Comparison between proton magnetic resonance spectroscopy and high-performance liquid chromatography to quantify muscle carnosine in humans

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Key points summary:

- Although proton magnetic resonance spectroscopy (1H-MRS) was developed to quantify carnosine in human muscle as a non-invasive alternative method to high-performance liquid chromatography (HPLC) in extracts from human muscle biopsy, a thorough assessment of 1H-MRS validity is lacking. Thus, we examined signal linearity *in vitro*, matrix effect *in vivo*, as well as reliability, convergent validity and discriminant validity of *in vivo* 1H-MRS for the determination of carnosine in human muscle using *in vitro* and *in vivo* experiments.

- An excellent 1H-MRS *in vitro* signal linearity was shown for carnosine across the physiological range, although broadening and signal losses were observed when 1H-MRS was performed *in vivo*.

- Free histidine and imidazole also emitted quantifiable signals at the same chemical shift of carnosine, which could constitute a source of error in carnosine quantification. Large protein (e.g., bovine serum albumin) did not emitted signal, thereby indicating they do not constitute a source of error.

- 1H-MRS can detect and quantify muscle carnosine *in vivo*, and it is sensitive to detect increases in muscle carnosine brought about by β-alanine supplementation.

- Despite being sensitivity, 1H-MRS showed poor test-retest reliability, especially due to voxel repositioning and re-shimming.

- A poor agreement was shown for muscle carnosine determination between 1H-MRS and HPLC performed in muscle biopsies taken at the closest possible site (*m. gastrocnemius*).

- Caution should be exercised when interpreting muscle carnosine data obtained with 1H-MRS.
Abstract
Proton magnetic resonance spectroscopy (1H-MRS) has been used as a non-invasive alternative to quantify carnosine in human muscle. It is unclear whether 1H-MRS is a valid and reliable method. 1H-MRS validity and reliability was examined in a series of *in vitro* and *in vivo* studies. In the *in vitro* study, phantoms containing different concentrations of carnosine, imidazole, histidine and bovine serum albumin (BSA) were submitted to 1H-MRS to verify: 1) signal linearity; 2) whether other sources of imidazole could contribute to carnosine signal. In the *in vivo* study, carnosine was determined in the m. gastrocnemius by 1H-MRS and by high-performance liquid chromatography (HPLC, a reference method) in muscle biopsy samples from 16 young men. Test-retest reliability was determined with (n=10) and without (n=5) voxel repositioning and re-shimming. Convergent validity (n=16) was determined by comparing carnosine values obtained with 1H-MRS vs. HPLC. Discriminant validity (n=14) was determined by measuring carnosine before and after 4 weeks of β-alanine supplementation. *In vitro* carnosine signal showed excellent linearity (Pearson correlation: r=0.999). Histidine and imidazole, but not BSA, emitted quantifiable signals in the same chemical shift of carnosine. A clear loss in signal quality was shown in the signal obtained *in vivo*. 1H-MRS coefficient of variation without repositioning voxel was 6.6% and increased to 16.9% with voxel repositioning. 1H-MRS was able to detect a significant increase in muscle carnosine after β-alanine supplementation, both a substantial disagreement with HPLC was shown. 1H-MRS showed adequate discriminant validity, but limited reliability and poor agreement with the reference method.
Introduction

Carnosine is a multifunctional dipeptide abundantly found in human skeletal muscle cardiac muscle and in some neuronal cells (Artioli et al, 2018). Carnosine has numerous properties that confers performance enhancing effects (Saunders et al., 2017), as well as a wide-range of potential therapeutic applications (Artioli et al. 2018). Such properties include hydrogen cation (H+) buffering (Dolan et al. 2018), scavenging of reactive species (Carvalho et al. 2018), and protection against glycation end products (Ghodsi & Kheirouri, 2018). Several studies have demonstrated the beneficial effects of increased muscle carnosine content (for a comprehensive review, please see Boldyrev et al. 2013), which can be easily achieved via dietary supplementation of β-alanine, the rate-limiting precursor of carnosine synthesis (Harris et al. 2006).

A reliable and valid method for tissue carnosine quantification is crucial for advancing the knowledge on biological processes involved with carnosine metabolism, including whether its properties translate into relevant roles for normal physiological function and disease prevention. In human skeletal muscle, carnosine has been quantified in biopsy samples followed by chromatography (Harris et al. 2006; De Salles Painelli et al. 2018) or mass-spectrometry (Carvalho et al. 2018). Even though obtaining muscle biopsies is a relatively simple and largely safe procedure (Neves Jr et al. 2012), the invasive nature of the muscle biopsy technique limits its application.

A non-invasive alternative method based on proton magnetic resonance spectroscopy (1H-MRS) has been developed to quantify carnosine in human skeletal muscle (Ozdemir et al. 2017). 1H-MRS has been considered advantageous to assess muscle carnosine because it is non-invasive, virtually free of risk and suitable to be used in any population. In 1H-MRS, carnosine is quantified from two unique detectable signals emitted by the carbon four (C4-H) and the carbon two (C2-H) of the imidazole ring, which resonate at seven and eight ppm of the magnetic resonance spectrum (Ozdemir et al. 2007).

Although 1H-MRS has been used to quantify carnosine in numerous investigations, there has not been any comprehensive investigation of the validity of 1H-MRS for muscle carnosine assessment against a reference method, such as the chromatographic determination in muscle biopsy samples.
Carnosine quantification by 1H-MRS has several limitations that warrant a thorough experimental examination. Firstly, in vivo carnosine signals are broad, of small amplitude, often close to the noise level, and tend to suffer dipolar coupling, in particular the signal emitted by C4-H. This makes C4-H quantification unfeasible in most cases (Boesch & Kreis, 2001). Also, the in vivo spectrum is crowded with metabolite peaks, thereby making carnosine identification particularly challenging, even when prior knowledge-based approaches are used (Kreis, 1997; Tkac et al, 2002) and, as such, carnosine quantification appears to be more difficult than other abundant muscle metabolites, such as creatine, taurine and lactate (Just Kukurová et al, 2016). Secondly, the signals emitted by the imidazole ring could, in theory, also be detected in other imidazole-containing molecules, such as free imidazole, free histidine, carnosine analogues and histidine residues in proteins. In fact, previous investigations have reported problems in differentiating signals from carnosine and its analogue homocarnosine in human brain (Solis et al. 2015). This could represent a confounding factor for carnosine quantification by 1H-MRS. Thirdly, carnosine concentrations are not homogenous in muscle tissue, since fibre type distribution may affect local carnosine concentrations (Hill et al. 2007; Kendrick et al. 2009; De Salles Painelli et al. 2018). Fourthly, fat and bone tissues surrounding the measurement area can suppress the 1H-MRS signal, adding another source of variation to the carnosine signal (Mon et al. 2013; Mon et al. 2016). Lastly, other variables, such as the signal-to-noise ratio, different angle and/or site of quantification, different machine operators and different data treatment can have major influences on metabolite quantification (for more details, see Alkemade et al, 1978; Hoult & Richards, 1976; Kreis, 1997; Boesch & Kreis, 2001; Tkac et al, 2002).

To address the potential limitations of 1H-MRS to quantify carnosine in human skeletal muscle, the present investigation examined the reliability, accuracy and sensitivity of 1H-MRS for the determination of muscle carnosine in humans using in vitro and in vivo experiments. Carnosine determination by high-performance liquid chromatography (HPLC) in extracts from human muscle biopsy samples was used as the reference method.

Methods

Experimental Design
The study was approved by the institution’s Ethics Committee and conformed to the 2013 version of the Declaration of Helsinki. This study comprised two investigations. In the first investigation, we performed a series of in vitro 1H-MRS acquisitions in phantoms aiming to 1) determine the linearity of the carnosine signal, 2) examine the influence of the presence of the imidazole ring in other compounds (i.e., in free histidine and in histidine residues in protein) to the carnosine signal, thereby gathering knowledge on the contribution of other sources of imidazole ring to the signal obtained in vivo, and 3) compare the signal quality obtained in vitro vs. in vivo.

The second investigation aimed to evaluate the test-retest reliability, as well as the discriminant and convergent validity of the 1H-MRS technique to measure muscle carnosine. HPLC quantification in muscle extracts was chosen as the reference method. To account for the major sources of error in both methods, test-retest reliability was assessed in two different conditions for 1H-MRS (i.e., with and without removing the participant from the scanner, repositioning and re-shimming the voxel) and in three different conditions for HPLC (i.e., same extract from the same sample analysed on two separated runs, different extracts from the same sample analysed on two separated runs and different extracts from two samples analysed on two separated runs). To assess discriminant validity, muscle carnosine was determined in a group of participants before (PRE) and after (POST) β-alanine supplementation. This intervention was intentionally chosen due to its highly consistent effects on muscle carnosine (Harris et al. 2006; Hill et al. 2007; Carvalho et al. 2018), thereby allowing the assessment of whether 1H-MRS is able to discriminate two knowingly different carnosine concentrations. To assess convergent validity, muscle samples were obtained from the same group of participants immediately after 1H-MRS, both before and after β-alanine supplementation, so that the results obtained with 1H-MRS could be compared with those obtained with HPLC. Muscle carnosine concentrations obtained with 1H-MRS were converted to the same unit of muscle carnosine content (i.e., from mmol·L⁻¹ to mmol·kg⁻¹ of dry tissue) for a clearer comparison between methods. Both 1H-MRS and HPLC techniques were performed by well-trained researchers, with large experience in carnosine determination. The 1H-MRS in vivo and muscle biopsy assessments were individually standardized so that each participant performed their PRE and POST-sessions at the same time of day. Participants were requested to abstain from alcohol and unaccustomed exercise in the 48 hours prior
to the experimental sessions. Participants were instructed to arrive at the laboratory at least 2 hours following their last meal. Ad libitum water consumption was allowed before and after the sessions.

In vitro investigation

Thirteen 0.5 L cylindrical bottles mimicking a human calf were filled with solidified solutions of carnosine, imidazole, histidine or BSA. Phantoms of 6 different carnosine concentrations (3.0, 4.5, 6.0, 12.5, 25.0 and 50.0 mmol·L⁻¹) were prepared to assess signal linearity within the physiological range and signal behaviour near to the lowest physiological range. Phantoms of 3 different imidazole and histidine concentrations (12.5, 25.0 and 50.0 mmol·L⁻¹ each) were prepared to examine whether the signals would differ between imidazole-containing substances. One phantom containing BSA (the equivalent to 12.5 mmol·L⁻¹ of imidazole) was prepared to assess whether imidazole-residues in large size molecules (e.g., protein) could emit a signal at the same chemical shift (7 and 8 ppm). All phantoms were solidified by melting agarose 2% w:v in autoclaved ultra-pure water prior to adding carnosine, imidazole, histidine or BSA. Phantom concentrations were calculate based on the imidazole content so that all concentrations were equimolar to 12.5 mmol·L⁻¹ of imidazole. The 12.5 mmol·L⁻¹ concentration for BSA was chosen because this is nearly the maximum achievable within the solubility of BSA and it represents a mid-range physiological concentration of carnosine in human skeletal muscle.

For the present investigation, a 3 Tesla whole-body magnetic resonance scanner (Achieva, Philips, Best, The Netherlands) equipped with an 8-channel knee coil was used. All the spectra were acquired using single voxel point-resolved spectroscopy (PRESS) localisation with the following parameters: TR/TE=6000/30 ms, voxel size=10×10×10 mm³, number of averages (NEX)=224, 2048 data points with a spectral width of 2000 Hz. The total acquisition time was 20 min for each phantom. All spectra were processed in jMRUI software. Residual water and lipid peaks were removed by a Hankel Lanczos Squares Singular Values Decomposition (HSVLD) algorithm from the carnosine, histidine and BSA spectra, and their C2-H and C4-H peaks were fitted with Advanced Method for Accurate, Robust and Efficient Spectral fitting (AMARES) using single
Lorentzian line shapes. Carnosine’s signal linearity was evaluated by linear regression of the “carnosine concentration vs. signal” calibration curve.

In vivo investigation

Sixteen young, healthy, physically active men volunteered to participate, two of whom could not complete the entire study due to personal reasons. Therefore, 14 participants (age: 27±5 years; body mass: 82.9±11.8 kg; stature: 1.77±0.06 m; body mass index: 26.3±2.4 kg·m$^{-2}$) completed all tests. Participants were fully informed of possible risks and discomforts associated with participation before providing informed consent. They were requested to maintain similar levels of physical activity and dietary patterns for the duration of the study. Exclusion criteria were: (i) use of supplements containing creatine or β-alanine in the 3 months and 6 months prior to the study; (ii) use of anabolic steroids; (iii) chronic use of glucocorticoids; (iv) chronic-degenerative disease and/or condition that affected the locomotor apparatus, and (v) any condition that would prevent them from undertaking the proposed tests (e.g., metallic prostheses that could interfere with 1H-MRS quality).

Participants were assessed for muscle carnosine before and after a 4-week period of β-alanine supplementation. β-alanine was provided in 800-mg tablets (CarnoSyn™, NAI, USA) and the participants were asked to take 2 tablets along with meals, four times per day, totalling 6.4g·d$^{-1}$ of β-alanine. All 16 participants completed a baseline assessment for carnosine quantification in the medial portion of $m$. gastrocnemius of the right leg using both 1H-MRS and HPLC. Carnosine quantification via 1H-MRS was not possible in one participant due to a peak of very small amplitude with baseline below zero. Therefore, the analysis of convergent validity was conducted on 15 participants. To minimise differences between methods owing to variations in sampling sites, biopsy sites were intentionally taken from the closest possible sites to those where the spectra were obtained. This was ensured with the physician examining the image of the voxel position before defining the location and the depth the biopsy needle would be inserted. Following supplementation, the 14 participants who completed the entire study were again assessed for muscle carnosine using both 1H-MRS and HPLC. The responses to supplementation were used to compare the discriminant validity between methods.
A sub-sample of 10 participants volunteered for the test-retest reliability assessment of 1H-MRS with voxel repositioning and re-shimming. They undertook the first 1H-MRS, left the room, waited for 5 minutes and then were repositioned back on the machine for the second 1H-MRS. The voxel was repositioned as closely as possible to the site where it was positioned in first test; this was achieved using an image of the voxel position obtained in the first 1H-MRS as a guide. Another sub-sample of 5 participants volunteered for the test-retest reliability assessment of 1H-MRS without voxel repositioning and re-shimming. They undertook the first 1H-MRS and stood still for the second 1H-MRS, which was performed immediately after the first.

To assess inter-assay reliability of HPLC determination of muscle carnosine, muscle extracts obtained from 15 biopsy samples randomly chosen from a collection of containing 144 muscle samples, using an online random number generator. These were analysed in duplicate in two independent runs performed on different days. To assess “inter-extract” reliability of HPLC, two different muscle extracts obtained from 11 biopsy samples randomly chosen from this same collection were analysed in duplicates on different days. To assess “inter-biopsy” reliability of HPLC, two consecutive muscle samples were obtained from the same incision in a sub-sample of 7 participants who volunteered for this study. The second biopsy location was changed by rotating the needle’s window guillotine in 90 degrees, thereby sampling the collateral site of the first biopsy.

1H-MRS in vivo assessment

Spectra were acquired using single voxel point-resolved spectroscopy (PRESS) localisation with the following parameters: TR/TE=3000/30 ms, voxel size=10×10×30 mm³, number of averages (NEX)=256, 2048 data points with a spectral width of 2000 Hz. The total acquisition time of the 1H-MRS was 13.9 min. The 50 mmol·L⁻¹ carnosine phantom was used as an external reference. To that end, an acquisition was made using the same parameters as those used in vivo, except for the TR, which was 12000 ms. In each in vivo measurement, the right leg of each participant was positioned and was firmly immobilised in the knee coil, such that the gastrocnemius muscle was in the centre of the coil. The left leg was supported outside the coil to improve comfort and thus minimise leg movement. Voxel location was standardised on the larger calf region in the centre of
the medial portion of the gastrocnemius muscle of the right leg. The same well-trained and experienced biomedical technician was responsible for placing the voxel in all conditions. After placing the voxel, a set of images depicting individual voxel location was saved and used to guide positioning in all further exams of that individual.

**Quantification**

Absolute quantification of the carnosine resonance was determined using the following equation (Ozdemir et al, 2007):

\[
SV = \frac{S_M F_{MT1} F_{MT2} \times 0.66}{S_{H2Ov} F_{H20MT1} F_{H20MT2}} \\
ST = \frac{S_R F_{RT2}}{S_{H2Ov} F_{H2OvRT2}} \\
CM = \frac{SV}{ST P_t} \times 50 \text{ mmol/L}
\]

CM is the concentration of the metabolite *in vivo*, SV and ST are the signals of the water-corrected metabolite *in vivo* and *in vitro*; SM is the integral of the carnosine peak *in vivo* and SR is the integral of the carnosine peak *in vitro*; F\(_{MT1}\) is the correction factor for T1 relaxation of the metabolite *in vivo*; F\(_{MT2}\) and F\(_{RT2}\) are the correction factors for metabolic T2 relaxation *in vivo* and *in vitro*; S\(_{H2Ov}\) is the integral of the water peak *in vivo* and S\(_{H2Ov}\) is the integral of the water peak *in vitro*; F\(_{H20MT1}\) is the correction factor for T1 relaxation of water *in vivo*; And F\(_{H20MT2}\) and F\(_{H2ORT2}\) are the correction factors for T2 relaxation of water *in vivo* and *in vitro*. Pt is the temperature correction factor applied as the signal decreases by 6% between the room temperature (*i.e.*, phantom temperature) and body temperature (Davies, 2003). For the *in vitro* signal, it is not necessary to correct the T1 relaxation, since the acquisition was performed with a sufficiently long TR (TR=12000 ms) to neglect this factor. Signals were also corrected by water content; since the water content in phantoms is ~100%, a correction factor=1 was used. For the *in vivo* analyses, a correction factor=0.66 was used, assuming that ~2/3 of the muscle is water (Schoeller, 1989).

The relaxation correction factors were calculated using the following equations:

\[
F_{T1} = \frac{1}{1 - \exp\left(-\frac{TR}{T1}\right)}
\]
\[ F_{T2} = \frac{1}{-\exp\left(\frac{TE}{T2}\right)} \]

T1 and T2 values for water and carnosine were taken from the literature, and were assumed to be 1420 ms and 32 ms for water in muscle (Gold et al., 2004) and 520 ms and 66 ms for in vivo carnosine (Ozdemir et al., 2007). The T2 values of in vitro water and carnosine in vitro (52 ms and 200 ms) were measured using different TEs (31, 61, 99, 150, 228, and 400 ms) with TR of 6000 ms and calculated using MATLAB® software (MathWorks, Natick, MA, USA) by fitting data points determined using the peak areas of metabolites to a mono-exponential function \( M_s = M_0 \exp(-TE/T2) \). To further convert concentration values in mmol·L\(^{-1}\) into the content equivalent in mmol·kg\(^{-1}\) of dry muscle, results were multiplied by 3.3, a factor which assumes that for every 1 kg of dry muscle there is 3.3 kg of water. Spectrum figures are presented with the raw (untreated) spectrum on the left side and a cut window (framework of the target the frequency) on the right. The cut window signal was normalized by the baseline offset to facilitate visual comparison between both situations (Kohl et al. 2012).

**Muscle biopsies**

Muscle samples (~70-100 mg) were obtained under local anaesthesia (3 mL, 1% lidocaine) from the mid-portion of the *m. gastrocnemius* using the percutaneous needle biopsy technique with suction (Bergstrom, 1962). Samples were obtained from the same leg for all experiments. PRE and POST supplementation biopsies were taken from incisions made as close as possible to one another. Samples taken for the inter-biopsy reliability analyses were obtained from the same incision, but from slightly different sites, as described above. All samples were snap frozen in liquid nitrogen and were subsequently stored at -80°C until analyses. Samples were freeze-dried and dissected free of any visible blood, fat and connective tissue before being powdered and further submitted to HPLC determination of carnosine.

**Chromatographic determination of histidine-containing dipeptides in whole muscle**

Deproteinised muscle extracts were obtained from 3-5 mg freeze-dried samples according to the protocol described by Harris et al. (2006). Briefly, samples were deproteinised with 0.5M HClO\(_4\) and neutralised with 2.1M KHCO\(_3\). The extracts were
then filtered through a 0.22 µm centrifugal PVDF filter unit and stored at -80°C until analysis. Total muscle carnosine content was quantified by HPLC (Hitachi, Hitachi Ltd., Tokyo, Japan), according to the method described by Mora et al. (2007). Mobile phases consisted of solvent A, containing 0.65 mM ammonium acetate, pH 5.5, in water/acetonitrile (25:75); and solvent B, containing 4.55 mM ammonium acetate, pH 5.5, in water/ acetoni-

crile (70:30). The chromatographic separation was developed using an Atlantis HILIC silica column (4.6 x 150 mm, 3 µm; Waters, Milford, MA, USA) attached to an Atlantis Silica column guard (4.6 x 20 mm, 3 µm), at room temperature. The analysis conditions comprised of a linear gradient from 0 to 100% of solvent B in 13 min at a flow rate of 1.4 mL·min⁻¹. Separation was monitored using a U.V. coupled detector at a wavelength of 214 nm.

Statistical analyses

Signal linearity of the in vitro analysis was verified by interpolating signal intensity by concentration and calculating Pearson correlation coefficient (r). Test-retest reliability was assessed using: 1) a paired sample t-test to check for systematic errors, 2) intraclass correlation coefficient (ICC - two-way random, absolute agreement, single-measures) with their respective 95% confidence intervals (95%CI) and 3) within-subject mean square root coefficient of variation (CV) (Hyslop & White, 2009). Test-retest data were plotted to individually display the distance between data pairs and the identity line. Convergent validity was assessed using: 1) an unpaired sample t-test to check for differences between HPLC and 1H-MRS values, for both PRE and POST supplementation data sets, and 2) the Bland-Altman plot for percentage differences against mean values for the overall data set. HPLC vs. 1H-MRS data was plotted to individually display the distance between data pair and the identity line. Discriminant validity was assessed using a paired sample t-test to compare mean carnosine values between PRE and POST supplementation period. Effect sizes (ES) for the muscle carnosine content increase after β-alanine supplementation were calculated using Cohen’s d. All analyses were conducted in the IBM SPSS software (version 20). Bland-Altman was build using the GraphPad Prism software (version 5.03).

Results
In vitro analyses

Figure 1 displays a spectrum obtained in vitro with a 12.5 mmol·L⁻¹ carnosine phantom alongside a spectrum obtained in vivo from human calf of similar concentration (10.42 mmol·L⁻¹). It is possible to visualise that the signal obtained in vitro is sharp and has low noise, whereas the signal obtained in vivo is broadened, less sharp and has increased noise.
Figure 1. Signal obtained *in vitro* (top panel) and *in vivo* (bottom panel). On the left, the signals are plotted as raw data; water peak is circled and the frequency area containing the carnosine peaks is indicated by the vertical dashed lines. On the right is the cut window of the frequency area containing the carnosine peaks, indicating the signal after normalisation.

Signals obtained *in vitro* from phantoms containing carnosine, imidazole and histidine displayed excellent linearity within the concentration range assessed (Figure 2). The carnosine signal was sharp and of low noise (Figure 3, panel A), whereas the imidazole (Figure 3, panel C) and histidine (Figure 3, panel B) were less sharp and had larger noise in comparison with the signal amplitude. Yet, all signals were quantifiable, except for the BSA signal (Figure 3, panel D).
Figure 2. Signal linearity obtained from phantoms containing imidazole, histidine and carnosine. Concentrations are equimolar in imidazole.
Figure 3 – Spectra obtained from phantoms containing 12.5 mmol·L⁻¹ of carnosine, histidine and imidazole (panels A, B and C, respectively) and bovine serum albumin (BSA) (panel D). Signals were quantifiable in all spectra, except BSA.

Reliability of HPLC for carnosine quantification in human skeletal muscle

Inter-assay reliability showed very similar values for both test and retest (mean ± 1SD difference=0.6±4.0%). No statistically significant differences between measures were shown (t=0.144; p=0.887), suggesting that HPLC is free of systematic errors when the same muscle extracts are analysed. A high ICC and low CV were seen between test and retest values (ICC=0.996, 95%CI=0.987-0.999; CV=2.72%) (Figure 4, panel A). Thirteen of the 15 samples had less than 5% variation between measurements, with the remaining two samples having less than 10% variation.

Inter-extract reliability also showed very similar measurements between test and retest (mean±1SD difference=1.0±4.5%). No statistically significant differences were shown between measures (t=0.519; p=0.615). A high ICC and low CV were seen between test and retest values (ICC=0.988, 95%CI=0.956-0.997; CV=3.17%) (Figure 4, panel B).
Eight of the 11 samples were below 5% of variation between measurements, with the remaining 3 samples being below 10% variation.

Inter-biopsy reliability analysis also showed very similar measurements (mean ± 1SD difference =1.1±6.0%). No statistically significant differences were shown between measures (t=-0.588; p=0.578). A high ICC and low CV were seen between test and retest values (ICC=0.957, 95%CI=0.750-0.993; CV=3.95%) (Figure 4, panel C). Five of the seven samples were below 5% of variation between measurements with the remaining 2 samples being either below or at 10% of variation.
Figure 4. Repeatability analysis of the chromatographic determination of carnosine in muscle samples considering the intra-assay variability (same extraction from the same samples measured twice – panel A), inter-assay variability (different extractions from the same samples, measured in duplicate – panel B) and the inter-biopsy variability (different extractions from different samples, measured in duplicate – panel C). The left charts depict test-retest agreement for each individual sample (in comparison with the line of identity) along with indexes of reliability. The right charts depict the mean ±1SD for test and retest conditions.

Reliability of 1H-MRS determination of muscle carnosine

The mean carnosine values obtained via 1H-MRS for test and retest without voxel repositioning showed similar measurements (mean±1SD difference=5.0±6.7%) (Figure 5, panel A). No statistically significant differences were shown (t=-1.0; p=0.37), indicating that 1H-MRS is free of systematic errors. Intraclass coefficient correlation was 0.924 (95%CI=0.451-0.992) and the CV was 6.6%. The variation between tests was below 5% in 4 out of the 5 participants.

Reliability indexes of 1H-MRS were poorer when the individuals were repositioned on the equipment, and the voxel was repositioned and re-shimmed (ICC=0.775, 95%CI=0.325-0.939; CV=16.9%). The mean ± 1SD difference between tests was 2.4±26.8%. No statistically significant differences were shown (t=0.72; p=0.49). The variation between tests was below 5% in only one participant, between 5-10% in only two participants, and above 10% in the remaining 7 participants (Figure 5, panel B).
Figure 5 – Repeatability analysis of the 1H-MRS determination of carnosine in muscle samples considering test and retest without voxel repositioning (individuals laid still on the equipment between tests – panel A) and with voxel repositioning and re-shimming (individuals were repositioned on the equipment between tests – panel B). The left charts depict test-retest agreement for each individual test (in comparison with the line of identity) along with indices of reliability. The right charts depict mean ± 1SD for test and retest.

*Convergent validity of 1H-MRS vs. HPLC*
Both methods can detect the increase in muscle carnosine in response to β-alanine supplementation (both p<0.05; figure 6, panel A). Although no statistically significant differences for the POST-PRE deltas were shown between methods (neither for the absolute, nor for the relative delta – Figure 6, panel B), a large disagreement was shown between methods regarding the ability to detect changes in muscle carnosine in response to supplementation (Figure 6, panels C and D).

When both PRE- and POST-supplementation measures were pooled, the Bland-Altman plot showed a visible disagreement between HPLC and 1H-MRS, which increased when carnosine values were <10 mmol·kg⁻¹ dm (Figure 6, panel E). Only two of the 27 measures were below 5% difference between techniques; 17 out of 27 measures were above 20% difference, and 7 measures were above 50% (Figure 6, panel F).
Figure 6. Convergent validity of 1H-MRS vs. HPLC for muscle carnosine determination. Panel A: Individual (represented in letters) and mean±1SD values for muscle carnosine measured using both methods before and after β-alanine supplementation. Panel B: Absolute (left chart) and relative (right chart) post-pre delta values for muscle carnosine measured using both methods in response to β-alanine supplementation. Panel C: Absolute post-pre delta values obtained using both methods plotted against the identity line (i.e., representing 100% agreement between methods). Panel D: Relative post-pre
delta values obtained using both methods plotted against the identity line (i.e., representing 100% agreement between methods). Panel E: Bland-Altman plot for percent differences between methods. Panel F: pooled pre and post data for muscle carnosine values obtained using both methods plotted against the identity line (i.e., representing 100% agreement between methods).

Discussion

In light of the growing attention that muscle carnosine has been receiving due to its potential ergogenic and therapeutic properties, quantifying this dipeptide in muscle tissue is becoming an increasingly necessary procedure. As such, the use of an accurate, reliable and sensitive method for carnosine quantification is of the utmost importance. Although 1H-MRS has emerged as a non-invasive alternative for analytical methods that require muscle biopsies, no study to date has examined its validity against a well-established reference method. In the present study, we performed a series of in vitro and in vivo experiments to examine several aspects of 1H-MRS validity (i.e., signal linearity, matrix effect, reliability, discriminant and convergent validity). In order to be certain that HPLC is a reliable method for muscle carnosine determination and could be used as the reference in this study, we also conducted a thorough reliability examination of the its reliability, which showed excellent repeatability in all instances (i.e., inter-assay, inter-extract, and inter-biopsy).

The present investigation revealed important methodological issues that must be considered when interpreting muscle carnosine values obtained in vivo by 1H-MRS. Due to the small signal amplitude (Boesch & Kreis, 2001; Kreis, 1997; Tkac et al. 2002; Just Kukurová et al., 2016), we sought to be certain that the carnosine signal is quantifiable across the entire physiological range, including the expected values for the lowest and highest extremes of human population, such as those reported in vegetarians (De Salles Painelli et al. 2018) and bodybuilders (Tallon et al. 2005). In this respect, we showed an excellent linearity in vitro, as well as a clear ability to detect and quantify carnosine even in the lowest range. However, it must be noted that this does not necessarily imply that quantifying the carnosine signal in vivo would be equally feasible, as the in vivo signal is clearly broader, of lower amplitude, and presented higher baseline noise. Such
phenomenon is similar to the matrix effect often seen in analytical methods and can be explained by the potential influence of fat and bone tissues to the carnosine signal (Mon et al. 2013; Mon et al. 2016), as well as the large number of compounds with magnetic nuclei present in human muscle. The magnetic interaction among neighbouring, non-equivalent, magnetic nuclei causes a phenomenon known as spin-spin coupling, where the magnetic field generated by each proton interferes with the other one, resulting in splitting and broadening of signal peaks (Due et al. 1998). This phenomenon can be better observed for the C4-H peak, since its position in the imidazole ring and its proximity with the nearest protons makes its signal more susceptible to spin-spin coupling effects (Boesch & Kreis, 2001). These features represent a challenge when trying to use 1H-MRS to quantify carnosine in human muscle. As it was herein demonstrated, carnosine peaks already present a small amplitude in the 1H-MRS (Just Kukurová et al, 2016); when these signals are divided or further weakened by the spin-spin coupling, a decrease in signal, resulting in lower signal-to-noise ratio (Hoult & Richards, 1969). In individuals with low muscle carnosine content, increased error is to be expected, since peak amplitude is naturally lower and, therefore, very close to the basal noise. This is supported by the increased disagreement between 1H-MRS and the reference method shown in the Bland-Altman plot when carnosine concentrations are near to the lowest range. One could suggest to measure, as an alternative, the carnosine subpeaks. However, identifying all subpeaks in the spectrum may not be possible, since they might be totally covered by noise, especially in volunteers who present low muscle carnosine levels.

To investigate the potential impact of the imidazole ring present in other molecules (e.g., free imidazole, free histidine, carnosine analogues and histidine residues in proteins) on the carnosine signal detected by 1H-MRS, the in vitro signal was compared between carnosine, imidazole, histidine, and BSA. Although the best signal quality was obtained with carnosine, quantifiable signals were also obtained with imidazole and free histidine. This indicates that small imidazole-containing molecules might constitute a potential source of error, although they are likely of low relevance for the skeletal muscle since they are expressed in very low concentrations in comparison with carnosine (Parkhouse et al. 1985). Conversely, no signal was obtained with BSA, probably due to its large size (~66 kDa). Increasing molecular size leads to slower tumbling and correspondingly shorter spin-spin relaxation times (T2), resulting in to a more complex
spectrum with very broad peaks of low amplitude that do not surpass noise level. Accordingly, 1H-MRS experiments become unreliable at room temperature for proteins larger than 30 kDa and largely fail for proteins above 35 kDa in the absence of elevated temperature (Wand et al. 1998). These results indicate that imidazole-rich proteins such as haemoglobin and other large proteins do not represent a source of error. However, it is still possible that other smaller histidine-rich proteins, such as myoglobin (17 kDa, 4.7 mg·g\(^{-1}\) wet muscle, 11% histidine) might contribute to the in vivo signal (Moller & Sylven, 1981). Unfortunately, purified myoglobin is not easily accessible and we could not prepare a phantom containing myoglobin for further verification. Hence, whether myoglobin constitutes a source of error requires future clarification.

To assess reliability, convergent and discriminant validity of in vivo 1H-MRS, a series of analyses were conducted. No significant differences were shown between test and retest values, indicating that 1H-MRS is free of systematic errors and that the variation is explained by random error. Importantly, a remarkable increase in test-retest variation (6.6% vs. 16.9%) was shown when the retest was performed with the participant being removed from and then relocated to the scanner, which is a more “real-world” representation of studies assessing muscle carnosine before and after an intervention. Such an increase in variation indicates that voxel positioning and shimming are major sources of random error in 1H-MRS. In the present study, all possible measures were taken to ensure that voxel would be positioned in the same location (i.e., the same experienced technician was responsible for voxel positioning in all exams; voxel positioning at “retest” was done with the image depicting voxel position at “test” as a guide). Nonetheless, a large variation was observed between test and retest measurements, indicating that small differences in voxel position have a large impact on the results. Such a large variation with voxel repositioning and re-shimming is somewhat expected for a metabolite with a broad signal and of low amplitude, such as carnosine, since similar levels of variation have been reported for other metabolites of much sharper and high amplitude signals (Al-iiedani et al., 2018). In addition, the ~17% variation reported in this study is not too dissimilar to the ~23% previously shown by Ozdemir et al. (2007).

One explanation for the larger variation of 1H-MRS is the non-homogeneity of carnosine distribution in the skeletal muscle (Baguet et al, 2018). Carnosine content is
greater in type II than type I muscle fibres (Painelli et al., 2018); therefore, the inevitable change in muscle site when performing two 1H-MRS may cause sampling sites to have different fiber type composition, adding a source of measurement error. However, slight changes in sampling sites likely occurred with muscle biopsies, but the variation for HPLC was lower, nonetheless. This means that other factors may play a role in the increased variability in 1H-MRS, such as the proximity with tissues that may cause signal interference (e.g., adipose tissue). The fat signal often appears bright in many important clinical imaging sequences and can obscure other signals (Bley et al. 2010; Maudsley et al. 2012). Thus, adipose tissue near or inside the data acquisition site can contribute to the increased variability. Additionally, 1H-MRS appears to be more sensitive to changes in sampling sites because shimming and spin-spin coupling are dependent on the angle between spins and the magnetic field, which may alter with slight changes in sampling sites. Such orientation-dependence is particularly true for the carnosine signal in human skeletal muscle (REF). Finally, participants' motion during the exam could also disrupt data acquisition, thereby contributing to 1H-MRS variability (Marshall et al. 1996).

The discriminant validity study showed that 1H-MRS, despite having large variation owing to random errors, is sensitive to detect group-mean increases in muscle carnosine in response to β-alanine supplementation. The changes shown with 1H-MRS are similar to those reported in other studies (Derave et al. 2007; Chung et al. 2014). Yet, when comparing the ability of 1H-MRS to detect changes in muscle carnosine with HPLC, a high degree of disagreement between methods was shown. Likewise, a large degree of disagreement was shown in all instances where 1H-MRS and HPLC were compared, which becomes particularly evident when the results are analysed at the individual level (Figure 6). The disagreement seemed to increase when participants showed smaller carnosine content, possibly due to 1H-MRS signal characteristics. As discussed, the carnosine peak amplitude in 1H-MRS makes it very prone to suffer noise interference, which is more pronounced at lower concentrations. On the other hand, carnosine peaks in the HPLC chromatogram are large, sharp and easily quantifiable across the entire physiological range (Mora et al. 2007). Previous studies in the literature also appear to support the discrepancy between 1H-MRS and HPLC measurements of muscle carnosine, since HPLC studies consistently show 40-80% increases in muscle carnosine in response to 4 weeks of a 6.4 g.day⁻¹ dosage of β-alanine supplementation (Harris et al.
2006; Hill et al. 2007; Saunders et al. 2017), whereas studies using 1H-MRS show increases of 140-160% in muscle carnosine (Chung et al. 2014), or no increase at all (Black et al. 2018) following the same β-alanine supplementation protocol. Indeed, the differential sensitivity of the magnetic resonance scanners (e.g., 3 vs. 1.5 Tesla) used in the aforementioned studies (Chung et al. 2014; Black et al. 2018) contributed to the heterogeneous findings, since 1H-MRS using a 1.5-Tesla scanner is unlikely to have the sensitivity to detect changes in muscle carnosine, even if substantial as expected with β-alanine supplementation.

Interestingly, muscle carnosine could not be quantified by 1H-MRS in one participant, since there were no detectable peaks in the area of carnosine frequency. This lack of carnosine signal was observed only for this subject, who happened to present the largest amount of calf subcutaneous fat tissue among all participants. The relationship between local fat depots and sizable frequency-dependent signal attenuation in 1H-RMS is still debatable. While evidence by Mon et al. (2013) and Mon et al. (2016) supports that an increase in body mass index may lead to a degradation in spectral quality, Kyathanahally et al. (2015) could not demonstrate this effect. More research is needed to clarify the influence of local fat depots on the signal acquired by 1H-RMS, as well as on carnosine quantification.

A potential limitation of this study is that the results were obtained from the human calf muscle (i.e., gastrocnemius medialis), known to have a mixed proportion of type I and II fibers; hence, it remains to be elucidated if 1H-MRS shows better measurement performance for carnosine quantification in a more homogeneous muscle tissue. In addition, considering that physically active participants were recruited for the present study and that local adipose tissue appeared to be an important factor interfering with 1H-MRS signal, we cannot rule out the possibility that 1H-MRS is more reliable in individuals with low body fat, such as athletes.

**Conclusion**

1H-MRS is capable of measuring carnosine in muscle tissue and is sensitive to detect overall changes in muscle carnosine brought about by β-alanine supplementation. However, 1H-MRS has a high-degree of variation due to random error associated with voxel positioning, and poor convergent validity. This makes quantification problematic,
particularly in regions surrounded by fat tissue, in individuals with high levels of body fat, and in individuals with low muscle carnosine levels. Caution should be exercised when interpreting muscle carnosine quantification data obtained with 1H-MRS.

References

1. Alkemade CTJ, Snelleman W, Boutilier GD, Pollard BD, Winefordner JD, Chester TL, & Omenetto N (1978). A review and tutorial discussion of noise and signal-to-noise ratios in analytical spectrometry—I. Fundamental principles of signal-to-noise ratios. Spectrochimica Acta Part B: Atomic Spectroscopy, 33(8), 383-399.

2. Artioli GG, Sale C & Jones RL (2018). Carnosine in health and disease. Eur J Sport Sci 4, 1-10.

3. Baguet A et al. (2011). A new method for non-invasive estimation of human muscle fiber type composition. PLoS One 6.7, e21956.

4. Baguet A, Bourgeois J, Vanhee L, Achten E & Derave W (2010). Important role of muscle carnosine in rowing performance. J Appl Physiol 109, 1096-1101.

5. Bergstrom J (1962). Muscle electrolytes in man determination by neutron activation analysis on needle biopsy specimens. A study on normal subjects, kidney patients and patients with chronic diarrhea. Scand J Clin Lab Inv 14, 100–110.

6. Black MI, Jones AM, Morgan PT, Bailey SJ, Fulford J & Vanhatalo A (2018). The Effects of β-Alanine Supplementation on Muscle pH and the Power-Duration Relationship during High-Intensity Exercise. Front Physiol 9, 111.

7. Bley TA, Wieben O, François CJ, Brittain JH & Reeder SB (2010). Fat and water magnetic resonance imaging. J Magn Reson Imag 31, 4-18.

8. Boesch, C., & Kreis, R. (2001). Dipolar coupling and ordering effects observed in magnetic resonance spectra of skeletal muscle. NMR in Biomedicine: An International
Journal Devoted to the Development and Application of Magnetic Resonance In Vivo, 14(2), 140-148.

9. Carvalho VH, Oliveira AHS, de Oliveira LF, da Silva RP, Di Mascio P, Gualano B, Artioli GG & Medeiros MHG (2018). Exercise and β-alanine supplementation on carnosine-acrolein adduct in skeletal muscle. Redox Biol 18, 222-228.

10. Chung W, Baguet A, Bex T, Bishop DJ & Derave W (2014). Doubling of muscle carnosine concentration does not improve laboratory 1-hr cycling time-trial performance. Int J Sport Nutr Exerc Metab 24, 315-324.

11. Chung W, Shaw G, Anderson ME, Pyne DB, Saunders PU, Bishop DJ & Burke LM (2012). Effect of 10-week beta-alanine supplementation on competition and training performance in elite swimmers. Nutrients 4, 1441-1453.

12. Davies GR, Ramani A, Dalton CM, Tozer DJ, Wheeler-Kingshott CA, Barker GJ & Tofts PS (2003). Preliminary magnetic resonance study of the macromolecular proton fraction in white matter: a potential marker of myelin? Multiple Sclerosis Journal, 9(3), 246-249.

13. De Salles Painelli V, Nemezio KM, Pinto AJ, Franchi M, Andrade I, Riani LA, Saunders B, Sale C, Harris RC, Gualano B & Artioli GG (2018). High-Intensity Interval Training Augments Muscle Carnosine in the Absence of Dietary Beta-alanine Intake. Med Sci Sports Exerc 50, 2242-2252.

14. Derave W, Ozdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, Wise JA & Achten E (2007). Beta-alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. J Appl Physiol 103, 1736-1743.

15. Dolan E, Saunders B, Dantas WS, Murai IH, Roschel H, Artioli GG, Harris R, Bicudo JEPW, Sale C & Gualano B (2018). A Comparative Study of Hummingbirds and Chickens Provides Mechanistic Insight on the Histidine Containing Dipeptide Role in Skeletal Muscle Metabolism. Sci Rep 8, 14788.
16. Due CO, Weber OM, Trabesinger AH, Meier D & Boesiger P (1998). Quantitative 1H MRS of the human brain in vivo based on the simulation phantom calibration strategy. Magn Reson Med 39, 491-496.

17. Ghodsi R, & Kheirouri S (2018). Carnosine and advanced glycation end products: a systematic review. Amino acids, 1-10.

18. Gollnick PD, Sjödin B, Karlsson J, Jansson E & Saltin B (1974). Human soleus muscle: a comparison of fiber composition and enzyme activities with other leg muscles. Pflügers Archiv 348, 247-255.

19. Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill CA, Sale C & Wise JA (2006). The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. Amino Acids 30, 279-289.

20. Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK & Wise JA (2007). Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. Amino Acids 32, 225-233.

21. Hoult DI & Richards RE (1969). The signal-to-noise ratio of the nuclear magnetic resonance experiment. J Magn Reson 1, 71-85.

22. Hyslop NP & White WH (2009) Estimating precision using duplicate measurements. J Air Waste Manag Assoc 59, 1032-1039.

23. Just Kukurová I, Valkovič L, Uкроpec J, de Courten B, Chmelík M, Uкроpová, B & Krššák M. (2016). Improved spectral resolution and high reliability of in vivo 1H MRS at 7 T allow the characterization of the effect of acute exercise on carnosine in skeletal muscle. NMR in Biomedicine, 29(1), 24-32.

24. Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT & Wise JA (2009). The effect of 4 weeks beta-alanine supplementation and isokinetic training on carnosine concentrations in type I and II human skeletal muscle fibres. Eur J Appl Physiol 106, 131-138.
25. Kohl SM, Klein MS, Hochrein J, Oefner PJ, Spang R & Gronwald W (2012). State-of-the-art data normalization methods improve NMR-based metabolomic analysis. Metabolomics 8, 146-160.

26. Kreis R & Boesch C (2000). Orientation dependence is the rule, not the exception in 1H-MR spectra of skeletal muscle: the case of carnosine. ISMRM Proc Intl Soc Magn Reson Med, 31.

27. Kreis R (1997). Quantitative localized 1H MR spectroscopy for clinical use. Progress in nuclear magnetic resonance spectroscopy 31, 155-195.

28. Kyathanahally SP, Fichtner ND, Adalid V & Kreis R (2015). Does superficial fat affect metabolite concentrations determined by MR spectroscopy with water referencing? NMR in Biomedicine 28, 1543-1549.

29. Marshall I, Wardlaw J, Cannon J, Slattery J & Sellar RJ (1996). Reproducibility of metabolite peak areas in 1H MRS of brain. Magn Reson Imaging 14, 281-292.

30. Maudsley AA, Govind V & Arheart KL (2012). Associations of age, gender and body mass with 1H MR-observed brain metabolites and tissue distributions. NMR Biomed 25, 580–593.

31. Möller P & Sylven C (1981). Myoglobin in human skeletal muscle. Scand J Clin Lab Invest 41, 479-482.

32. Mon A, Abé C, Durazzo TC & Meyerhoff DJ (2013). Effects of fat on MR-measured metabolite signal strengths: implications for in vivo MRS studies of the human brain. NMR Biomed 26, 1768–1774.

33. Mon A, Abé C, Durazzo TC & Meyerhoff DJ (2016). Potential effects of fat on magnetic resonance signal intensity and derived brain tissue volumes. Obes Res Clin Pract 10, 211–215.

34. Mora L, Sentandreu MA & Toldrá F (2007) Hydrophilic chromatographic determination of carnosine, anserine, balenine, creatine, and creatinine. J Agric Food Chem 55, 4664-4669.
35. Moss CL (1991). Comparison of the Histochemical and Contractile Properties of Human Gastrocnemius Muscle. J Orthop Sports Phys Ther 13, 322-328.

36. Neves M Jr, Barreto G, Boobis L, Harris R, Roschel H, Tricoli V, Ugrinowitsch C, Negrão C & Gualano B (2012). Incidence of adverse events associated with percutaneous muscular biopsy among healthy and diseased subjects. Scand J Med Sci Sports 22, 175-178.

37. Ozdemir MS, Reyngoudt H, De Deene Y, Sazak HS, Fieremans E, Delputte S, D'Asseler Y, Derave W, Lemahieu I & Achten E (2007). Absolute quantification of carnosine in human calf muscle by proton magnetic resonance spectroscopy. Phys Med Biol 52, 6781-6794.

38. Parkhouse WS, McKenzie DC, Hochachka PW & Ovalle WK (1985). Buffering capacity of deproteinized human vastus lateralis muscle. J Appl Physiol 58, 14-17.

39. Saunders B, Elliott-Sale K, Artioli GG, Swinton PA, Dolan E, Roschel H, Sale C & Gualano B (2017). β-alanine supplementation to improve exercise capacity and performance: a systematic review and meta-analysis. Br J Sports Med 51, 658-669.

40. Schoeller, DA (1989). Changes in total body water with age. The American journal of clinical nutrition 50.5, 1176-1181.

41. Solis MY, Cooper S, Hobson RM, Artioli GG, Otaduy MC, Roschel H, Robertson J, Martin D, S Painelli V, Harris RC, Gualano B & Sale C (2015). Effects of beta-alanine supplementation on brain homocarnosine/carnosine signal and cognitive function: an exploratory study. PLoS One 10, e0123857.

42. Tallon MJ, Harris RC, Boobis LH, Fallowfield JL & Wise JA (2005). The carnosine content of vastus lateralis is elevated in resistance-trained bodybuilders. J Strength Cond Res 19, 725-729.

43. Tkac I, Ugurbil K, & Gruetter R (2002). On the quantification of low concentration metabolites by 1H NMR spectroscopy in the human brain at 7 Tesla. In Proceedings of the 10th Meeting of the International Society of Magnetic Resonance in Medicine, Honolulu, USA (p. 528).
44. Wand AJ, Ehrhardt MR & Flynn PF (1998). High-resolution NMR of encapsulated proteins dissolved in low-viscosity fluids. Proc Natl Acad Sci 95, 15299-15302.

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Competing Interests

The authors declare that they have no competing interests.

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Imidazole

$R^2 = 0.9719$

$r = 0.986$

Histidine

$R^2 = 0.9207$

$r = 0.960$

Carnosine

$R^2 = 0.9981$

$r = 0.999$
