SECONDARY PROTEIN IN THE MUSCLE OF EXERCISED RATS THAT RECEIVED CREATINE SUPPLEMENTATION

PROTEÍNA SECUNDÁRIA NO MÚSCULO DE RATOS EXERCITADOS QUE RECEBERAM SUPLEMENTO DE CREATINA

PROTEÍNA SECUNDARIA EN EL MÚSCULO DE RATONES EJERCITADOS QUE RECIBIERON SUPLEMENTO DE CREATINA

ORIGINAL ARTICLE
ARTIGO ORIGINAL
ARTÍCULO ORIGINAL

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ABSTRACT

Introduction: Currently there is a lack of clarity around the use of Fourier transform infrared (FT-IR) spectroscopy to analyze the effect of creatine (Cr) supplementation on the secondary structures of skeletal muscle tissue protein subjected to exercise. Objective: The objective of this study was to evaluate the spectral characteristics of the tibialis anterior muscle in rats subjected to exercise in a pool and to Cr supplementation. Methods: Experiment 1. First, an experiment was conducted to ensure that FT-IR would be able to detect change in the secondary structures of skeletal muscle tissue protein in the group of sedentary rats (SED) and in the group of rats that received creatine supplementation (CRE). Experiment 2. Next, the effect of physical exercise on the spectral characteristics of muscle tissue, especially when compared to the groups without exercise practice, was examined. Results: It was possible to verify that the peaks centered on 1658 cm-1 (amide I) and 1546 cm-1 (amide II) are characteristic spectra and indicated as markers of protein content. Conclusion: Thus, FT-IR spectroscopy proved to be able to monitor changes in secondary structures of skeletal muscle protein in both animals that received supplements and in those subjected to exercise and both cases reconciled. Furthermore, the FT-IR technique proved to be a viable method for the nondestructive evaluation of skeletal muscle protein structures. ***Level of evidence II, Investigation of treatment results.***

Keywords: Spectroscopy, Fourier transform infrared; Biotechnology; Creatine; Dietary supplements.

RESUMO

Introdução: Atualmente, não há clareza no que diz respeito ao uso da técnica de espectroscopia de infravermelho com transformada de Fourier (FT-IR) para análise do efeito da suplementação de creatina (Cr) sobre as estruturas secundárias da proteína do tecido muscular esquelético submetido a exercício. Objetivos: O objetivo deste estudo foi avaliar as características espectrais do músculo tibial anterior de ratos submetidos a exercício em piscina e à suplementação com Cr. Métodos: Experimento 1. Em primeiro lugar, foi realizada uma experiência para assegurar que a FT-IR seria capaz de detectar a variação nas estruturas secundárias da proteína do tecido muscular esquelético no grupo de ratos sedentários (SED) e no grupo de ratos que só receberam suplemento de creatina (CRE). Experimento 2. Em seguida, foi examinado o efeito do exercício físico sobre as características espectrais do tecido muscular, especialmente quando comparado com os grupos sem prática de exercício. Resultados: Foi possível verificar que os picos centrados em 1658 cm^{-1} (amida I) e 1546 cm^{-1} (amida II) são espectros característicos e indicados como marcadores do teor proteico. Conclusão: Assim sendo, a técnica de espectroscopia de FT-IR mostrou ser capaz de monitorar as variações nas estruturas secundárias da proteína do tecido muscular esquelético tanto em animais que receberam suplementos, quanto nos que foram submetidos a exercício e ambos os casos conciliados. Além disso, a técnica FT-IR provou ser um método viável para a avaliação não destrutiva de estruturas proteicas no músculo esquelético. ***Nível de evidência II, Investigação dos resultados do tratamento.***

Descritores: *Espectroscopia de infravermelho com transformada de Fourier; Biotecnologia; Creatina; Suplementos nutricionais.*

RESUMEN

Introducción: Actualmente, no hay claridad en lo que se refiere al uso de la técnica de espectroscopia de Infrarrojo con transformada de Fourier (FT-IR) para análisis del efecto de la suplementación de creatina (Cr) sobre las estructuras secundarias de la proteína del tejido muscular esquelético sometido a ejercicio. Objetivos: El objetivo de este estudio fue evaluar las características espectrales del músculo tibial anterior de ratones sometidos a ejercicio en piscina y a la suplementación con Cr. Métodos: Experimento 1. En primer lugar, fue realizada una experiencia para asegurar que la FT-IR sería capaz de detectar la variación en las estructuras secundarias de la proteína del tejido muscular esquelético en el grupo de ratones sedentarios (SED) y el grupo de ratones que sólo recibieron suplemento de creatina (CRE). Experimento 2. A continuación, fue examinado el efecto del ejercicio físico sobre las características espectrales del

tejido muscular, especialmente cuando comparado con los grupos sin práctica de ejercicio. Resultados: Fue posible verificar que los picos centrados en 1658 cm^{-1} (amida I) y 1546 cm^{-1} (amida II) son espectros característicos e indicados como marcadores del tenor proteico. Conclusión: Siendo así, la técnica de espectroscopia de FT-IR mostró ser capaz de monitorizar las variaciones en las estructuras secundarias de la proteína del tejido muscular esquelético, tanto en animales que recibieron suplementos, como en los que fueron sometidos a ejercicio y ambos casos conciliados. Además, la técnica FT-IR probó ser un método viable para la evaluación no destructiva de las estructuras proteicas en el músculo esquelético. ***Nivel de evidencia II, Investigación de los resultados del tratamiento.***

Descriptores: *Espectroscopía infrarroja por transformada de Fourier; Biotecnología; Creatina; Suplementos dietéticos.*

DOI: http://dx.doi.org/10.1590/1517-869220212702145670 Article received on 02/05/2015 accepted on 11/11/2020

INTRODUCTION

Creatine (Cr) is an organic compound synthesized mainly in the liver and kidneys from the amino acids glycine, arginine and methionine[1] and plays an important role in providing rapid energy during muscle contraction involving the transfer of a phosphate group from phosphorylcreatine (PCr) for ADP, regenerating ATP through a reversible reaction catalyzed by phosphocreatine kinase (CPK).[2,3] Physiologically, Cr is used predominantly by tissues with high energy demand.[4,2] The main storage site for Cr is the skeletal muscle tissue that accounts for about 95% of the body's total Cr. Inserted in the sports context, since the 90s, Cr supplementation has become an ergogenic resource that helps increase performance in physical exercises.[5,6]

Different methods have been used to assess the Cr concentration in skeletal muscle tissue.[6,7] Some direct techniques include muscle biopsy and nuclear magnetic resonance (1H and 31P-MRS).

In recent years, the Fourier Transform (FT-IR) technique has been used in prognosis and diagnosis of diseases and experimental models of biological systems.[8,9] FT-IR allows measuring the frequency and intensity at which a given sample absorbs radiation infrared, providing the identification of functional groups such as carboxyl, amine, carbonate, hydroxyl among others.[10] Over the last 15 years, the use of this vibrational spectroscopy technique in sports science has increased exponentially.[11] Its usefulness as a tool to provide new perspectives on the heterogeneity and regulation of skeletal muscle tissue metabolism.[9,11] To date, no study has been found relating the FT-IR technique, Cr supplementation on secondary protein structures of skeletal muscle tissue submitted to exercise. However, recent studies have been using the FT-IR technique to verify the structural stability of proteins, their secondary structures, conformational changes, among others.[12,13,14]

Thus, due to the lack of information and clarity in the use of FT-IR to analyze the effect of Cr supplementation on secondary protein structures of skeletal muscle tissue submitted to exercise, the aim of this study was to evaluate the spectral characteristics of the anterior tibial muscle of rats submitted to swimming and supplementation with Cr.

MATERIALS AND METHODS

The experiments were carried out in accordance with the Ethical Principles for Animal Experimentation approved by the Brazilian College of Animal Experimentation (COBEA) and approved by the Animal Research Ethics Committee of the Vale do Paraíba University (n. A36 / CEP / 2008).

Experiment 1

Eighteen male Wistar rats (270.14 ± 10.76g) were used, weighing. They were kept in a light / dark cycle in a temperature-controlled environment throughout the whole study, at the Bioterium of the Physiology and Pharmacodynamics Laboratory (IP&D, UNIVAP, São José dos Campos - SP, Brazil). The rats remained throughout the study in individual cages and the groups were randomly separated, supplemented with Creatine (CRE n=9) and sedentary group (SED n=9), animals that did not receive Cr supplementation or performed physical exercise.

Food and Supplementation Protocol

The animals were fed with standard ration (Labcil, Nutri Forte, Uberaba, MG, Brazil) and water ad libitum. The animals received Cr supplementation by gavage (micronized Creatine, Integral Medical, Embu-Guaçu, SP, Brazil) at a dose of 5g.kg.day^{-1} for 1 week (loading phase) and 1g.kg.day^{-1} for 8 weeks after the loading phase (maintenance phase). Considering that a daily dose of 300 mg of Cr per kilogram of body weight is routinely used in other animal studies[15,16] and is equivalent to the dose used of 20 g.day^{-1} in a 70 kg person, producing the maximum effects in 5 days, so the supplementation regime adopted in the present study must be considered supra physiological.

Muscle Sample Extraction

The animals were anesthetized with intramuscular administration of 40mg.Kg^{-1} of xylazine HCl (Xylazine 2%, 50 mL; Syntec do Brasil Ltda., SP, Brazil) and 50mg.Kg^{-1} of ketamine HCl (Ketamine 10%, 50mL; Syntec do Brasil Ltda., SP, Brazil) and euthanized, with intracardiac injection of KCl solution (Potassium Chloride 10%, Laboratório Ariston Ltda., SP, Brazil). Subsequently, the right anterior tibial muscle was extracted, immediately frozen in liquid nitrogen and stored at -80ºC until the study with FT-IR spectroscopy.

Muscle Preparation for FT-IR Spectroscopy Study

The frozen muscle samples were lyophilized in high vacuum equipment (Eppendorf do Brasil, São Paulo, Brazil) for 8 hours, in order to remove dry water. The dried samples were then ground in a nitrogen-cooled colloid mil (SpexIndustries, Metuchen, Nj, USA) to obtain tissue powder. This powder was mixed with dry potassium bromide (KBr) tissue in a mortar (in a proportion of 0.5mg: 150mg) and dried in the lyophilizer for 18h to remove all traces of remaining water. The mixture was compressed on a thin KBr disk under a pressure of ~ 100kg / cm^{-2} for 6 min in an evacuated mold producing a transparent disk for use in the FT-IR spectrometer.[17]

FT-IR spectroscopy

Infrared spectra were obtained using a Perkin-Elmer spectrum and an FT-IR spectrometer (Perkin-Elmer Inc., Boston, MA, USA), equipped with a TGSMIR detector. The air interference spectrum and the transparent KBr disk were recorded together, as a background and automatically subtracted by using the software (Spectrum One Software).

The spectra of the muscle sample were recorded at room temperature, in the region of 4000 to 900 cm^{-1}. Each interferogram was collected with 50 scans 4 resolution cm^{-1}. Three different samples were digitized under the same conditions, all of which resulted in identical spectra, the average of the three spectra being used in the statistical analysis. To remove noise, the spectra were first analyzed with ninepoint Savitzky-Golay smooth function. In determining the average values for the band area, the spectra belonging to each group were considered being calculated from the smoothing, baseline of the spectra, corrected

and normalized in relation to the region of the band of amide I (1700 - 1600 cm^{-1}) and amide II (1600 - 1500 cm^{-1}) using the Origin 8.0 software (Microcal Software, Inc., Northampton, MA, USA).[18]

Experiment 2

Since it was demonstrated that the supplementation protocol with Cr promoted variation in the secondary structures of skeletal muscle tissue protein and the FT-IR spectroscopy technique was effective in detecting this variation, a second experiment was carried out to evaluate the effects of a high intensity exercise protocol combined with Cr supplementation using the FT-IR spectroscopy technique. In this protocol, thirty-six male Wistar rats, weighing 251.32 ± 3.54 g, were kept in the same conditions as previously described for experiment 1.

Exercise Protocol

The animals were divided into two groups: only exercised (EXE) group and exercised and supplemented with Cr (CRE + EXE) group. Both two groups were submitted to a swimming protocol where the adaptation period occurred (30 minutes daily without load, for five consecutive days), in order to decrease the factors related to the stress promoted by the swimming exercise.[19] During this period, Cr was not administered. After the adaptation phase the animals were individually subjected to the maximum load test (MLT).[20] The load cells were used with weights corresponding to 1, 2, 3%, etc. of the mass of the rat, where they were increased at 3-minute intervals. The load cells were attached to the animal's tail until the maximum working load was reached, which was determined at the time the animal became tired (unable to remain on the surface after about 8 to 10 seconds).

This test allowed the correct adjustment of 80% of the maximum load to suit the physical exercise protocol. This protocol was adopted due to the promotion of more vigorous intensity when compared to individual swimming protocols.[19] The training occurred five times a week with sessions daily of 30 minutes for the entire duration of the eight weeks. The swimming protocol was carried out in an asbestos tank with a capacity of 250 liters of water kept at 34 ± 2 ºC of temperature. After the experimental period, a new MLT was performed to verify the effects of Cr supplementation in a training regime. At this time the animals in the SED group also underwent MLT to serve as a control group.

Statistical analysis

One-way ANOVA was used to analyze the area of the peak band 1656 cm^{-1} (amide I), related to the C = O bond and 1546 cm^{-1} (amide II) related to the NH / CN bonds. These structures are correlated with protein content where we can monitor changes in tissue structure generated after the exercise protocol.[13] Tukey-Kramer test analysis was used to determine the location of significant differences when necessary. Statistical analysis was performed using the SPSS program (version 17.0). The results were used with mean and standard deviation (mean ± SD), with values of $p<0.05$ being considered statistically significant.

RESULTS

Experiment 1

Figure 1 shows the prominent FT-IR spectrum of the vibrational bands related to the SED and CRE groups, this typical spectrum showed the main FT-IR bands characterized by two distinct regions: (A), indicating bands assigned to the NH link stretching vibrations (~ 3000 to 3500 cm^{-1}) and bands (B) indicating CH bonding and CHO stretching vibrations (~ 900 to 1800 cm^{-1}).

Figure 2 shows the spectra overlapping of the CRE and SED groups. It is possible to observe the spectra of the groups exhibit characteristic peaks centered on 1546 cm^{-1} and 1656 cm^{-1}, however the intensities of the FT-IR signal show important differences between the two experimental groups. Since the C = O bond (1615 -1700 cm^{-1}) peak amide I and the NH / CN (1500 -1600 cm^{-1}) peak amide II bonds are sensitive to conformation, these bands are very useful for determining secondary protein structures, the results obtained being consistent with previous research results.[2,8,13]

The quantitative estimate of the secondary structure of the protein is based on the assumption that any protein can be considered as the sum of a linear derivative of structural elements, and the percentage of each element is only related to the spectral intensity.[8,13]

ANOVA statistical analysis followed by Tukey post-test indicated that the animals that received Cr supplementation (CRE) showed an area of greater FT-IR band centered in 1546 and 1656 cm^{-1}

Experiment 2

Figure 3 shows the overlapping spectra of the CRE, SED, EXE and CRE + EXE groups, so the spectra of the tibial muscle of the rats shows the frequency of vibration of the bands 1546 and 1656 cm^{-1} sensitive to the conformation NH/CN and C = O respectively, determining of secondary structures of the protein. [2,13] The differences in the areas of the FT-IR bands centered between the experimental groups, animals from the CRE + EXE groups showed a significant difference ($p<0.05$) in the 1546 cm^{-1} band when compared to SED, CRE and EXE (Figure 4A).

In addition, in Figure 4B it was shown that there was a significant difference in the 1656 cm^{-1} band between CRE+EXE and EXE ($p<0.05$) and

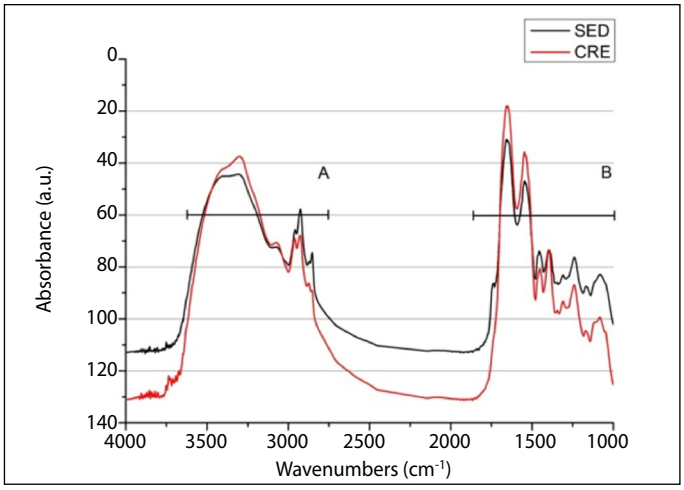

Figure 1. Prominent vibration spectrum bands (A) NH stretching vibrations (~ 3000-3500 CM21) and (B) related to amide I and II, CH CHO and stretching vibrations (~ 900-1800 CM21).

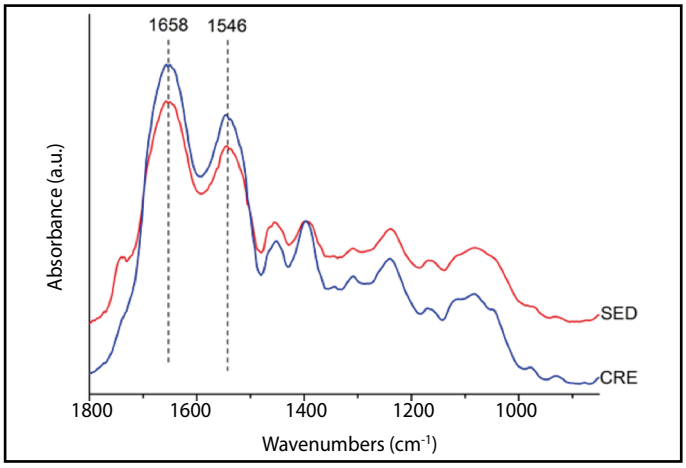

Figure 2. FT-IR spectra of skeletal muscle tissue of the Cr-supplemented (blue) and sedentary (red) groups in the 1800-900 CM21 region. The vertical lines show the correspondence between the corresponding Amide I and II signature lines in the skeletal muscle tissue spectrum.

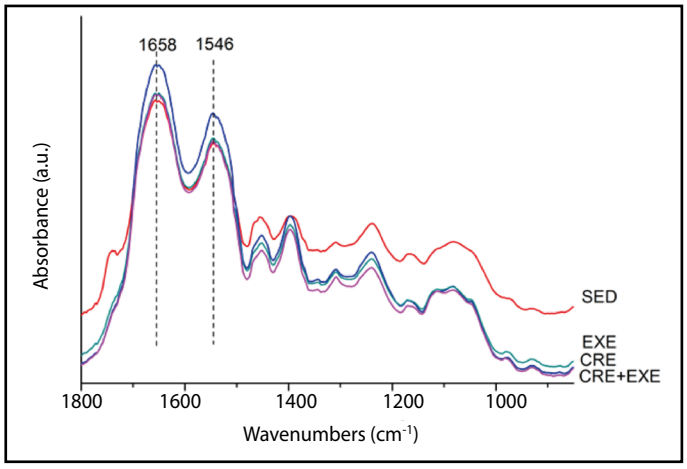

Figure 3. FT-IR spectra of skeletal muscle tissue of the Cr-supplemented (blue) and sedentary (red), exercised (green) and Cr-supplemented and exercised (pink) groups in the 1800-900 CM21 region. The vertical lines show the correspondence between the corresponding Amide I and II signature lines in the skeletal muscle tissue spectrum.

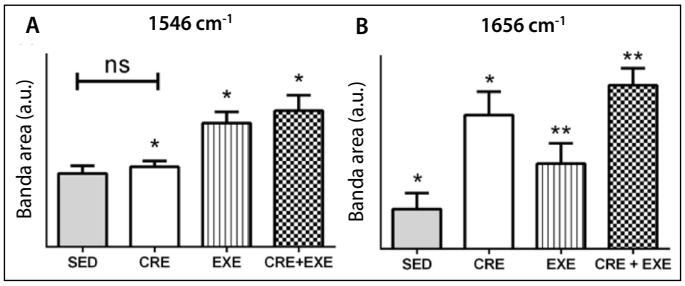

Figure 4. Means and standard deviations of the integrated area of the FT-IR bands centered on the 1546 Amide II (a) and 1656 Amide I (b) spectral regions of muscle tissue in the animals that received creatine supplementation (CRE), did not receive either creatine supplementation or perform physical exercise (SED), exercised (EXE), and received creatine supplementation and performed exercise (CRE + EXE). In (a), " * " indicates a statistically significant difference ($p < 0.05$) between groups compared with CRE, EXE and CRE + EXE, and n.s. indicates that no significant difference exists ($p > 0.05$). In (b) " * " and " ** " indicate a statistically significant difference ($p < 0.05$) between the groups marked as such.

between CRE and SED ($p<0.05$), showing the effects of supplementation and the training regime. A significant difference was observed between the groups exercised after the experimental period.

DISCUSSION

The normal content of total Cr in skeletal muscle is ± 120mmol.Kg^{-1} dry weight.[20,21] Harris et. al. (1992), in its research with Cr supplementation, indicated a content of 155-160 mmol.Kg^{-1} of dry weight representing the maximum limit for the storage of Cr in skeletal muscle tissue. Fast-twitch fibers (*e.g.*, anterior tibial) have greater concentration and sensitivity to Cr supplementation when compared to slow-twitch fibers.[2,22]

In our understanding, this is the first time that the action of Cr supplementation with physical exercise has been monitored using the FT-IR spectroscopy technique. the starting point of the present study was to identify in the spectra of the SED and CRE groups the characteristic peaks of the amide I and amide II groups, a characteristic spectral region for analysis of secondary proteins. [13,18] As shown in figure 2, the spectrum of the SED groups and CRE characterized by two distinct peaks, which were consistent with other results found results.[18] Thus, from the data from experiment 1 it was possible to identify that the bands centered on 1546 and 1658 cm^{-1}, making it possible to use the bands as a marker of the characterization of secondary protein structures in skeletal muscle tissue. It is important to note in Figures 2 and 3 that the FT-IR spectrum of skeletal muscle is composed of several bands from other functional groups of various macromolecules: carbohydrates, lipids and proteins, as well as a study in which the detection of Cr in tissue muscle of rats using FT-IR spectroscopy, characteristic in the amide III bands in 1396 and 1308 cm^{-1}.[2,11,14,23]

The main bands attributed to FT-IR are shown on Table 1, considering the band located at 1546 cm^{-1} overlaps with NH bend, CN stretch, stretching due to the protein content, the band located at 1658 cm^{-1} was considered the marked the most sensitive marker correlated to protein content in relation to Cr supplementation, since the amide I and amide II bands are located in an interference-free region, thus facilitating the identification of the protein.[18,24]

Since the intensity of the FT-IR signal is directly proportional to the present concentration of markers in skeletal muscle tissue, it is possible to determine that there was a significant change in secondary proteins after the experimental protocol EXE and CRE + EXE. Considering that Cr supplementation promotes increased cellular hydration and thus stimulates protein synthesis or decreases protein degradation[4] and these structural changes were characteristic in the FT-IR spectra, it is possible to infer that Cr supplementation (CRE group) promoted an increase in protein content and when combined with supplementation, physical exercise (CRE + EXE group) enhanced the intensity of the spectrum signal, which indicates a positive change in the concentration of secondary proteins in skeletal muscle tissue.

However, in the present study, it was demonstrated that in the EXE group showed a greater intensity in the amide band II (1546 cm^{-1}), in relation to the CRE and SED groups, thus corroborating the efficiency of physical exercise.

Using other analysis techniques, studies in humans and animals have shown that Cr supplementation can increase the intracellular content of this product,[17] thus favoring the protein content or by its synthesis or delaying its depletion.[10,26]

Table 1. Overall assignment of the FT-IR spectral band of skeletal muscle.

Wavenumber (cm^{-1})	Definition of the spectral assignment
3307	Mainly N–H stretching (amide A) of amide groups of proteins, with the little contribution from O–H stretching of polysaccharides and intermolecular H bonding
3012	Olefinic =CH stretching vibration: unsaturated lipids, cholesterol esters
2962	CH3 asymmetric stretching: lipids, protein side chains, with some contribution from carbohydrates and nucleic acids
2929	CH2 asymmetric stretching: mainly lipids, with some contribution from proteins, carbohydrates, nucleic acids
2874	CH3 symmetric stretching: protein side chains, lipids, with some contribution from carbohydrates and nucleic acids
2855	CH2 symmetric stretching: mainly lipids, with some contribution from proteins, carbohydrates, nucleic acids
1656	Amide I (protein C=O stretching)
1540	Amide II (protein N–H bend, C–N stretch)
1452	CH2 bending: lipids
1392	COO^- symmetric stretching: fatty acids
1261	$PO2^-$ asymmetric stretching, nonhydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids
1236	Sulfate stretch from proteoglycans, collagen amide III vibration with significant mixing with CH2 wagging vibration from the glycine backbone and proline side chain
1170	CO–O–C asymmetric stretching: phospholipids, triglycerides and cholesterol esters
1080	$PO2^-$ symmetric stretching: nucleic acids and phospholipids. C–O stretch: glycogen, polysaccharides, glycolipids
976	C–N+–C stretch: nucleic acids, ribose-phosphate main chain vibrations of RNA

CONCLUSION

The present study demonstrates that FT-IR used in the bands of amide I (1656 cm-1) and amide II (1546 cm-1) is a viable and non-destructive method for assessing the concentration of secondary proteins in muscle tissue, providing additional support for the theory that performance can be positively influenced by exercise and Cr supplementation. The biggest advantage is linked to the ability to analyze samples in native conditions, which allows new perspectives on samples without the need for fixation, dyes, or additional marker.

All authors declare no potential conflict of interest related to this article

AUTHORS' CONTRIBUTIONS: Each author made significant individual contributions to this manuscript. DPJ: general data collection, preparation of the Excel spreadsheet with the inclusion of the data, translation of the abstract to English and Portuguese, and preparation of the format and submission of the article; DPJ and HLM: statistical analysis and table layout; DPJ and RAS: translation of the article into English; WR: critical review of the article.

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