nitsos I, Hale N, Walker DW, Ellery SJ. The physiological effects of creatine supplementation in fetal sheep before, during, and after umbilical cord occlusion and global hypoxia. J Appl Physiol. 2021 Aug 12;131(3):1088–99.

22. Gordon MJ, Huang X, Pentoney SL, Zare RN. Capillary electrophoresis. Vol. 242, Science. American Association for the Advancement of Science; 1988. p. 224–8.

23. Ouimet CM, D'amico CI, Kennedy RT. Advances in capillary electrophoresis and the implications for drug discovery. Vol. 12, Expert Opinion on Drug Discovery. Taylor & Francis; 2017. p. 213–24.

24. Smith-Palmer T. Separation methods applicable to urinary creatine and creatinine. J Chromatogr B Anal Technol Biomed Life Sci. 2002 Dec 5;781(1–2):93–106.