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Original article

Applicability of vibrational spectroscopy in the analysis of liquid biopsy after a cycling time-trial test

Aplicabilidade da espectroscopia vibracional na análise de biópsia líquida após teste de contra-relógio no ciclismo

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ABSTRACT

Introduction: Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) is a technique that analyzes biochemical changes and monitors physiological responses; however, chemometric methods are required for its analysis. **Aim:** To investigate whether ATR-FTIR, combined with multivariate analyses, can be used to characterize and distinguish the biochemical profile of athletes before and after a cycling test. **Methods:** Cross-sectional study with 10 cyclists performing a 20 km time trial. **Results:** The results revealed that ATR-FTIR, in conjunction with pattern recognition approaches, allowed the identification of biochemical differences between pre- and post-test moments. After the removal of two outlier samples, principal component analysis (PCA) revealed a distinct separation in the fingerprint region of the spectrum. An analysis using a Monte Carlo sampling associated with genetic algorithm-based discriminant analysis (MC-GA-LDA) identified specific spectral regions related to these differences, indicating that the athletes' physiological variations were reflected in the spectra. The most relevant regions were in the bands of 1338-1308, asymmetric C-H stretching that can be assigned to amide III bond, and 1125-1108, asymmetric C-O stretching assigned to lactate biomolecule. These results demonstrate the sensitivity of ATR-FTIR in detecting metabolic changes and suggest its applicability as a tool for monitoring physiological responses. The technique can be useful in personalized training load monitoring and the identification of specific performance, fatigue, or physiological stress markers. **Conclusion:** ATR-FTIR technique combined with multivariate analyses can be a promising approach to characterize and distinguish the biochemical profile of athletes in response to physical stimuli.

Keywords: bicycling; spectroscopy; athlete; FTIR; chemometrics

RESUMO

Introdução: A espectroscopia de infravermelho com transformada de Fourier e reflectância total atenuada (ATR-FTIR) é uma técnica utilizada para analisar alterações bioquímicas em amostras biológicas. Entretanto, são necessários métodos quimiométricos e uso de ferramentas de inteligência artificial (IA) para análise desses dados. **Objetivo:** Investigar se o ATR-FTIR pode ser usado para caracterizar o perfil bioquímico de atletas antes e após um teste de ciclismo. **Métodos:** Estudo transversal com 10 ciclistas realizando um teste contra o relógio de 20 km. **Resultados:** Os dados de ATR-FTIR combinado com abordagens de reconhecimento de padrões permitiram identificar diferenças bioquímicas entre os momentos pré e pós-teste. Após remoção de duas amostras outliers, análise de componentes principais (PCA) revelou uma separação distinta de pré e pós-teste a partir da região espectral fingerprint (1800 – 900 cm-1). A análise com amostragem pelo método de Monte Carlo associado ao algoritmo genético e análise discriminante (MC-GA-LDA) identificou regiões espectrais específicas relacionadas a essas diferenças, indicando as variações bioquímicas mais relevantes (bandas de 1338-1308, estiramento C-H assimétrico – amida III; e 1125-1108, estiramento assimétrico C-O - lactato). Esses resultados demonstram a sensibilidade do ATR-FTIR em detectar alterações metabólicas e sugerem sua aplicabilidade como ferramenta para monitorar respostas fisiológicas em atividades esportivas. A técnica pode ser útil no acompanhamento personalizado da carga de treinamento e identificação de marcadores específicos de desempenho, fadiga ou estresse fisiológico. **Conclusão:** A técnica espectroscópica ATR-FTIR associada à quimiometria pode ser uma abordagem promissora para caracterizar e distinguir o perfil bioquímico de atletas em resposta a estímulos físicos.

Palavras-chave: ciclismo; espectroscopia vibracional; ATR-FTIR; quimiometria

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Introduction

Infrared (IR) spectroscopy is a method that measures the absorption of radiation in the IR region depending on the specific functional groups of the molecules present in the sample. IR radiation excites these molecules, and the frequency of these vibrations corresponds to the frequency of the absorbed light. The theoretical basis of IR is described in detail in several reviews [1-3], and the ability to identify the presence of functional groups is one of the advantages of this technique. It is a rapid analysis, requiring minimal sample preparation, with the ability to analyze any biofluid in less than 1 minute. It is considered a viable option for analyzing chemical changes in biological processes and evaluating metabolites in biofluids [4]. Although infrared spectroscopy is not as specific as other techniques, it is capable of analyzing the sample as a whole, in the set of all macromolecules present (carbohydrates, proteins, lipids, DNA, RNA...), forming a type of metabolic fingerprint of the sample [2,3].

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) is a type of infrared spectroscopy that has been used for a wide variety of health studies. Recent applications include analysis of whole blood [5], tears [6], and specific isolates such as exosomes [7]. ATR-FTIR has also proven useful in monitoring oxidative stress under conditions of chronic psychological stress in rat mononuclear cells [8]. Bujok *et al*. used ATR-FTIR to assess protein oxidation in the blood plasma of horses after physical exercise. The results obtained from the analysis of the ATR-FTIR spectra were similar to those obtained from the gold standard carbonyl spectrophotometric assay using DNPH, thus suggesting ATR-FTIR as a cheaper and faster tool for the study of exercise-induced protein oxidation [9].

High-performance athletes rely on specialized training to achieve the highest levels of efficiency in their sports. To better results, excessive training can lead these athletes to a state known as overtraining, which results in the opposite effect than expected, such as loss of performance [10]. Identifying the training moment that induces better results or a drop in productivity is difficult. Therefore, evaluating these individuals at the end of their activities, whether competitions or training, is essential to monitor their responses. There are many methodologies to assess the biochemical reactions of athletes. It is common to measure them based on cardiorespiratory variables, such as oxygen consumption and metabolites in serum and urine (lactate, urea, creatinine, creatine kinase, and ketone bodies) [11]. These metabolites are related to metabolic responses during activity. However, they require methods that are sometimes expensive and time-consuming, in addition to being a method for each metabolite analyzed.

The standard technique for evaluating these metabolites, such as urea, creatinine, glucose, and ketone bodies in urine, is based on colorimetric measurements (absorption). In this process, a specific reagent reacts with the molecule of interest, which has absorption at a particular wavelength, and this is used to identify and

quantify the desired component. The major disadvantage is that the results can take hours or days and are nonspecific for some analytes (mainly proteins). There is an ongoing search for a fast, minimally invasive technique with high sensitivity and specificity that can be used in this situation. One of the advantages of using ATR-FTIR in this context is that it allows the analysis of the modifications of all these substances (macromolecules) at once instead of analyzing them individually [12]. The ATR-FTIR spectrum contains vast information, so applying artificial intelligence and chemometric tools is essential for its analysis.

Thus, this study aimed to verify whether ATR-FTIR spectroscopy, together with AI and chemometric analyses, can provide a new biochemical view of characterizing and distinguishing the profile of athletes before and after a cycling test.

Methods

Participants

Ten male recreational cyclists, master category (42 ± 6 years, 75 ± 7 kg, 174 ± 7 cm) who had been cycling for 20 ± 10 years were invited to participate in the study. They participated in cycling races (competitive) and trained for an average of 11 ± 2 hours per week. Participants were instructed to abstain from strenuous activities at least 72 hours before the 20 km Cycling Test (TT20), avoid any analgesic (anti-inflammatory) medications, and maintain their regular dietary intake and lifestyle habits throughout the study. A written informed consent form was provided, and all subjects completed a clinical history questionnaire. The procedures were approved by the Human Research Ethics Committee (59773616.0.0000.5153). Male cyclists with at least five years of experience in regional-level competitive sports activities were included in the study. Exclusion criteria were those who used any anabolic steroid, drugs of abuse, or medications with a potential effect on sports performance.

Cycling Test (TT20)

The cyclists performed a 10-minute warm-up with free pedaling at their own pace, followed by a 5-minute rest. Then, the participants performed an individualized 20 km time trial using their bicycles coupled to a CompuTrainer ProLab 3D (Racermate), which measured performance during the test. All participants were instructed to finish the TT20 as quickly as possible. Verbal encouragement was provided throughout the event, but they were blinded to feedback such as time, cadence, power, and heart rate, as these could interfere with the stimulation effort. The course was configured in the Computrainer 3D software with automatic control of the constant load mode and an individual weight (bicycle + cyclist). The cycling test was performed at the *Laboratório de Força e Condicionamento* (Strength and Conditioning Laboratory – LAFEC) of the Federal University of Espírito Santo with a temperature controlled between 20°C and 22°C.

Heart rate analysis

Heart rate (HR) was monitored during the test using the H7 Bluetooth heart rate transmitter worn around the chest below the pectoralis major (Polar, USA) and connected to the HRV® software. The recorded data were subsequently analyzed using a computer program (Kubios software, HRV standard 3.3.0®), which allows the selection of specific run periods. Maximum heart rate (HR_{max}) was determined by the highest HR achieved and maintained for 30 seconds during the test.

Subjective perception of effort

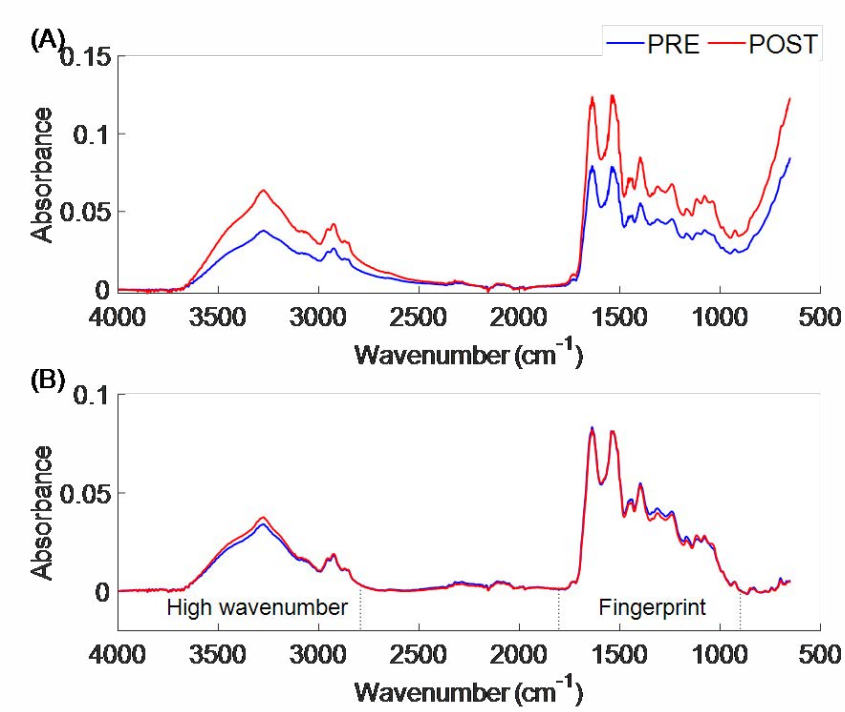
At the end of the TT20, participants were asked about their subjective perception of effort using the Borg scale (1-10), with 1 corresponding to "no effort" and 10 to "maximal/extreme effort" [13].

Vibrational spectroscopy

The instrumentation for mid-infrared vibrational spectroscopy comprises a spectrometer (Cary 630 FTIR, Agilent Technologies) coupled with an ATR complement and diamond crystal. The obtained spectra were recorded with a wavelength range of 650 to 4000 cm^{-1}, using 32 spectra for the background and sample analysis. Each spectrum contains 1798 analysis points (spectral resolution of 1.86 cm^{-1}).

To perform the spectroscopy, 10ul of plasma from the pre-intervention (PRE) and post-intervention (POST) moments were pipetted three times onto a sheet of aluminum foil on its shiny side; the samples were left at room temperature overnight to dry. After drying, the samples were analyzed directly on the crystal, in triplicate, using the equipment's press, which exerts continuous and equal pressure on all samples. At the end of each analysis, the crystal was cleaned with deionized water and 70% alcohol to remove residues from the previous sample.

Figure 1 shows the raw and pre-processed spectra before and after the TT20. Figure 1A represents the average of the raw spectra at the PRE and POST test moments, and Figure 1B represents the average of the pre-processed spectra with baseline correction, Savitzky-Golay smoothing, and vector normalization.



(A) Raw average spectrum. (B) Average spectrum after preprocessing (baseline correction, Savitzky--Golay smoothing, and vector normalization) with identification of high wavenumber (4000 - 2800 cm^{-1}) and Fingerprint (1800 - 900 cm^{-1}) regions. Each spectrum consists of 1798 analysis points
Figure 1 - Representation of plasma spectra before TT20 (PRE) and immediately after (POST)

Statistical analysis

Biological data are presented as mean and standard deviation. MathLab2020 software was used for AI and chemometric analyses. All spectra were preprocessed with baseline correction, Savitzky-Golay smoothing, and normalization.

Principal Component Analysis (PCA) is a pattern recognition model developed to identify possible anomalous samples, visualize similarities and possible natural groupings between samples, and analyze the behavior and dispersion of spectral variables [14].

For variable selection, the genetic algorithm method based on discriminant analysis with Monte Carlo sampling (MC-GA-LDA) was used, which is an association of the Monte Carlo sampling method (MC) with the genetic algorithm based on discriminant analysis (GA-LDA).

The MC method is a statistical method for solving various problems through random sampling using the probability distribution of the sample set. GA-LDA uses Fisher's ratio as a metric for selecting subsets of variables that maximize separation between classes [15,16]. The association of MC sampling with GA-LDA was applied in the present study to choose variables that discriminate physiological variations in PRE and POST TT20 individuals and that are reflected in the infrared spectrum of the samples. For this purpose, the data set with all average spectra was subjected to 800 random samplings and selection by GA-LDA iteratively. At each iteration, GA-LDA selected variables, identifying the variables that maximize separation between classes. Each variable's relative selection frequency was calculated at the end of the

iterations (N= 800). The final selection corresponds to the variables with the highest relative frequency values at the end of the 800 iterations.

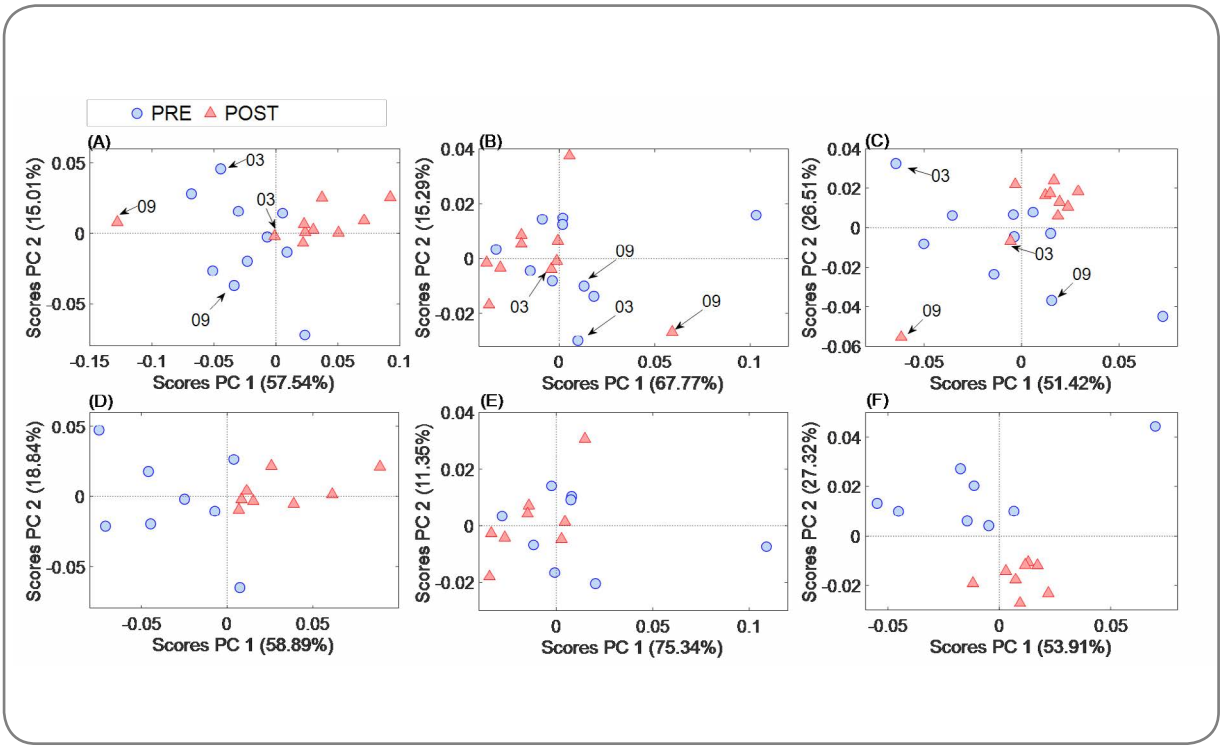
Results

Initially, we needed to ensure that the volunteers had exerted themselves to their maximum during the TT20. The ten subjects in the study completed the test with an average time of 33.4 ± 1.7 minutes. The subjective perception of effort, assessed by the Borg scale, at the end of the TT20 was 9.1 ± 0.9, and the maximum HR reached was 182 ± 13 bpm, which is equivalent to 102 ± 3% of the maximum HR estimated by age (220 - age). These data suggest that the subjects exerted maximum effort during the TT20.

The first analysis was the unsupervised PCA to identify whether ATR-FTIR can differentiate the samples from the PRE and POST TT20 moments based only on the characteristics of the infrared spectrum in the plasma. The analysis was performed considering all 1798 features of the spectrum (Figure 2A 4000-900 cm^{-1}) and in specific regions called the High Wavenumber region (Figure 2B, 4000–2800 cm^{-1}) and the Fingerprint region (Figure 2C, 1800-900 cm^{-1}). In neither case was a clear separation between the PRE and POST moments. However, after an individualized analysis, it was possible to observe that in all situations, samples 3 and 9 of the POST were the ones that did not allow a complete separation of the moments. In other words, the PCA analysis on the spectra of these two individuals did not match the rest of the groups (arrows in Figures 2A, 2B, and 2C).

Therefore, as the next step, these samples were removed, and the PCA was repeated (total spectrum, Figure 2D; high wavenumber, Figure 2E and fingerprint, Figure 2F). With the total spectrum (Figure 2D), we observed the separation between the groups (PC1 axis explaining 58.8% of the variance of the samples).

The next question was about which region of the spectrum is responsible for this separation, and so the spectrum was analyzed again as two separate parts (high wavenumber and fingerprint). We then observed that the separation was only observed in the fingerprint region (Figure 2F, PC2 axis, 27.3% of the variance) and not in the high wavenumber region (Figure 2E). It is important to highlight this result since this fingerprint region (1800-900 cm^{-1}) is the region that contains the most significant amount of information in biological samples [3].



(A and D), high wavenumber region (B and E), and fingerprint region (C and F).
(A), (B) and (C): models from spectra of all 10 subjects; (D), (E), and (F): models from spectra after removing samples 03 and 09 (n = 16). In blue: PRE moment, and in red: POST moment
Figure 2 - Graph of PC 1 versus PC2 scores of PCA models from full spectral variables

After this series of unsupervised exploratory analyses, we investigated, through MC-GA-LDA, which of the 1798 variables (spectrum regions) were responsible for this distinction between the PRE and POST TT20 moments. MC-GA-LDA analysis is an AI technique that combines two algorithms: GA, an optimization technique inspired by natural selection, where candidate solutions evolve through mutation and recombination to find the best model, and LDA, a probabilistic algorithm that identifies latent variables in a data set. Finally, the MC method refers to the number of times a random simulation is repeated to estimate the real value that a spectrum variable has relevance. The more iterations are performed, the more accurate the estimate will be. It is a widely used probabilistic technique that simulates random scenarios to calculate the probability of different outcomes. In our study, we performed 800 iterations; the model was repeated 800 times, and the two most prominent spectral regions (highest relative frequency values) were ~1338-1310 cm^{-1} and ~1125-1108 cm^{-1}. The other regions with median relative frequency values (~0.4 and 0.3) were ~1041-1026 cm^{-1} and ~965-922 cm^{-1} (Figure 3). Table I shows the chemical assignments of these identified regions.

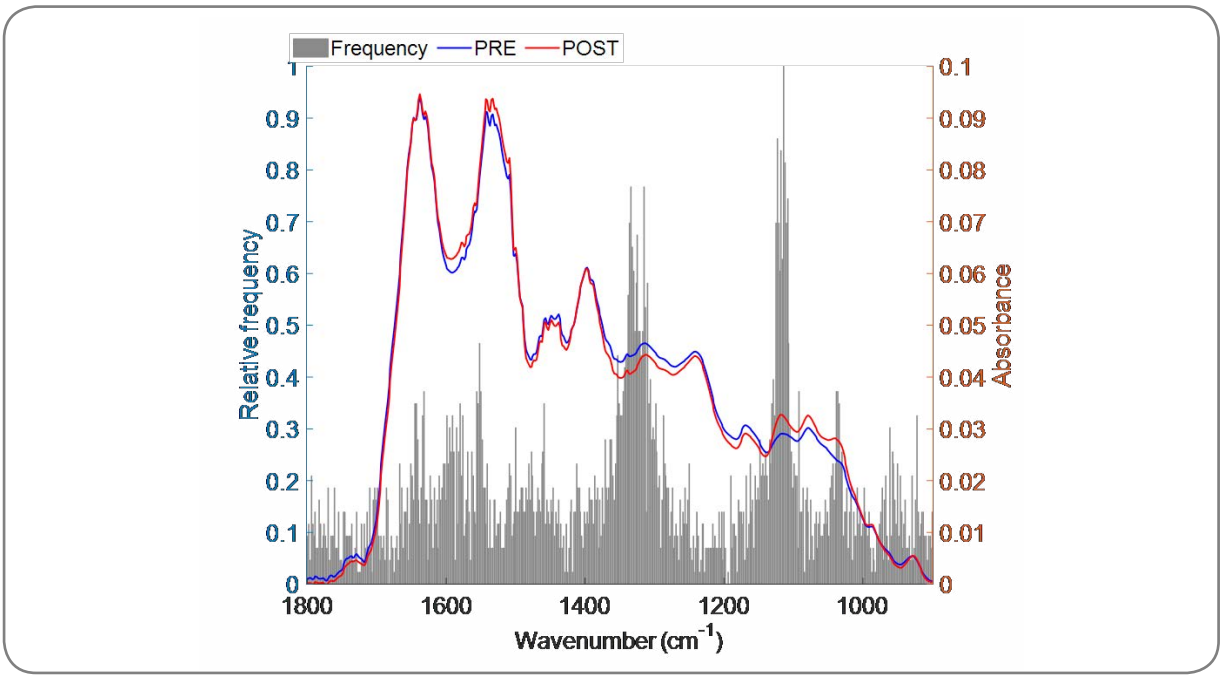


Figure 3 - Relative frequency of variable selection by the Monte Carlo GA-LDA method with 800 iterations

the most prominent regions (highest relative frequency values) are 1338 to 1310 cm^{-1} and 1125 to 1108 cm^{-1}. The other regions that stand out are those from 1041 to 1026 cm^{-1} and 965 to 922 cm^{-1} (Table 1).

Table I – Chemical assignment of the main selected FTIR regions by the Monte Carlo GA-LDA with 800 iterations

Wavenumber (cm^{-1})	Molecular vibration	Probable assignment
1338-1310 cm^{-1}	CH_2	Collagen, Amide III
1125-1108 cm^{-1}	C-O	Lactate
1041-1026 cm^{-1}	C-OH	Carbohydrate
965-922 cm^{-1}	PO_4^-	DNA, RNA, phospholipids

Discussion

As a result of the study, it was possible to observe that the infrared vibrational spectroscopy technique, ATR-FTIR, could identify differences in the biochemical profile of the sample between the PRE and POST TT20 moments. Furthermore, through the AI and chemometric analyses, it was possible to indicate that these differences are predominantly in the fingerprint region of the spectrum, more specifically in the wavelengths of 1338 to 1310 cm^{-1} (CH_2), 1125 to 1108 cm^{-1} (C-O), 1041 to 1026 cm^{-1} (C-OH), and 965 to 922 cm^{-1} (PO_4^-).

In 2003, Petibois and Déléris [17] used ATR-FTIR to obtain a global analysis of the energy metabolism of swimmers during a 400m race by analyzing the plasma by ATR-FTIR every 100 meters. The authors concluded that FTIR allowed for a global description of changes in blood content during the race. One of the significant ad-

vantages of using FTIR is that it can be performed using capillary blood collection (blood collected from the fingertip rather than via venipuncture), which respects the athlete's comfort and allows successive analyses to be obtained in short periods. The authors concluded that the region of most significant change was 1300-900 cm^{-1}, similar to the data found in the present study, a region also known to represent the majority of circulating bioenergetic molecules such as sugars.

Khaustova *et al*. [18] used saliva to monitor physiological stress in 48 conditioned athletes (VO_{2max} = 58.9±10.1 $ml.min^{-1}.g^{-1}$). The FTIR spectrum was obtained in saliva before, immediately after, and 30 minutes after a maximal step test. The authors showed that the method allows determining the concentrations of substances present in saliva, but in a cheaper way and without sample preparation and reagents, from the minimum sample volume and (almost) immediately after sample collection. This study analyzed and compared changes in the concentration of total proteins, cortisol, alpha-amylase, immunoglobulin A, urea, and phosphate with the gold standard methods.

Similar to the data found by our group, Caetano Junior *et al*. also identified two individuals among 13 rugby athletes using the FTIR technique, collected in saliva, whose spectrum behavior in the post-test moment was not discriminating from the pre-test moment. The two individuals in this study had lower HR responses than the group average, which suggested that these individuals exerted less effort during the test than the other volunteers [19].

In the present study, analyzing the individual data of the two individuals (03 and 09), it was also observed that they had characteristics that were distinct from the rest of the group: individual 03 was the oldest (54 years old), had a lower BMI (20.6 vs. average of 25.2), had trained longer (40 years vs. average of 22 years), and finished the test with the lowest $\%HR_{max}$ (91% vs. average of 102%). Individual 09 had the most discrepant result, having had the worst performance among the 10 volunteers, finishing the test in 36.5 minutes (study average of 33.5 minutes). Thus, it is believed that their chemical profile at the POST moment did not differ from the PRE moment in the PCA analysis because the individuals had not reached their limit. We then identified a possible application of this technique. Were these athletes unmotivated for the test or overtrained? Although we do not have much data to explain this, it is a fact that FTIR identified these two individuals with only 10ul of plasma in less than 1 hour.

In 2022, Chrimatopoulos *et al*. [20] used ATR-FTIR coupled with AI (PCA and PLS-DA) to determine biochemical changes after exercise using spectra obtained in the saliva of athletes with different fitness levels. The authors also identified regions similar to ours in this work, with 921 cm^{-1} (membrane lipids/phospholipids/carbohydrates) and 1080 cm^{-1} (sugars) being the main ones modified after physical exercise. The authors suggest that ATR-FTIR analysis of saliva samples will be able to distinguish the fitness level of athletes accurately.

More recently, in 2024, Souza *et al*. [21] used ATR-FTIR to distinguish biochemical changes induced by different types of exercise: high-intensity interval training,

continuous exercise, and strength training. The authors used more robust machine learning algorithms such as Naive Bayes, Random Forest, K-NN, AdaBoost, Support Vector Machine, Neural Network, and Logistic Regression to interpret the spectra. The authors observed that the biochemical components changed explicitly according to each type of exercise. Thus, spectral vibrational modes were identified as potential biomarkers for every exercise performed.

The present research group has already been working with the use of this tool (ATR-FTIR) to identify pathological conditions such as iron overload, COVID, and sepsis [6,22-25], but this study was the first to use the tool in a physiological condition such as physical exercise.

This study demonstrated that with FTIR data and an unsupervised multivariate analysis, it was possible to distinguish the PRE- and POST-TT20 moments. In addition, it was possible to observe the regions of the spectrum responsible for this differential identification of the two moments, being in the fingerprint region and not in the high wavenumber region. Therefore, the study opens the possibility for the applicability of FTIR as a personalized training load monitoring tool or even as a marker of a specific biological response such as performance, fatigue, damage, or physiological stress since the tool proved to be sensitive to detect individual variations from moment to moment. Once this digital signature has been identified in an overtraining condition, with a simple collection of capillary blood or saliva, for example, followed by an analysis on the equipment for no more than 15 minutes followed by computational analysis, it will be possible to diagnose the individual's condition almost instantly.

Conclusion

The results showed that it is possible to biochemically differentiate and classify the physiological state of athletes undergoing physical training by ATR-FTIR using PCA and GA-LDA. In addition, through multivariate analysis, it is possible to identify the peaks of the spectra that underwent alteration after physical stress, and these alterations are related to variations in organic molecules due to the change in the physiological state. These results demonstrate the sensitivity of the technology in detecting changes in metabolism in a generalized manner and suggest the possibility of being used to monitor adaptations in one athlete throughout training. However, it is necessary to conduct other studies with larger sample sizes to better evaluate the patterns in the spectra associated with improvement or worsening in performance, greater or lesser muscle damage, or better or worse cardiovascular response, for example, in practitioners of some regular physical exercise.

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Conflicts of interest
The authors declare no conflict of interest

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Authors' contributions
Conception and research design: Barauna VG, Silvatti AP, Leite RD; Data collection: Leal LB, Baiense IM, Lopes ALC; Data analysis and interpretation: Leal LB, Baiense IM, Lopes ALC, Santos L, Nascimento MH; Statistical analysis: Nascimento MH, Leal LB; Manuscript writing: Santos L, Barauna VG; Critical revision of the manuscript for important intellectual content: Leal LB, Baiense IM, Lopes ALC, Santos L, Nascimento, MH, Barauna VG, Silvatti AP, Leite RD

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