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Detection of Creatine in Rat Muscle by FTIR Spectroscopy

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Abstract—There is a current lack of clarity regarding the use of Fourier-transform infrared spectroscopy (FT-IR) to evaluate intramuscular concentrations of creatine (Cr). Thus, the aim of this study was to assess the FT-IR spectral features of *tibialis anterior* muscle in rats submitted in conditions that were expected to perturb the Cr pool. First, an experiment was performed to ensure that FT-IR was able to detect the Cr intramuscular in sedentary and supplemented rats (Experiment 1). The effect of physical exercise on spectral muscle features was then examined, especially in relation to the spectroscopy markers (Experiment 2). Using pure Cr (control), it was possible to verify that only the peaks centered at 1308 and 1396 cm^{-1} of all the spectra showed the same peak positions, indicating these FT-IR shifts as indirect markers of Cr intramuscular content. Experiment 2 revealed a higher Cr content for the Cr-supplemented and exercised animals than the rats of other groups. In conclusion, it was demonstrated that FT-IR spectroscopy using 1396 cm^{-1} and mainly 1308 band was able to monitor Cr muscle content in rats sedentary, Cr-supplemented, and submitted to physical training. Besides, FT-IR could be a feasible method for the nondestructive assessment of Cr skeletal muscle content.

Keywords—Vibrational imaging spectroscopy, Biomedical applications, Creatine supplementation, Analysis of nitrogenous compounds.

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INTRODUCTION

Creatine (Cr) is an organic compound synthesized mainly in the liver and kidneys from the amino acids—glycine, arginine, and methionine.[1,38] Cr plays an important role in rapid energy provision during muscle contraction involving the transfer of phosphoryl group from phosphorylcreatine (PCr) to ADP to regenerate ATP through a reversible reaction catalyzed by phosphorylcreatine kinase (PCK).[36] Physiologically, Cr is used predominantly by tissues with high energy demands.[9,16] The main storage site of Cr is the skeletal muscle, which corresponds to approximately 95% of the body Cr.[36] Introduced in this context, especially since the 1990s, Cr supplementation became a popular ergogenic aid to increase exercise performance.[26] In 2000, the American College of Sports Medicine estimated that 2500 metric tons were consumed every year.[33]

Different methods have been employed to evaluate muscle Cr content.[27,28,32,34] Direct techniques include muscle biopsy, magnetic resonance nuclear (MRN), or magnetic resonance nuclear spectroscopy (^{1}H- and ^{31}P-MRS). In addition, because the ingestion of Cr markedly reduces the urine volume during the initial days of supplementation, presumably because of increased retention of intracellular water, urine volume measurements have been used as an indirect marker of increased intramuscular Cr content. Similarly, the observation of increased body mass has been identified as a result of muscle uptake of Cr supplementation. Although these techniques are recommended to monitor Cr content, studies that observe the effects of Cr

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supplementation on skeletal muscle have not used these procedures to determine how the protocol supplementation affects the intramuscular concentrations of Cr.

In recent years, Fourier-transform infrared spectroscopy (FT-IR) has been used in prognosis and diagnosis of diseases and experimental models of biological systems.[18,21,29] FT-IR allows measuring the frequency and intensity at which a given sample absorbs infrared radiation, providing the identification of functional groups like carboxyl, amine, hydroxyl, carbonyl, and others.[6] Over the last 15 years, the use of this vibrational spectroscopy technique in exercise and sports science has increased exponentially.[22] This has been undertaken to determine its utility as a suitable tool to provide new insights into the heterogeneity and regulation of local tissue metabolism in skeletal muscle tissue.[22] Thus, it is important to improve the knowledge on how this technology has been applied to exercise training and sport, and how it can be used to design training programs for athletes.

Until now, no study in relation to FT-IR and Cr supplementation on skeletal muscle and exercise has been found in the literature. However, microcrystalline deposits of Cr, suggestive of perturbed energetic status, were detected by FT-IR in the amyloid precursor protein (APP) of transgenic mice and post-mortem Alzheimer-diseased human brain, indicating feasibility of this molecular technique to monitor Cr content.[8] Thus, owing to the current lack of clarity in the use of FT-IR to evaluate intramuscular Cr storage, the aim of this study was to assess the spectral features of *tibialis anterior* muscle in rats submitted in conditions that were expected to perturb the Cr pool. First, an experiment was performed to ensure that FT-IR was able to detect intramuscular Cr in sedentary and supplemented rats. Then, the effect of physical exercise on spectral muscle features, especially in relation to the Cr bands was examined.

MATERIALS AND METHODS

The experiments were conducted according to the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee for Ethics in Animal Research of the Universidade do Vale do Paraíba (no. A36/CEP/2008).

Experiment 1

Animals

Eighteen male Wistar rats weighing 270.14 ± 10.76 g were kept on a normal light/dark cycle in a climate-controlled environment for the duration of the study in the bioterium of the Physiology and Pharmacodynamics Laboratory (IP&D, UNIVAP, São José dos Campos, SP, Brazil). The rats were maintained in individual cages and were randomly assigned to either the Cr supplementation group (CRE, $n = 9$) or the sedentary group (SED, $n = 9$). In the SED group, the animals did not receive the Cr supplementation or perform physical exercise.

Feeding and Supplementation Protocols

Animals were fed ad libitum standard chow (Labcil, Nutri Forte, Uberaba, MG, Brazil) and water. The animals received the Cr supplementation by gavage (Micronized Creatine, Integral Médica, Embu-Guaçu, SP, Brazil) at a dose of 5 g kg day^{-1} for 1 week (loading phase) and 1 g kg day^{-1} for 8 weeks (maintenance phase) after the loading phase. Considering that a daily dose of 300 mg of Cr per kilogram of body weight is routinely used in other animal studies,[4,7,20,39] which is equivalent to the customary loading dose of 20 g day^{-1} in a person with a weight of 70 kg and which produces maximal effects in 5 days, the Cr supplementation regimen adopted in the present study must be considered as supraphysiological.

Muscle Samples Extraction

Animals were anesthetized with intramuscular administration of 40 mg kg^{-1} of xylazine HCl (Xilazin 2%, 50 mL; Syntec do Brasil Ltda., São Paulo, Brazil) and 50 mg kg^{-1} of ketamine HCl (Cetamin 10%, 50 mL; Syntec do Brasil Ltda.) and euthanized with an intracardiac injection of KCl solution (Cloreto de potássio 10%; Laboratório Ariston Ltda., São Paulo, Brazil). Right *tibialis anterior* muscles were extracted, immediately frozen in liquid nitrogen, and kept at −80 °C until FT-IR spectroscopy study.

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 h to remove water. The dried samples were then ground in a liquid nitrogen-cooled colloid mil (Spex Industries, Metuchen, NJ, USA) to obtain tissue powder. The tissue powder was mixed with dried potassium bromide (KBr) in a mortar (at a ratio of 0.5 mg:150 mg) and re-dried in the freeze drier for 18 h to remove all traces of remaining water. The mixture was compressed into a thin KBr disk under a pressure of ~100 kg cm^{-2} for 6 min in an evacuated die producing a transparent disk for the use in the FTIR spectrometer.[37]

FT-IR Spectroscopy

Infrared spectra were obtained using a Perkin-Elmer Spectrum One FT-IR spectrometer (Perkin-Elmer Inc., Boston, MA, USA) equipped with a MIR TGS detector. The interfering spectrum of air and the KBr transparent disk was recorded together as background and subtracted automatically by means of the appropriate software (SpectrumOne software).

Collections of spectra were carried out using SpectrumOne software (Perkin-Elmer). The spectra of muscle samples and pure Cr (Sigma-Aldrich, St. Louis, MO, USA) were recorded at room temperature in the 4000–900 cm^{-1} frequency region. Each interferogram was collected with 50 scans at 4 cm^{-1} resolution. Three different aliquots of each sample were scanned under the same conditions, all of which gave identical spectra. The average spectra of these three replicates were used in statistical analysis. To remove the noise, the spectra were first smoothed with a nine-point Savitzky-Golay smooth function. In order to determine the mean values for the band area, the spectra belonging to each individual of the groups were considered. These procedures were calculated from smoothed, baseline-corrected, and normalized spectra with respect to amide I band region (1700–1600 cm^{-1}) using Microcal Origin 8.0 software (Microcal Software, Inc., Northampton, MA, USA).[3,30]

Experiment 2

Once it was demonstrated that the proposed Cr supplementation protocol had effectively improved Cr detection in the IR spectra, a second FT-IR experiment was carried out to evaluate the effects of a high-intensity exercise protocol on Cr muscle content.

Animals

Thirty six male Wistar rats, weighing 251.32 ± 3.54 g were kept under the same conditions as previously described for Experiment 1. The procedures for randomization and group assignment (CRE $n = 9$; SED $n = 9$; EXE $n = 9$ and CRE + EXE $n = 9$), feeding and supplementation protocols, tissue preparation, and FT-IR spectrometry evaluation were also identical to those of Experiment 1.

Exercise Protocol

The animals of EXE and CRE + EXE groups were submitted to a swimming adaptation period (daily 30 min without load, during five consecutive days) to decrease factors related to the stress promoted by the swimming activity.[24,25] During this period, the Cr was not administered. After adaptation, these animals were individually submitted to the maximum load test (MLT).[10,12] Load cells (lead fish sinkers) were increased at 3-min intervals by weights corresponding to 1%, 2%, 3%, etc. of the rat's mass. The load cells were attached to the tail of the animal until the maximal work load was reached, which was determined at the moment when the animal became exhausted (unable to surface after approximately 8–10 s). This test allowed the working load adjustment for the physical training at 80% of the maximal load. This training was performed in groups of nine animals because of the more vigorous exercise promotion compared with the individual swimming.[24,25] Such training occurred five times a week with daily training sessions of 30 min throughout the duration of the 8 weeks. The swimming protocol was performed in an asbestos tank with 250 L of water kept at 34 ± 2 °C temperature. After the experimental period, a new MLT was performed to verify the effects of Cr supplementation on the training regimen. At this moment, sedentary animals also underwent the MLT to serve as control group.

Statistical Analysis

One-way ANOVA was used to analyze the band area of peaks related to Cr content changes, and the maximal work load, among the experimental groups. A post hoc analysis (Tukey–Kramer test) was used to determine the location of significant differences when necessary. The statistical analyses were conducted utilizing the program SPSS (version 17.0). The results were registered with the media ± standard deviation, with the values of $p < 0.05$ being considered as statistically significant.

RESULTS

Experiment 1

Figure 1 shows the FT-IR spectrum of prominent vibrational bands related to pure Cr. This typical spectrum showed the main FT-IR bands of Cr, which was characterized for two distinct regions: (a) indicating bands assigned to the NH stretching vibrations (~3000–3500 cm^{-1}) and (b) indicating bands assigned to the CH and CHO stretching vibrations (~900–1800 cm^{-1}).

Figure 2 demonstrates that the spectra of CRE and SED groups superimposed on the spectrum of pure Cr in the regions (a) and (b). It was observed that the muscle spectra did not exhibit all the characteristic peaks of pure Cr in region of NH stretching vibrations (Fig. 2a). On the other hand, it was possible to verify that the peaks centered at 1308 and 1396 cm^{-1} of three spectra showed the same peak positions, but the

intensities at specific FT-IR shifts were different, evidencing important differences between the two experimental groups (Fig. 2b). Since these results were consistent with previous results,[13] the 1308 and 1396 cm^{-1} bands were used as indirect markers of Cr content.

ANOVA statistical analysis followed by *Tukey* post-test indicated that the Cr-supplemented animals (CRE) showed a higher band area of the FT-IR bands centered at 1308 and 1396 cm^{-1} than that of the SED animals and only at 1396 cm^{-1} for the pure Cr group (Figs. 3a and 3b). Also, it was demonstrated that the spectral values of SED group were higher than those of pure Cr (Fig. 3b).

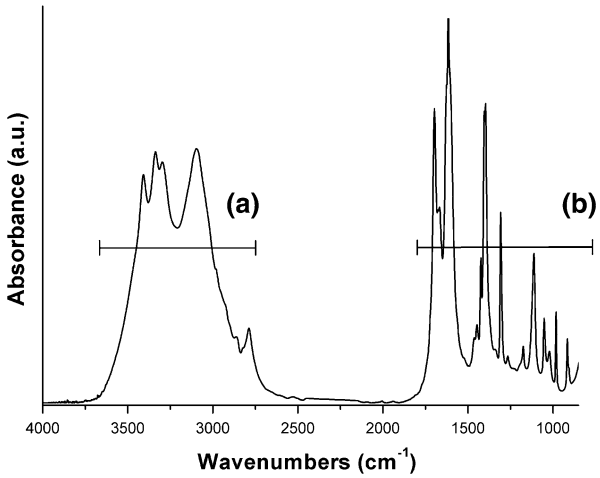

FIGURE 1. The representative spectrum of prominent vibrational bands related to pure creatine in the (a) NH stretching vibrations (~3000–3500 cm^{-1}) and (b) CH and CHO stretching vibrations (~900–1800 cm^{-1}).

Experiment 2

Figure 4 demonstrates that the spectra of CRE, SED, EXE, and CRE + EXE groups superimposed on the spectrum of pure Cr in regions (a) and (b). Again, no similarity was observed between muscle and pure Cr spectra in the region of NH stretching vibrations (Fig. 4a), and the intensities at specific FT-IR bands (1308 and 1396 cm^{-1}) were different (Fig. 4b).

Figure 5 shows the differences of band area of the FT-IR bands centered at 1308 and 1396 cm^{-1} between all the experimental groups. In the 1308 cm^{-1} region, the Cr-supplemented and exercised animals (CRE + EXE) presented significant difference compared with pure Cr, SED, and CRE groups. In addition, sedentary animals presented lower values than CRE and EXE groups (Fig. 5a). In the 1396 cm^{-1} region, with exception to CRE and EXE groups ($p > 0.05$), all other comparisons were significantly different (Fig. 5b).

Figure 6 presents the effects of Cr supplementation on the training regimen. A significant difference was observed between exercised groups for the maximal work load reached after the experimental period. The CRE + EXE group performed a significantly higher maximal work load than EXE and SED group. The EXE group also had a significantly higher maximal work load than the SED group.

DISCUSSION

Recently, Cr has become the nutritional supplement of choice for athletes. This compound has accounted for most of the supplement sales during the past few years, and the market continues to grow as a result of

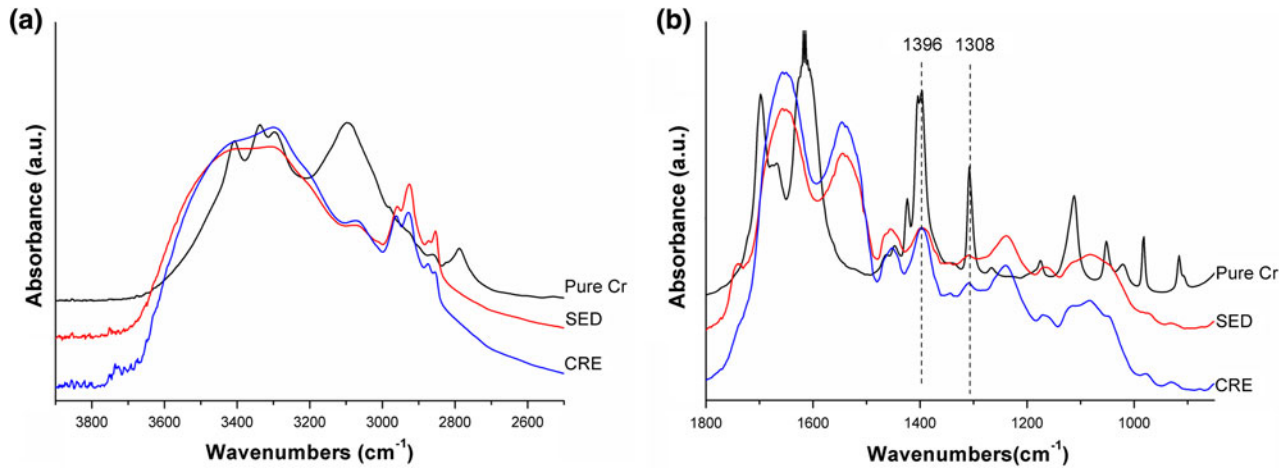

FIGURE 2. Spectra of skeletal muscle tissue and pure creatine. (a) FT-IR spectrum in the 3900–2500 cm^{-1} region of the muscle spectra of Cr-supplemented (blue) and sedentary (red) animals not exhibiting all the characteristic peaks of pure creatine (black). (b) FT-IR spectrum in the 1800–900 cm^{-1} region. Vertical lines show the correspondence between the signature lines of pure creatine and the matching lines in the skeletal muscle tissue spectrum.

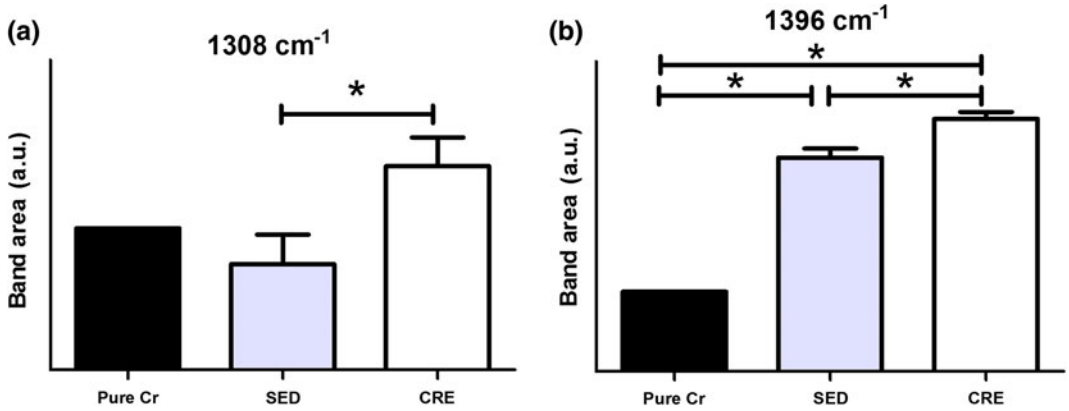

FIGURE 3. Means and standard deviations of integrated area of the FT-IR bands centered at 1308 cm^{-1} (a) and 1396 cm^{-1} (b) regions of those animals that received supplementation with creatine (CRE); did not receive the Cr supplementation or perform physical exercise (SED); and pure Cr. "*" Indicates a statistically significant difference between the groups indicated by the horizontal bar ($p < 0.05$).

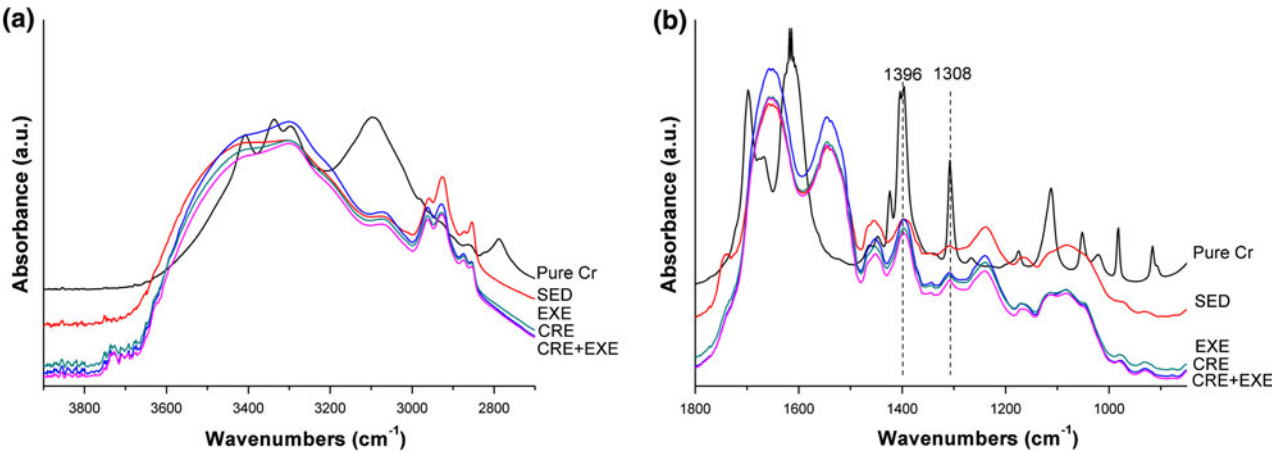

FIGURE 4. Spectra of skeletal muscle tissue and pure creatine. (a) FT-IR spectrum in the 3900–2500 cm^{-1} region of the muscle spectra of Cr-supplemented (blue), sedentary (red), exercised (green), and Cr-supplemented and exercised (pink) not exhibiting all the characteristic peaks of pure creatine (black). (b) FT-IR spectrum in the region of the 1800–900 cm^{-1} region. Vertical lines show the correspondence between the signature lines of pure creatine and the matching lines in the skeletal muscle tissue spectrum.

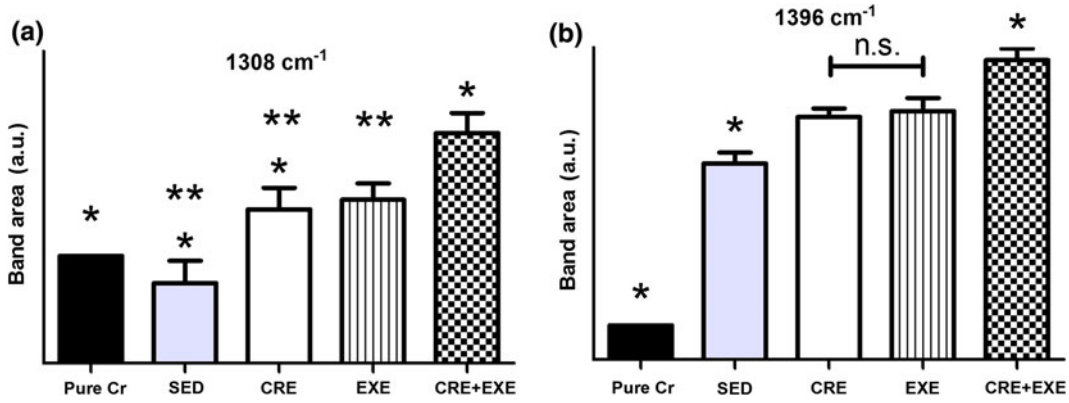

FIGURE 5. Means and standard deviations of integrated area of the FT-IR bands centered at 1308 cm^{-1} (a) and 1396 cm^{-1} (b) regions of the animals those that received supplementation with creatine (CRE); did not receive the Cr supplementation or perform physical exercise (SED), exercised (EXE), Cr-supplemented and exercised (CRE + EXE), and pure creatine. In (a), "*" indicates a statistically significant difference ($p < 0.05$) between (CRE + EXE) vs. pure Cr, SED and CRE groups; "" indicates a statistically significant difference ($p < 0.05$) between SED and pure Cr. In (b) "*" indicates a statistically significant difference ($p < 0.05$) between the groups signed; n.s. indicates no significant difference ($p > 0.05$).**

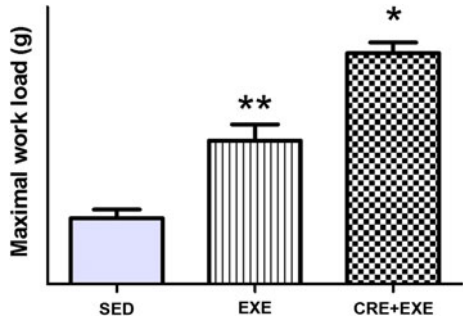

FIGURE 6. Effects of Cr supplementation on the training regimen. "*" Indicates a statistically significant difference ($p < 0.05$) between Cr-supplemented and exercised (CRE + EXE) group vs. exercised (EXE) and sedentary (SED) groups; "" indicates a statistically significant difference ($p < 0.05$) between EXE vs. SED group.**

endorsement by professional athletes. In addition, Cr does not appear on the banned list of substances of any sports federation.[38] In this sense, there is an increased need for advanced technologies to assess Cr compounds on their main storage tissue, the skeletal muscle.

The normal content of total Cr in skeletal muscle, is close to 120 mmol kg^{-1} of dry weight.[19] In their research with Cr supplementation, Harris *et al.*[11] indicated that a content of 155–160 mmol kg^{-1} of dry weight can represent the upper limit for the storage of muscle Cr. Considering that 1 mmol of Cr corresponds to about 131 mg of Cr into the muscle, the muscle content of Cr represents about 4 g kg^{-1} of the body weight. However, the fast twitch fibers (e.g., *tibialis anterior*) have higher content and higher sensibility to supplementation when compared with to slow twitch fibers.[5,15] Anyway, the absolute content of Cr in isolate muscle fibers is in low physiological concentration,[36] which denotes the necessity of more efficient analytic methodologies to enable their characterization. In this way, several spectroscopy techniques have been considered as basis for minimally invasive and nondestructive measuring systems, as the use of these instrumental analytic tools is becoming a very interesting and auspicious medical alternative.

The purpose of the present study was to use FT-IR spectroscopy to evaluate Cr muscle content in sedentary, supplemented, and exercised rats. FT-IR spectroscopy is a very versatile instrument technique for the analysis of biological materials.[17] This spectroscopy tool of vibrational characterization is well known as a resource with great number of structural details, allowing qualitative, quantitative, and physicochemical characterization. Furthermore, FT-IR spectroscopy allows less invasive and nondestructive biological analysis, which can be optimized for biochemical characterization and diagnosis of several diseases.[31]

Infrared spectroscopy has been studied as a potential tool to play an important role in monitoring applied sports science and exercise prescription.[22] However, to the best of our knowledge, it was the first time that the Cr muscle content was monitored using FT-IR spectroscopy. The starting point of the current study was to identify FT-IR spectra of pure Cr. As shown in Fig. 1, the typical spectrum of Cr was characterized for two distinct regions, which were consistent with previous results.[13] Then, the Experiment 1 identified that only bands centered at 1308 and 1396 cm^{-1} of pure Cr and skeletal muscle sample showed the same peak positions. Thus, these bands were used as indirect markers of Cr content in skeletal muscle. For Cr detection in the IR maps of brain tissue, Kastyak *et al.*[13] have used the integrated intensity of the sharp band appearing at 1304 cm^{-1}, since it lies in a region that is relatively free of interfering bands in normal tissue and can be readily identified.

It is important to note in Figs. 2 and 4 that the FT-IR spectrum of skeletal muscle is composed of several bands originating from functional groups of several macromolecules: carbohydrates, lipids, and proteins. The main FT-IR bands assignments are demonstrated in Table 1.[3] Considering that the band located at 1396 cm^{-1} overlaps with COO^- symmetric stretching because of fatty acids and the pure Cr is a protein compound, the 1308 cm^{-1} band was considered the more sensible marker of Cr, since it refers to the symmetric stretching present in the amino acids (Amide III). Moreover, 1308 cm^{-1} band is in a region free of the intense bands of amides I and II proteins, thus facilitating their identification.[8,13]

Since the intensity of the IR signal is directly proportional to the amount present, we can state with confidence that the amount of Cr represented by their FT-IR markers in the skeletal muscle tissue was changed after experimental protocols (supplementation and physical exercise). Considering that Cr-supplemented animals (CRE) showed higher band area in 1308 and 1396 cm^{-1} bands than that of the non-supplemented animals (SED), it is possible to infer the existence of higher Cr intramuscular content after supplementation. However, in the present study, it was used as supraphysiological Cr dosage, which could favor our findings.

Other interesting findings are the results of band area of pure Cr compared with the area of Cr bands in skeletal muscle. The pure Cr should have a higher area under a band, since absorption of pure molecules is higher than the ones presented in tissues. However, this event occurred only for the 1308 cm^{-1} band when comparing pure Cr and sedentary animals in both experiments (Figs. 3 and 5). It was considered that the band area of pure Cr was lower than all other groups, including the SED, because the 1396 cm^{-1} is

TABLE 1. General band assignment of FT-IR spectrum 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	CH_3 asymmetric stretching: lipids, protein side chains, with some contribution from carbohydrates and nucleic acids
2929	CH_2 asymmetric stretching: mainly lipids, with some contribution from proteins, carbohydrates, nucleic acids
2874	CH_3 symmetric stretching: protein side chains, lipids, with some contribution from carbohydrates and nucleic acids
2855	CH_2 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	CH_2 bending: lipids
1392	COO^- symmetric stretching: fatty acids
1261	PO_2^- 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 CH_2 wagging vibration from the glycine backbone and proline side chain
1170	CO–O–C asymmetric stretching: phospholipids, triglycerides and cholesterol esters
1080	PO_2^- 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

associated with fatty acids, and there is a large storage of lipids in skeletal muscles. These results support the inference that the Amide III band is the best marker to evaluate intramuscular concentrations of Cr.

Using other analysis techniques, studies in humans and animals demonstrated that Cr supplementation can increase the Cr muscle content.[35–37] In fact, after Professor Roger Harris and his colleagues have demonstrated that Cr loading is able to enhance muscle Cr and PCr content,[11] Cr supplementation has been largely used to improve exercise capacity in healthy individuals and athletes. Thus, a growing body of evidence indicates that Cr exerts ergogenic effects, especially in supra-maximal short-term efforts, in which PCr–PCK plays an essential energetic role.[33]

To better evaluate the use of FT-IR spectroscopy to monitor Cr presence in skeletal muscle submitted to physical exercise, a second experiment was conducted. Again, the Cr-supplemented animals (CRE) showed higher values of Cr spectroscopies markers than the SED group. However, the higher band areas in 1308 and 1396 cm^{-1} bands were obtained by CRE + EXE group. In accordance with another study that used ^{31}P-RMS,[2] our findings should be taken as follows: the Cr supplementation associated with a training program increases the muscle capacity to stock this organic compound. Besides, considering that Cr supplementation in humans and rats has been proven to increase physical performance,[1,14,23] the findings showed in Fig. 6 supports the idea that Cr-supplemented animals had increased the Cr muscle content.

In summary, the present study demonstrated that FT-IR could be a feasible method for the nondestructive assessment of Cr skeletal muscle content, providing additional support to the theory that the physical performance may be positively influenced by Cr supplementation. The greatest benefit of this technique lies in the high molecular sensitivity combined with a spatial resolution down to a few micrometers. Another advantage is its ability to probe samples under native conditions, which allows infusing new insights into samples without the need for fixation, stains, or an additional marker. The advent of news technologies (e.g., fiber optic FT-IR spectroscopy optimization) will enable the detection of more subtle differences in specific spectral features. Furthermore, to better estimate Cr supplementation-induced alterations on the protein secondary structure in the skeletal muscle and other tissues, new statistical analysis, as second derivative, will provide more sensitive results.

CONCLUSIONS

It was demonstrated that FT-IR spectroscopy using 1396 cm^{-1} and mainly 1308 band could monitor Cr muscle content in rats—sedentary, Cr-supplemented, and submitted to physical training. If confirmed by other techniques considered as gold standard, FT-IR spectroscopy could prove to be an alternative technique to explain the ergogenic effect of Cr supplementation in skeletal muscle.

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