Intramuscular lactic acid assessment through Raman spectrography: new perspectives in sports medicine*

Fabiano de Barros Souza[1], Marcos Tadeu T. Pacheco[2], Antônio B. VilaVerde[2], Landulfo Silveira Jr.[2], Rodrigo L. Marcos[1] and Rodrigo Alvaro B. Lopes-Martins[1]

ABSTRACT

The infrared Raman spectroscopy is a technique that could be employed in the future in physical evaluations, specifically to non-invasive determinations of the muscle amounts of lactate. Raman spectroscopy supplies a wealth of details provided by the molecule vibrational levels of energy, representing the "fingerprint" of the molecules. In previous studies the Raman spectra of lactic acid in human plasma and rat blood were obtained, where characteristic peaks of this compound in the biological matrix were clearly observed. In this study, Raman spectroscopy was used in order to determinate the skeletal muscle amounts of lactic acid using the experimental model of rat tibial muscle. For this purpose the authors used a system composed by a Ti:saphire laser tuned at 830 nm, a spectrometer Kaiser f/ l.8 and a CCD detector refrigerated with liquid nitrogen. Holographic Kaiser filters were employed to reduce back-scattered radiation by the sample. The Raman spectrum of the lactic acid presented several sharp and well-resolved peaks in the frequency range between 700 and 1,500 cm^{-1}. The principal Raman peak at 830 was used to detect the presence of the lactic acid in the rat tibial muscle. Raman spectra of tibial muscles of rats were taken *in vivo* and *in vitro*. The diffusion of the lactic acid through the muscle, *in vitro*, was also monitored. The results suggest that near infrared Raman spectroscopy, in the future, could be an alternative technique for physical evaluation, allowing measurements of the lactic acid concentration in skeletal muscle, through a non-invasive method.

Key words: Lactic acid. Raman spectroscopy. Skeletal rat muscle.

INTRODUCTION

For an individual to perform physical activities, his/her organic functions should be fully adjusted, to better endure the intense demands from the exercises[1]. Thus, from a guided training one may adjust one's physiological systems to endure physical exertion[1].

As voluntary muscle contractions depend on a number of factors, such as the central nervous system, peripheral neurons, neuromuscular junction, and striated skeletal muscles, it is hard to establish a strict definition for muscle fatigue. According to Edwards[2], muscle fatigue is the inability to sustain the required or expected muscle contraction strength. In principle, any of such factors may be involved in the muscle fatigue process; thus the significant physiological factor for the development of fatigue may be of mechanic, metabolic or electrophysiological nature.

In spite of muscle fatigue being a complex phenomenon related to multiple causes, some authors classify it whether from peripheral or central origin[3,4]. Accumulation of metabolites such as lactic acid takes place during intense physical activity, and is followed by a fall in tissue pH. There are, however, evidences that the increase of hydrogen ions concentration may have an inhibitory effect on contractile filaments, including reduction of calcium troponin sensitivity[5].

A number of studies have investigated biological factors that influence fitness of athletes, seeking for reference pat-

* Instituto de Pesquisa e Desenvolvimento – IP&D. Universidade do Vale do Paraíba – UNIVAP.

1. Laboratório de Fisiologia e Farmacodinâmica.
2. Laboratório de Terapia Fotodinâmica.

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Correspondence to:
Rodrigo Alvaro B. Lopes Martins, Ph.D.
Laboratório de Fisiologia e Farmacodinâmica
Instituto de Pesquisa & Desenvolvimento – IP&D
Universidade do Vale do Paraíba – UNIVAP
Av. Shishima Hifumi, 2.911 – Urbanova
12244-000 – São José dos Campos, SP
E-mail: rlopes@univap.br

terns to prescribe training. Another important aspect is to physiologically characterize a metabolic zone from which an unbalance occurs between metabolite production and clearance, which may cause muscle fatigue. Among such metabolites, lactic acid has been mentioned as an excellent indicator of the energy system chiefly used during exercise[1,6]. Lactic acid is constantly produced in the body, and its concentration increases in muscle areas during a high intensity physical activity[7].

Since Fletcher and Hopkins[8] showed how lactic acid is formed during muscle contraction, much heed has been paid to the probable mechanisms that control lactic acid production and clearance during exercise. In the late 50 s and early 60 s, Hollmann *et al.*[9] developed the idea of "the onset of anaerobic metabolism to measure cardiorespiratory performance". In their studies, they observed that during exercises with load increments at every three minutes, one would reach a point where pulmonary ventilation (PV) had a higher increase than the oxygen uptake ($\dot{V}O_2$). As PV and plasma lactic acid changes were similar, Hollmann defined this moment of the exercise as "the point of optimal ventilatory efficiency". Later on, Wasserman and McLlory[10], based on a study with cardiac patients, presented the expression "anaerobic threshold", proposing that ventilatory parameters could be used to estimate the inflexion point of the plasma lactic acid curve.

The "anaerobic threshold" was the focus of a number of investigations over the past decade, and is one of the most polemic and controversial subjects in the recent history of Exercise Physiology. Even though investigators disagree on its basic mechanisms[10], the anaerobic threshold (AnT) has been broadly used by investigators, physiologists, physical trainers and physicians. Practical applications for determining AnT include exercise prescription at an adequate intensity[13], performance prediction[14], and longitudinal assessment of aerobic training effects[15].

In spite of the significant number of expressions and references used to establish thresholds, these can be divided into two major categories: OPLA (onset of plasma lactate accumulation) as the exercise intensity prior to an exponential plasma lactic acid increase. Even though some authors use the same basics of the previous study, for them exercise intensity is defined as lactate threshold (LT)[16]. There are authors, who use the same expression (LT), define it differently, as LT being the exercise intensity that causes a 1 mM increase in plasma lactic acid above base line values (Δ1mM). Coyle[17] supports his methodology from finding in LT exercise intensities 5% higher than OPLA's, and very close to the speed athletes use at a marathon. Furthermore, for cyclists, employing an LT-matching intensity results in a frequency similar to muscle glycogenolysis, leading to a similar time for fatigue among subjects due to glycogen depletion (three hours)[18].

A number of studies investigated the relationship among lactic acid and noradrenaline and adrenaline concentrations, suggesting a strong causative relationship among them[19]. Mazzeo and Marshall observed that the behavior of catecholamines during progressive load exercises is similar to lactic acid behavior, and the adrenaline concentration inflexion point (adrenaline threshold) may be used to predict LT.

It is well known that there are some difficulties and hurdles to directly assess the level of lactic acid during exercise. The first is that the lactic acid measured is in the blood, and not actually in the muscles used for performing a physical activity. As the exercise-generated lactic acid is produced and cleared particularly by the active muscles, and may be cleared by active and inactive muscles, heart and liver, lactic acid concentrations may be different for exercises of similar intensities, depending on the site from where blood is drawn. Thus, comparison of results from different studies should take into account the site blood sample was taken from, and the handling (plasma or total blood) of the samples. Secondly, these are invasive methods, and, on a higher or lower degree, there is a risk of contamination of both, the athlete and the evaluator. A third important reason is that typically it is not possible to take samples in real time, during the physical activity, but only before or after the exercise. Considering these factors, from a fitness assessment perspective, further studies of new technologies that provide risk reduction and reliability enhancement in lactic acid measurements in the muscle during exercise are rather interesting.

Spectroscopy techniques have been used for assessment of biologic tissues, with quite reliable results, as long as one manages to adjust the spectroscopy technique to the necessary information for an accurate assessment of the sample[20].

When the light falls over any substance, it can either be absorbed or elastically scattered. *The infrared spectroscopy* (IR) measures the frequency in which a given sample absorbs IR radiation, and the intensity of such absorption. Thus, the infrared spectrum identifies a sample with peak absorption matching the frequency of the vibration among that material's atoms. Determining the frequencies allows identification of the sample's chemical components, considering that any chemical group absorbs light at a given frequency. The intensity of the absorption is related to the concentration of a specific component, thus one has a quantitative analysis. *Raman Espectroscopy* with Fourier transformation uses a laser beam with a power close to the infrared to charge a given sample and measure its light

emission. Most of the scattered light may have the same frequency that the incident light (Rayleigh scattering – elastic). However, a small fraction of the incident light ($\mathbf{h}\upsilon_i$) might have its power reduced ($\mathbf{h(\upsilon_i-\upsilon_R)}$ stokes) or increased ($\mathbf{h(\upsilon_i+\upsilon_R)}$ anti-stokes) (Raman scattering – inelastic) (figure 1). Considering that the power of light is proportional to the frequency, the change in frequency of the light inelastically scattered is equal to the vibrational frequency of the scatter molecule. Such energy-exchange process among the molecule, the scatter and the incident light is known as Raman effect. From the energy perspective, the Raman scattering process may be considered as the transition of a molecule from a fundamental stage to a charged vibrational stage, followed by a simultaneous absorption of an incident photon, and emission of a scattered photon (Raman). The scattered Raman light may be assessed by a spectrometer, and its intensity is shown as a function of its changed frequency (Raman displacement). As each molecule sample has its own molecular vibrational set, the Raman spectrum of a particular sample would be a series of peaks, each one displaced by the characteristic vibrational frequency of that molecule, thus allowing identification of the molecule under investigation. Raman displacement is typically measured in wavelength (cm^{-1}), a convenient unit to relate the frequency change of the scattered light to the frequency of the incident light.

Cells and tissues are made of proteins, nucleic acids, polysaccharides, lipids, vitamins and other components that form the molecular compound, with a highly complex structure. All diseases cause changes in cell and/or tissue biochemistry. The current challenge of modern medicine is to find non-invasive and non-destructive analytical techniques to investigate such changes. Few analytical methods meet these requirements and are sensitive enough to reveal composition and structural details. The most outstanding techniques in this field are the NMR, infrared (IR), and Raman spectroscopy. Among them the Raman and the infrared spectroscopy are currently emerging as powerful methods for medical diagnosis, considering that microscopic changes characteristic of the disease may be detected by optical spectroscopy, thus allowing a non-invasive *in vivo* diagnosis.

Raman and IR spectroscopy may provide detailed biochemical information that may be used to detect diseases in their early stages. As the vibrational spectroscopy technique may be employed with the use of optical fibers, sample collection is not necessary, which is a major advantage in face of conventional biopsy techniques and further histopathological testing[21]. Once optical diagnosis is made, the assailed organ may be effectively treated before it reachs an advanced stage. Vibrational spectroscopy techniques, such as Raman and IR, have been recently used to investigate human and animal skin cancer[22,23], and for atherosclerosis diagnosis[24,25]. Raman spectroscopy has also been used to study skin inflammatory diseases, and to assess the effect of drugs on the skin[25]. According to the vibration nature, which is determined by the molecule symmetry, infrared or Raman vibrations may be allowed or not.

The purpose of this investigation paper is to assess infrared Raman spectroscopy as a possible method to detect musculoskeletal lactic acid.

MATERIAL AND METHODS]

For the experiments, male Wistar rats with weight ranging from 250 to 350 g, from UNIVAP's Research and Development Institute were used. The rats were kept at an average temperature of 25°C, light/darkness cycle of 12 h, with water and *ad libitum* food until the time of experiment.

At the time of the experiment, the animals were sedated with sodium pentobarbital (40 mg kg-1 i.v.) via intraperitoneum, and fastened on a surgical bed. Anterior tibial muscle and tibial nerve were dissected and isolated. The animal was placed on the Raman spectroscopy equipment (figure 2), to acquire the characteristic spectra.

The Raman system

Raman spectroscopy (RS) is a vibrational spectroscopy technique, used to determine molecular structure and material identification and appraisal. The main application of this technique in the chemical-pharmaceutical are is the non-destructive assessment of solid, liquid and gas products, and a swift identification of the sample.

The Raman system block diagram is shown in figure 2. In short, a 5 W argon laser is used to pump a solid Ti:saphire laser. Argon laser was installed and aligned as to provide maximum power. The Ti:saphire laser was installed an aligned as to provide maximum laser power in wave length

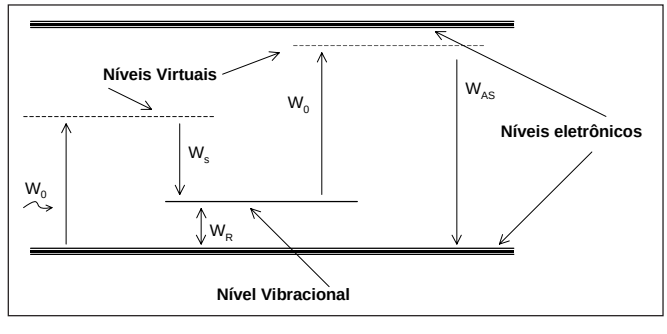

Fig. 1 *– Sketch of electron transition for obtaining stoke and anti-stoke components*

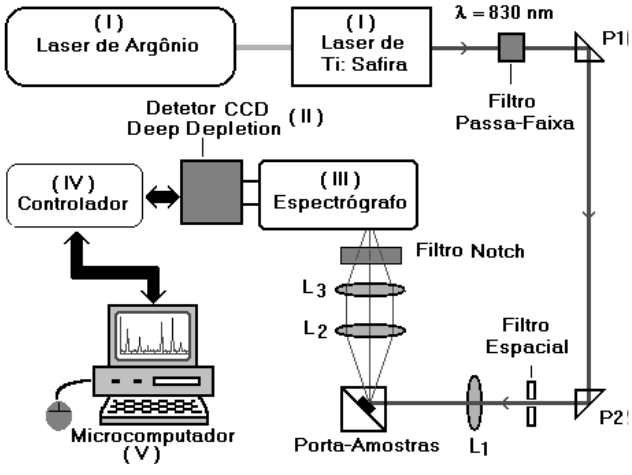

***Fig. 2** – Block diagram showing the bench Raman spectroscopy system*

tune ranging between 750 and 950 nm. Raman signal scattered on the sample is collected at 90 degrees, with notch-type filters, and focused at the entrance of the spectrograph breach. Notch filters eliminate the scattered Rayleigh light and transmit the Raman signal to the spectrograph, to be dispersed. The light scattered by the spectrograph is detected by a liquid nitrogen-refrigerated *Deep Depletion* CCD. The Ti:saphire laser wavelength passes through a holographic Kaiser filter to eliminate unwanted light and transmitting the desired wavelength only. The charging laser is then focused on the sample, after passing the dispersion optic device.

After preparation of the whole system, a fifty-second acquisition procedure was performed to obtain the Raman spectrum of the muscle without lactic acid (basal). Then 50 µl of lactic acid at 86% in a 9.4 mol/l concentration was injected with a 1 µl syringe in the proximal muscle area. After the injection, one waited for three minutes and then the acquisitions in the Raman system were made, each one of them for 50 seconds.

Injection of lactic acid and spectrum acquisition in the *in vitro* experiment

The muscle was prepared as previously described and fastened to the sample display carrier, for continuous 200 s acquisitions at a 4-minute interval.

To assess acid diffusion in the muscle, a 50 µl lactic acid injection at a concentration of 9.4 mol/l was given. After the injection, seven acquisitions at 4-minute were further made, in a total of 11 acquisitions.

This allowed the design of a chart showing diffusion of lactic acid in the muscle. The distance from the injection site to the laser incidence site on the muscle was of 1.5 cm,

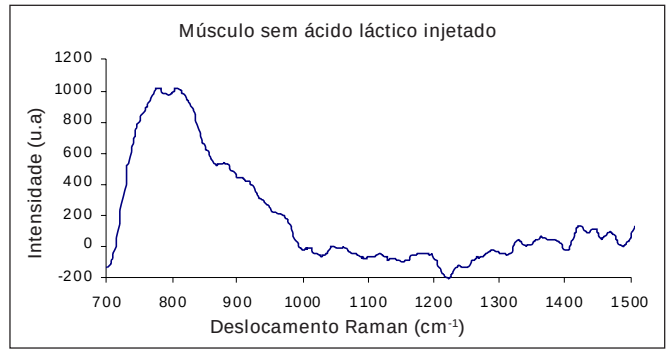

***Fig. 3** – Spectrum characteristic of the rat tibial muscle without lactic acid. The plotting represents the six animals used.*

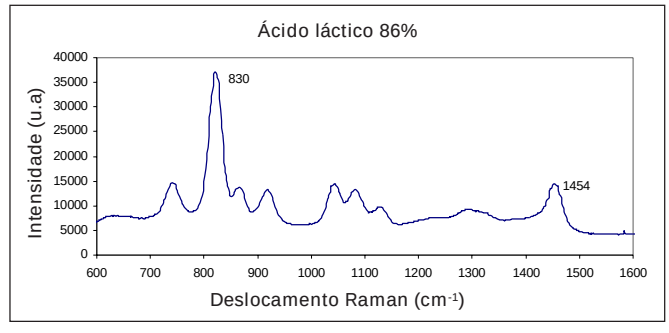

***Fig. 4** – Raman spectrum of lactic acid at 86%. One can see two well-marked characteristic peaks at 830 e 1,454 cm^{-1}. The plotting represents the six animals used.*

the maximum distance the length of the rat tibial muscle allowed.

Handling of the spectra

The spectra from the acquisitions in the Raman system were filtered by a digital filter, and implemented with use of *Matlab* software. A good portion of the spectra noises was deleted, for a better observation of the desired peak, and for measurement of peak intensity, in order to compare diffusion at different moments after lactic acid injection.

RESULTS

Raman spectrum of rat tibial muscle

Raman spectra of the control rats tibial muscle (i.e., with no lactic acid injection) were performed. Figure 3 shows a characteristic spectrum of rat tibial muscle without lactic acid injected.

Raman lactic acid spectrum in quartz bowl

The Raman lactic acid spectrum presented several and well-resolved peaks within the frequencies of our system, which reaches 1,500 cm^{-1}. These frequencies relate to the

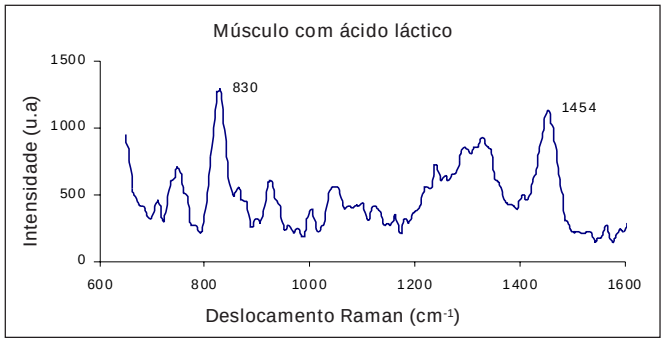

***Fig. 5** – Characteristic spectrum of the rat tibial muscle with injected lactic acid. One can observe lactic acid peaks at 830 e 1,454 cm^{-1}. The plotting represents the six animals used.*

different lactic acid molecule vibration mode, and their main characteristic peaks were 830 and 1,457 cm^{-1}.

Figure 4 shows the spectrum of lactic acid at 86% (*Sigma Chemical Co.*, St. Louis, USA), evidencing the several sample peaks in quartz bowl.

Raman lactic acid spectrum in the tibial muscle of rats after lactic acid injection

Figure 5 shows Raman spectrum of rats tibial muscle after lactic acid injection. We can clearly see the presence of characteristic lactic acid peaks at 830 and 1,457 cm^{-1}.

Lactic acid diffusion seen through Raman spectra

Table 1 shows the values of serial acquisition for the assessment of lactic acid diffusion in the tibial muscle of rats. The result of the linear regression analysis shows a 0.95 positive correlation between time of acquisition and intensity of Raman signal, shown in the table.

TABLE 1
Intensity values in arbitrary units (a.u), in relation to the time of their acquisition after lactic acid injection. The outcome of the linear regression analysis is a positive 0.95 correlation between Raman signal time for acquisition and intensity Raman (p < 0.0001)

Acquisitions	Time (min)	Intensity (a.u)
1	4	9,433
2	8	10,566
3	12	12,830
4	16	17,358
5	20	20,377
6	24	15,700
7	28	20,507
8	32	22,962
9	36	25,185
10	40	33,750
11	44	31,666

DISCUSSION

Raman and infrared spectroscopy techniques are emerging as powerful methods for clinical diagnosis, as microscopic changes characteristic of a disease can be detected by optical spectroscopy, thus allowing a non-invasive *in vivo* diagnosis.

As previously mentioned, Raman and IR spectroscopy may provide detailed biochemical information that can be used to detect a disease at an early stage. As the vibrational spectroscopy technique can be used through optic fibers, excision of a sample is not necessary, being an advantage over conventional biopsy techniques for histopathological testing. Depending on the nature of vibration, which is determined by the symmetry of the molecule, they may or may not be done in infrared or Raman.

In some materials, the Raman signal may be neutralized by fluorescence of impurities if the samples are charged with visible light. However, with the recent development of IR and Raman with Fourier transformation spectrometers operating close to the infrared region (NIR-FT-Raman), this scenario has been totally changed. It was shown that most organic samples may be charged by a Nd:YAG laser with λ = 1,064 nm. Considering that many of the biological material components do not present absorption bands in this range, there is a very low probability of fluorescence charging. From this perspective, Raman spectroscopy has been constantly used in medicine and biology for clinical diagnosis, studies with plants, and assessment of environmental pollutants.

In our investigation, we assessed the use of Raman spectroscopy as a possible tool to identify metabolites such as lactic acid, based in the fact that each biochemical component in the tissues has a characteristic vibrational spectrum, with narrow and well-resolved bands. As the particular focus of our investigation was to identify intramuscular lactic acid, we showed that this technique allowed a significant resolution of vibrational bands, providing information on the presence of such metabolite when its concentration increased in the observed muscle.

In the protocols where exogenous lactic acid administration was done through intramuscular injection, it was possible to clearly identify this substance and separate out from other muscle components. However, Pilotto *et al.*[26], in their study, mention that the Raman system was able to detect physiologic levels of lactic acid in the blood, where the signal had higher intensity and lower noise, facilitating the computer reading of the obtained Raman spectrum. In

the muscle, the signal/noise relationship is changed, showing a significant increase of noise and a decrease of the studied substance signal, making hard the physiologic detection of lactic acid.

Using the Raman system to detect lactic acid, the need for more specific protocols became clear, where the system instability is lessened by the type of laser used and the type of system alignment before each experiment. There is no question that the type of system used in our investigation is a powerful tool for a qualitative assessment of biologic samples. However, its high complexity makes it hard to be used by exercise physiology experts outside the experimental lab. This technology is still under development, and one sees the need of a more robust and closed system, of higher specificity and lower cost, to be used in the practice by professionals of this field.

On the other hand, Pilotto *et al.*[21] described the difficulties in the use of the optic fiber Raman system, particularly regarding the intensity of the noise caused by the strong Raman signal from the optic fiber, which directly interferes in the lactic acid signal. For this reason, to reach our results we used the bench Raman system.

As to the Raman displacement of the peaks, a small experimental variability was seen, described by Silveira Jr.[20] as signal capture by the spectrograph, and the displacement variation is due to the position of the cut-off grade in search of the desired signal.

As for the intensity of the peaks, the system showed a more intense peak close to 830 cm^{-1}, as mentioned by Pilotto *et al.*[21], and a less intense one close to 1,456 cm^{-1}, being characteristic of the substance. Even though the second peak was not observed in the *in vitro* protocol results, because of signal impairment that required a high number of accumulations during acquisitions, such discrepancy was likely due, according to Silveira Jr.[20], to the low power from the Ti:saphire laser alignment.

In the *in vitro* protocol, the increase of peak intensity during lactic acid diffusion was relevant, i.e., it was possible to show the spreading of the substance from the site of administration until the site of signal retrieval, in an attempt to monitor lactic acid diffusion in skeletal muscles (table 1). On the other hand, there was a decrease in intensity of the Raman signal in two points, which contradicts Silveira Jr.[20], who says that even during constant accumulation and acquisitions, there may be small differences in the signal intensity retrieval by the spectrograph, chiefly caused by instability of laser power or geometry problems in the incidence of light on the sample. As the different composition of tissues accounts for the observed spectral difference, the diagnosis of a disease or tissue classification may be established by identifying such spectral differences, with the use of the entire spectrum, or the most important transitions and vibrations[20].

Raman spectroscopy is a quite interesting technique to be used in the medical field due to its molecular specificity. However, the intensity of the back-scattered Raman signal intensity is extremely weak compared to other radiation decay processes, particularly fluorescence. Typically, the intensity of the Raman signal is 1,000 times less intense than fluorescent emission. It is thus necessary the use of appropriate instruments to retrieve such weak signal and sometimes the experiment and results assessment are spoiled due signal intensity variation.

An interesting and very important aspect of applying Raman spectroscopy in biologic samples is the proper selection of the charging wavelength. Most biologic molecules present electronic level transitions in ultraviolet and visible wavelengths. With the use of visible lasers for Raman charging, such an argon ion laser, for instance, the fluorescence of these biomolecules prevails over the Raman signal. As these molecules do not present electronic transitions to be charged at the infrared, fluorescence is almost non-existent, the lasers with 700-900 nm wavelength should be preferred.

The Raman spectroscopy showed high potential to detect lactic acid in tibial muscle of rats, even though the protocols should be better adjusted to the type of system used, so that, in future investigations, there are no instability problems in the equipment, as in this investigation. The difficulties found in standardizing the experiments are natural, due to the stage of development of this new technology, showing that further studies are necessary for the technique to be fully applied.

However, the initial results from our investigation clearly show that the system is able to identify lactic acid in the skeletal muscles, in physiologic concentrations, as the vibrational definition power is of high resolution. Moreover, the system was able to assess lactic acid diffusion over time, even with interference from a low Raman signal.

On the other hand, this system must be adjusted to be used in actual physical exertion in humans, as obstacles are present, such as movement and human skin, which is a barrier to capture the signal.

All the authors declared there is not any potential conflict of interests regarding this article.

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