Noninvasive biochemical monitoring of physiological stress by Fourier transform infrared saliva spectroscopy†‡

Svetlana Khaustova,[a] Maxim Shkurnikov,[a] Evgeny Tonevitsky,[a] Viacheslav Artyushenko[b] and Alexander Tonevitsky[a]

Received 16th July 2010, Accepted 29th September 2010
DOI: 10.1039/c0an00529k

Physical stress affects the immune system, activates the sympathetic (SNS) and parasympathetic (PNS) subsytems of autonomic nervous system (ANS), and increases the activity of the hypothalamic-pituitary-adrenal axis (HPA). The specific response of the major regulatory systems depends on the human functional state. Saliva is a unique diagnostic fluid, the composition of which immediately reflects the SNS, PNS, HPA and immune system response to stress. A new method of saliva biomarker determination by Attenuated Total Reflection Fourier-Transform Infrared (ATR FTIR) spectroscopy has been developed to monitor the exercise induced metabolic changes in saliva from male endurance athletes. The method has been tested using a group of professional athletes by analysing saliva samples collected before and after the exercise, and the saliva composition monitoring by ATR FTIR spectroscopy was shown to be suitable for real-time checking of response to stress.

Introduction

Saliva is a complex biological fluid. Its components are produced mainly by acinar cells and are delivered to the oral cavity by a cell-lined duct system where the fluid and electrolyte components are subjected to the secondary modifications.[1] Normally saliva is a colourless, dilute fluid with a density ranging from 1002 to 1012 mg cm^{-3} and pH of about 6–7.[1,2] The autonomic nervous system (ANS) regulates saliva secretion and its composition, so the changes in the activity of ANS affect the salivation level.[3]

Saliva is mainly composed of water, the rest are organic (67%) and inorganic (33%) components.[4] Among the inorganic saliva components, osmotically active cations (Na^+, K^+, Ca^{2+}) and anions (Cl^-, HCO_3^-) are of particular importance.[2,4] The total protein level in saliva is 0.8–3.0 g l^{-1}, of which 90% are produced by salivary glands (glycoproteins, α-amylase, lysozyme, *etc.*) and 10% are metabolic products of bacteria from blood or cells of the oral cavity. The saliva proteins and glycoproteins belong to at least seven subfamilies: mucins, proline-rich proteins (acidic, basic and glycosylated), cystatins, histatins and statherins, amylases, carbonic anhydrases and salivary peroxidases.[1,4] In addition to protein components, saliva contains a large variety of low molecular weight substances, amino acids and hormones (Table S1, ESI†).[1]

Saliva analysis is a powerful diagnostic tool in sports and rehabilitational medicine.[5] The relationships between the functional state of the human body and the salivary gland's physiological activity offer the possibility to use saliva as a source of diagnostic information, with a number of advantages over analysis of other biological fluids.[1,5] The saliva contains several biomarkers that reflect the impact of stress and physical exercise on the most important body regulatory systems.[4]

One of them—the secretory immunoglobulin A (sIgA), the first line of organism defense—is the most convenient and easily accessible object to study the primary immune response to exercise.[6] Nevertheless, the contradictory data on the influence of physical activity onto saliva sIgA level are published. Thus, prolonged physical exertion may lead to decrease,[7] increase,[6] or have no effect on the affect of sIgA concentration.[8] The data for short-term acute exercise are also ambiguous: the sIgA level may decrease,[7,9–12] increase[13] or remain unchanged.[14] The mechanism of sIgA level changing during physical activity is currently not clear, and nervous and endocrine factors seem to influence the way of the immune response to stress.

Another component is α-amylase whose activity correlates with activation of the sympathetic autonomic nervous system (SNS) induced by the stress and with basal plasma catecholamines levels in particular, with norepinephrine level and its changes in response to stress.[15–18] Saliva α-amylase activity increases in response to both psychological and physiological stress,[14,16–19] like physical activity, heat and cold shocks, written examinations,[18] competition,[20] as well as viewing movies with negative content.[21] In the number of studies the α-amylase level dynamics in response to physical activity is correlated with heart rate[18,20] and systolic blood pressure.[21]

The third component to be mentioned is cortisol whose concentration changes during physical stress are widely studied.[21–25] Among all the hormones secreted in response to stress, cortisol responds most strongly, being the most biologically active glucocorticoid.[26] Cortisol affects many physiological

[a]*Russian Research Institute of Physical Education and Sport, Moscow, 105005. E-mail: svetakhaustova@gmail.com*
[b]*Prokhorov General Physics Institute, Russian Academy of Sciences, Moscow, 119991. E-mail: sa@fibrephotonics.com*

† This article is part of a themed issue on Optical Diagnosis. This issue includes work presented at SPEC 2010 Shedding Light on Disease: Optical Diagnosis for the New Millennium, which was held in Manchester, UK June 26th – July 1st 2010.

‡ Electronic supplementary information (ESI) available: Table S1 The main low molecular weight saliva substances. See DOI: 10.1039/c0an00529k

systems, including immune function, glucose regulation, vascular tone, bone metabolism and muscle contractility.[21,24,27] Any strong physical or physiological effect, experienced by the organism, leads to a change of cortisol level.[21–28] And saliva cortisol concentration reflects free cortisol level, which is biologically active.[28]

Among low-molecular saliva components phosphates and urea are of interest since their concentrations are also varied during physical activity.[19]

Despite the great number of studies devoted to changes of the above-mentioned saliva components levels, in our opinion, are the only integrated approach that could evaluate the impact of exercises on the physiological status of athletes during training and competitions. In this regard, the purpose of this research was to perform an integrated evaluation of the impact of short-term high-intensity physical activity on the concentration of cortisol, sIgA, α-amylase, urea and phosphates that can be measured in saliva and indicate changes in the main regulatory body systems.

Currently, a broad set of methods used to analyze saliva includes immunoassay, colorimetric, enzymatic, kinetic, chromatographic and mass spectrometric methods of analysis,[6,12,17,27,29] while the spectroscopic identification and quantification of saliva biochemical components in the middle infrared (IR) region (4000–700 cm^{-1}) are not used for diagnostic purposes, although these compounds are highly specific in this spectral region.[30,31] At the same time, the application of mid-IR spectroscopy for quantitative analysis of other biological fluids is known for a long time and has several advantages.[32,33] This method is successfully applied in the analysis of blood,[34] serum,[35] plasma[36] and urine.[34] The Fourier transform infrared (FTIR) spectroscopy method makes it possible to calculate with high accuracy the concentrations of albumin, lactate, urea, glycerol, glucose, triglycerides, apoliproteines, transferrin, haptoglobin, α_1-antitrypsin, α_2-macroglobulin, immunoglobulins and amino acids.[32–36]

The study was divided into two steps. First the ATR FTIR spectroscopy method was adapted for simultaneous calculation of concentration of α-amylase, cortisol, sIgA, urea, total protein and phosphates in saliva. And next, metabolic changes in the athletes' saliva composition during short-term high-intensity exercise were monitored using the previously obtained calibration models.

Generally, we have applied the ATR FTIR spectroscopy within the new noninvasive method of studying the athletes' physical stress reaction and major regulatory systems monitoring.

Materials and methods

Participants

The study included 48 well-trained regularly practicing male athletes. Age 22.1 ± 6.0 years, height 181.7 ± 7.3 cm, weight 78.5 ± 8.3 kg, body mass index (BMI) 23.9 ± 2.1 kg m^{-2}. Participants were informed about the nature, purpose and potential risks of the experiments. The experiment was approved by Ethical committee of Russian Research Institute of Physical Education and Sport.

Experiment design

Ramp test exercise was performed on the treadmill Venus (h/p/Cosmos, Germany) with gradually rising power. The tape start speed and angle of treadmill inclination were 3.0 m s^{-1} and 1 degree, respectively, the load level duration was 3 min, with discrete changes of 0.5 m s^{-1} in belt speed. Before the test exercise the subjects performed warm-ups on the treadmill for 5 min at the tape speed of 3 m s^{-1}. During the test exercise the gas exchange indicators (O_2, CO_2) were measured on an Oxycon Pro (Viasis, USA) analyzer. Pulse rates were recorded using Polar RS800 (Polar, USA). The test was continued until the subject can no longer maintain the tape speed. Lactate level during exercise was measured in the last 15 s of each load step using the automatic glucose and lactate analyzer Biosen C-Line (EKF Diagnostic, Germany).

Diagnostic material

Venous blood and saliva samples were collected before exercise, immediately after its completion and after 30 min rest (Fig. 1). The blood serum was isolated by a standard protocol and stored at −20 °C. A Salivette system (Sarstedt, Germany), consisting of a neutral cotton swab and test tube, was used to collect saliva. The patient chewed a cotton swab for three minutes, which was then placed in a container and covered with a plastic lid. Container was put into a plastic tube, and then saliva from the swab was removed by centrifugation and stored at −80 °C. Immediately before the analysis saliva samples were filtered using spin filters 0.22 μm (Agilent Technologies, USA).

Before saliva collection, athletes rinsed their mouth with mineral water. Participants did not smoke, did not eat and drink (except water), or chew gum, and do not use steroid creams and lotions for one hour before saliva sampling. Based on the health questionnaire, which patients filled out before the study, samples included in this study were obtained from healthy and nonsmoking athletes. Sampling was performed between 09:00 and 11:00 a.m.

Among the saliva samples, 154 were anonymous ones: 120 for developing new partial least square (PLS)[37] calibration models, 34 for validating these new techniques. Also there were 144 saliva samples for health athletes monitoring (48 athletes, 3 saliva samples: before, after ramp test and rest). Each saliva sample used for design, calibration or validation of the model was divided into two aliquots: one aliquot was used for routine biochemical analysis, while the other one was analyzed by FTIR spectroscopy.

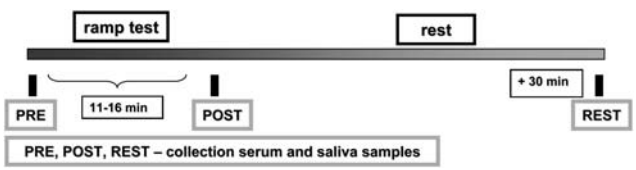


Fig. 1 Experiment design. PRE, POST, REST – collection of biological material (saliva and blood) before, after exercise and 30 min after its completion, respectively.

Assays

Determination of saliva total protein concentration was performed using the Bradford assay (Coomassie (Bradford) Protein Assay Kit (Thermo Scientific Pierce Protein Research Products, USA)). Bovine serum albumin (BSA) was used for calibration. The salivary sIgA and cortisol concentration were determined using a test-system "sIgA A8668" (Vector-Best, Russia) and "EIA 2930" (DRG Instruments, Germany), respectively, by enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions. The α-amylase activity, phosphates and urea levels in saliva were determined using an automatic biochemical analyzer HumaStar 300 (Human, Germany) according to the manufacturer's protocol using the recommended reagents. Preliminary results for α-amylase activity determination in saliva samples were diluted 200 times, for phosphate level calculation saliva samples were pre-diluted twice. Serum of all athletes before and after exercise was analyzed for creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and growth hormone (GH). Determination of CK, AST, ALT was performed on an automated biochemical analyzer HumaStar 300 (Human, Germany) using reagents and protocols recommended by the manufacturer. The GH concentration in blood serum was determined according to the method described in Ref. 38. All assays were performed three times.

Infrared spectroscopy

Spectra were recorded in the 4000–600 cm^{-1} region using FTIR-spectrometer Microlab (A2 technologies, USA) fitted with an ATR unit with a diamond disc as an internal-reflection element. In the diamond the infrared beam is reflected at the interface towards the sample. The volume of saliva sample was 2 μL. The sample was dried on ATR crystal for 3 min and then the spectra of saliva film were recorded. The spectrum of air was used as a background. Background and sample spectra were taken with 4 cm^{-1} of resolution and 32 scans.

Spectral data analysis procedures

The following steps were used to establish the model for the determination of concentrations of the six saliva compounds: 1) design and calibration of a model; 2) model validation. To obtain stable and reproducible results of multivariate analysis 1200 spectra were used for calibration, and 340 spectra—for validation. Concentrations of biochemical parameters in the calibration saliva samples were in the range of physiological norms. For each saliva sample 10 spectra were recorded, the obtained spectra were averaged and used for calibration plotting and further analysis. The spectra were adjusted to baseline (Rubberband correction) and normalized. To calculate the concentrations of analytes (total protein, sIgA, α-amylase, cortisol, phosphate, urea) the partial least square method (PLS, the algorithm PLS1) was used.[37]

We used two validation procedures which should not be mixed up. The first one was the cross-validation procedure which was used to find the optimal model parameters. The second one was the ordinary validation used to measure the model quality.

The leave-one-out cross-validation was used to find optimal model parameters. One spectrum was sequentially removed from the calibration spectra set and from the remaining spectra the regression coefficients were calculated. These, regression coefficients were used to predict the biomarker concentrations in a removed spectrum. Predicted concentration was compared with the concentration calculated from the classical method of biochemical analysis, and the root mean square error of cross-validation (RMSECV) was determined. Thus, the obtained parameters were used to create saliva component concentration measurement models.

Next, the second step for constructing the model consisted of the analysis of the sample spectra not included in the calibration set (test data set). Concentrations of substances in those samples were predicted using calibration models obtained previously. Root mean square error of prediction (RMSEP) characterizes the predictive force of model, along with the number of PLS vectors and the correlation coefficient R^2.

This calibration model was used to evaluate composition changes in the saliva samples obtained from 48 athletes. Saliva samples were taken as previously described before and after exercise and after 30 min recovery.

Statistical analyses

Results are expressed as mean ± SD. The Mann-Whitney U test was used to determine the reliability of the differences between the tested parameters levels. Differences considered significant at $p < 0.05$. Spearman's rank correlation coefficient was used for statistical association between parameters.

Results

Infrared spectroscopy

At the first step of the research we had to find optimal conditions to obtain reproducible, characteristic saliva spectra by ATR FTIR spectroscopy, to create further a model for determination of saliva component concentrations using multivariate calibration. IR-spectra of the saliva films were recorded in 4000–600 cm^{-1} region. Drying of the samples removes the water absorption band from spectra and increases the intensity of the compounds under investigation, thus allowing the determination of small concentrations of saliva components.

Saliva is a mixture of compounds each having their own absorption spectrum in the mid-IR region. Thus, the IR spectrum of saliva is a superposition of all these individual spectra and the intensities of the absorption bands in this spectrum are proportional to concentrations of the components (the Bouguer-Lambert-Beer law). The saliva spectrum contains several absorption bands in the mid-IR region (4000–600 cm^{-1}) which are typical for biological samples (Fig. 2).[31] The peaks with wave numbers 1636 cm^{-1} and 1541 cm^{-1} are usually identified in proteomic materials and characterize υC=O (Amide I) stretching and δ_NH (Amide II) bending vibrations, respectively.[30–36] In the region of 3600–2900 cm^{-1} the absorption bands of primary and secondary amines (NH_2, NHR) are observed; the peaks at 3200 cm^{-1} is assigned to N–H and/or O–H vibrations, at 3000 cm^{-1} and 2880 cm^{-1}—to asymmetric stretching vibrations of methyl (CH_3) and methylene (CH_2) groups, respectively; the

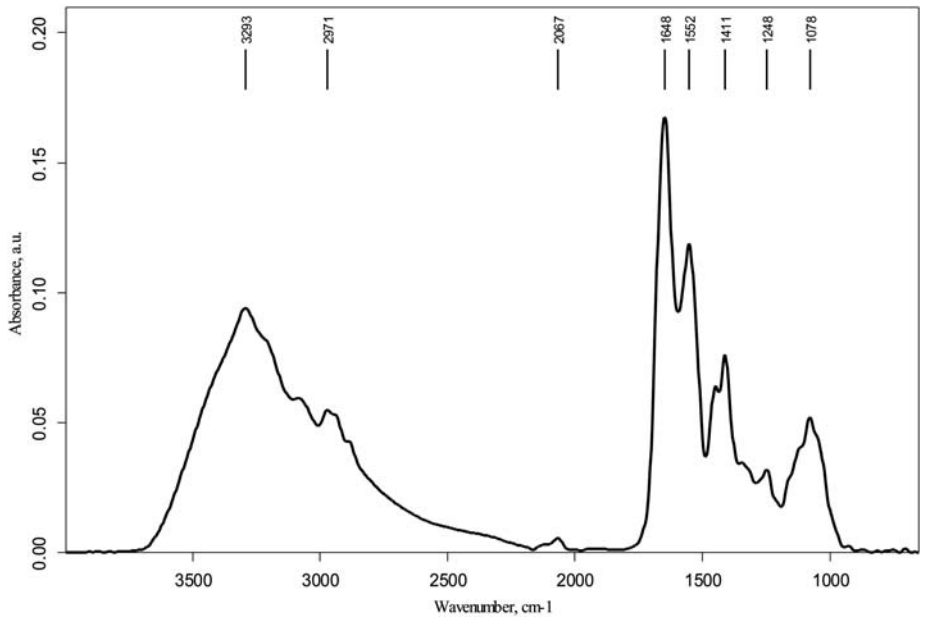


Fig. 2 Averaged spectrum of saliva recorded in 4000–600 cm^{-1} region. The main absorption bands are marked.

bands at 1450/1400 cm^{-1} are also attributed to vibrations of CH_2 and/or CH_3 groups. The absorption at 1246 cm^{-1} indicates CO groups (ester) stretching vibrations. The peak at 1348 cm^{-1} corresponds to the stretching vibrations of carboxyl group υCOO (Amide III), the absorption bands of sulfonic groups can also be founding this spectral area. Absorption band at 1075 cm^{-1} (generally, 1080–1047 cm^{-1}) may indicate the presence of glycosylated proteins and phosphorus-containing compounds.[30–32]

The obtained spectra were used to create calibration models. We analyzed several models using one or several spectral windows with variable RMSECV values and several factors. We chose a model with the highest R^2, the lowest RMSECV and one or several spectral windows corresponding to the molecular structures.

Infrared spectra of saliva samples with known total protein content were analyzed. The results of the cross-validation are shown in Fig. 3. The regression was performed with the two PLS-vectors (rank 2). As saliva molecules may have several specific bonds, the PLS 1 algorithm uses several spectral windows with correlations between the characteristic variations of the spectra and the values of known concentrations. It is therefore of utmost importance to select the correct window for the molecule being tested so that the model retains its predictive power. To design a model for total protein level calculation we used the following spectral regions: 1503–1440 cm^{-1}, 1317–1249 cm^{-1} and 1190–936 cm^{-1}. There is a reliable correlation between the total protein concentrations predicted by FTIR and those obtained by Bradford assay. The model has a high correlation coefficient $R^2 = 0.94$, and low RMSECV value (0.02 g l^{-1}). The total protein level in test saliva set samples is similar to the concentrations measured by Bradford assay, indicating a high predictive power of the model, RMSEP value is 0.04 g l^{-1}, $R^2 = 0.81$.

The model for the saliva sIgA concentration determination uses the spectral ranges of 1567–1526 and 1488–1406 cm^{-1} and has the following characteristics: $R^2 = 0.86$, RMSECV = 11 mg l^{-1} (Fig. 4), RMSEP = 8 mg l^{-1}, $R^2 = 0.83$. The results of the cross-validation are shown in Fig. 4.

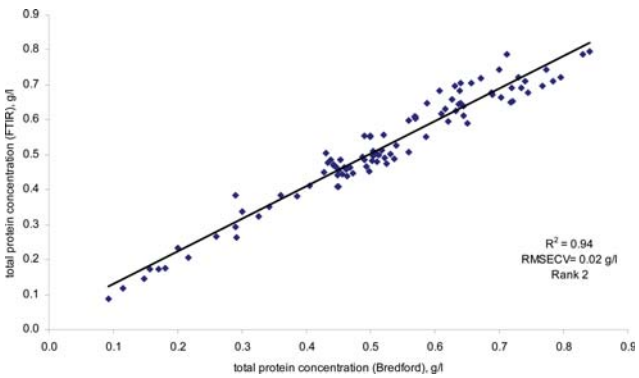


Fig. 3 Total protein concentration in saliva. Correlation between the total protein concentrations obtained by FTIR spectroscopy and Bradford assay.

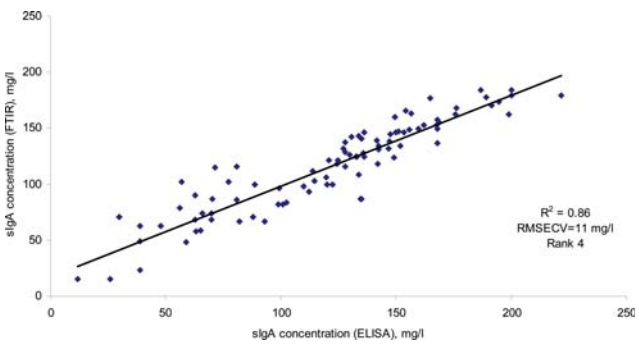


Fig. 4 sIgA concentration in saliva. Correlation between the sIgA concentrations obtained by FTIR spectroscopy and ELISA.

Spectral regions of 3500–3200 cm^{-1} and 1900–900 cm^{-1} are the most indicative for cortisol concentration determination, since the cortisol molecule contains three six-membered rings and one cyclopentane ring with ketone oxygens at C–3 and C–20, the double bond between C–4 and C–5, the β-hydroxyl group at C–11, the two-carbon chain in β-orientation, the hydroxyl group in at C–17, and β-methyl groups at C–18 and C–19. We used the 1900–900 cm^{-1} range as the basic one for the construction of gauge models and the search of more specific areas, the use of which gives the smallest error in the predictions of cortisol concentrations. The calibration model in 1943–1526 cm^{-1}, 1391–1249 cm^{-1} and 1115–973 cm^{-1} spectral regions has the lowest RMSECV value (2.21 mmol l^{-1}) and the highest $R^2 = 0.90$. The results of the cross-validation are shown in Fig. 5. Validation of the calibration model showed that RMSEP is 1.50 mmol l^{-1}, and $R^2 = 0.88$.

A model for the calculation of α-amylase level in saliva, which has the smallest RMSECV (24 U ml^{-1}) and the highest R^2 (0.91), was constructed for the following spectral regions: 1578–1548, 1526–1496, and 1444–1305 cm^{-1}. Validation showed that the model has good prediction accuracy and stability: RMSEP = 19 U ml^{-1}, $R^2 = 0.89$.

There is a correlation between the conformation and structure of proteins and their respective amide modes in IR spectra. The structure of sIgA consists of at least two IgA molecules covalently linked by a J-chain (15 kDa), and the glycoprotein secretory component (70 kDa), which is added as the antibody crossed the mucosal epithelial cells into the lumen. The structure of α-amylase is a 8-stranded α-β barrel containing the active site, interrupted by a ~70 amino acids, calcium-binding domain protruding between β-strand 3 and α-helix 3, and a carboxyl-terminal Greek key β-barrel domain. More than 25% of α-amylase secreted into saliva is assumed to be glycosylated and there are at least two major isoforms of α-amylase: 59 kDa and 56 kDa. Individual differences in the structures of these proteins are recognized in the saliva spectrum. Therefore it is possible to identify the specific narrow spectral areas of α-amylase and sIgA within the protein range of spectra (especially after a spectral preprocessing such as derivative analysis).

The calibration model for the urea level calculations has a high predictive ability (R^2 calibration 0.96, RMSECV = 0.1 mmol l^{-1}, R^2 validation 0.95, RMSEP = 0.1 mmol l^{-1}). Phosphate concentration can also be obtained by the spectral analysis: calibration R^2 is 0.87, RMSECV = 0.5 mmol l^{-1}, RMSEP = 0.3 mmol l^{-1}.

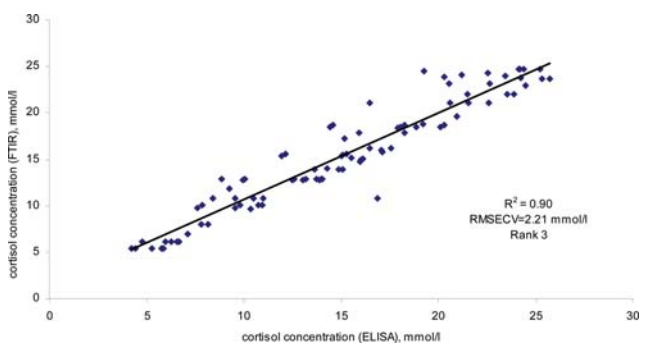


Fig. 5 Cortisol concentration in saliva. Correlation between the cortisol concentrations obtained by FTIR spectroscopy and ELISA.

Thus, the calibration model was established for 6 saliva components, and the results indicate that the determination of total protein level and concentrations of saliva substances such as cortisol, α-amylase, sIgA, phosphate and urea can be performed with high accuracy by ATR FTIR spectroscopy in mid-IR region. The results of the cross validation and test-set validation for these components are summarized in Table 1.

Together with PLS analysis, FTIR spectroscopy appears to be an easy-to-use and accurate method to determine multivariate concentrations of components in dried human saliva. Saliva biochemical profile can be determined without a special sample preparation in a few minutes after sample collection (no sample concentration, test component selection, or removal of interfering substances are required) with only ~10 μl of saliva needed. Besides, portable spectrometers are available from several manufactures, therefore it is possible to use this approach for the field studies. The accuracy and reproducibility of measurements are comparable with classical clinical approaches and limited by only the reference analysis used to derive the calibration model. The high accuracy is achieved by use of a large set of saliva calibration spectra (more than 1500), which are carefully selected according to physiological concentrations of various saliva biochemicals and uniformly cover the entire calibration range. So, we showed that the method is comparable with classical biochemical methods of analysis in terms of sensitivity, and could be an alternative tool for rapid quantitative analysis of many components after the development of the predictive model. Thus it was used in further experiments.

Ramp test

Participants were tested on a treadmill with gradually rising speed. Duration of exercise testing was 13:40 ± 02:13 min. The individual anaerobic threshold (AT) was determined using V-slope method based on gas analysis.[39] The average value of oxygen consumption at AT was 49.9 ± 9.2 ml min^{-1} kg^{-1}. The average value of maximum oxygen uptake (VO_{2max}) in response to exercise was 58.9 ± 10.1 ml min^{-1} kg^{-1}. Exercise duration before AT was 10:14 ± 02:07 min, after AT −03:26 ± 01:15 min. All the subjects were divided into two groups by the following main criteria: duration of stress testing and value of VO_{2max} (Table 2).

Athletes from the first group were able to perform the stress test for a longer duration (15:54 ± 01:05 min), had longer periods before AT (11:57 ± 01:44 min), and higher value of the VO_{2max} (65.9 ± 9.1 ml min^{-1} kg^{-1}). Whether corresponding values for the second group were significantly lower (12:05 ± 01:10 min, 09:01 ± 01:24 min, 53.8 ± 7.4 ml min^{-1} kg^{-1}, respectively). Thus, the first group includes athletes with a higher fitness level, while the second group includes less fit participants.

Serum parameters

The CK, AST, ALT and GH levels were analyzed in athletes' sera obtained before and after ramp test to standardize the results and to assess the overall physical state of the athletes. It is known that CK, AST and ALT are observed in the serum in the case of damage of cell membranes, in particular membranes of skeletal muscle myocytes.[40] The activity of CK, AST and ALT

Table 1 Determination of saliva biochemical profile by ATR FTIR method

	Total protein/g l^{-1}	sIgA/mg l^{-1}	Cortisol/mmol l^{-1}	α-amylase/U ml^{-1}	Urea/mmol l^{-1}	Phosphate/mmol l^{-1}
RMSECV	0.02	11	2.21	24	0.1	0.5
R^2 (calibration)	0.94	0.86	0.90	0.91	0.96	0.87
Number of PLS vectors	2	4	3	6	8	7
RMSEP	0.04	8	1.50	19	0.1	0.3
R^2 (validation)	0.81	0.83	0.88	0.89	0.95	0.9
Sample data set range (calibration)	0.17–2.10	40–350	5.42–34.74	44–830	2.5–9.0	0.9–7.9
Sample data set range (validation)	0.30–0.96	86–200	9.77–18.87	43–827	3.8–5.6	1.9–6.5

Table 2 Participants characteristics determined at the beginning of the study and after the ramp test[b]

Parameter	Group 1	Group 2
Number of athletes	20	28
Age/years	21.9 ± 5.1	22.2 ± 6.6
Body weight/kg	73.7 ± 5.1	82.6 ± 11.6[a]
Height/cm	179.1 ± 5.4	183.4 ± 7.9
BMI/kg m^{-2}	23.0 ± 1.5	24.5 ± 2.6
VO_{2max}/ml min^{-1} kg^{-1}	65.9 ± 9.1	53.8 ± 7.4[a]
Exercise duration/min	15:54 ± 01:05	12:05 ± 01:10[a]
Exercise duration after AT/min	03:58 ± 01:16	03:04 ± 01:06[a]

[a] $-p < 0.05$, significantly different from group 1. [b] Values are mean ± SD. BMI – body mass index, VO_{2max} – maximal oxygen uptake, AT – anaerobic threshold.

before and after the exercise testing was within the normal range, indicating that there were no damage of membranes of myocytes and hepatocytes. The significant increase in the blood GH concentration after the ramp test indicates that the athletes have experienced a strong physiological stress.[38]

Salivary parameters

The saliva composition depends on the saliva collection method, as well as on the nature and duration of salivation stimulation.[1,2,27] To get the correct experimental data, we used a standardized system and method for saliva sample collection. For the same reason, all the tests were performed in the morning, since concentrations of several substances in saliva (such as sIgA, α-amylase, cortisol) are known to have the lowest circadian fluctuations at this time of day.[15,16,27,41]

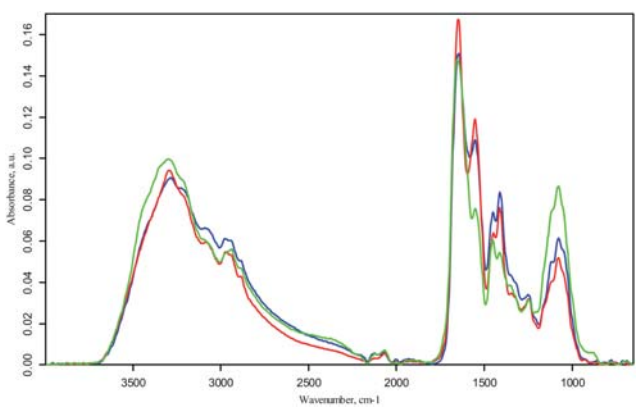


Fig. 6 A series of saliva spectra in mid-infrared region 4000–800 cm^{-1} before exercise (red line), after exercise (green line) and after the recovery (blue line).

The spectra from saliva samples taken before and after the ramp test and after 30 min of recovery, recorded in the mid-IR region, are significantly different (Fig. 6). Spectral analysis of saliva samples shows that short-term high-intensity exercise (ramp test) leads to changes in the saliva composition. The absorption bands which are specific for the samples containing the protein components with wave numbers 1648 and 1553 cm^{-1} are not only shifted, but also have different peak intensities. Apparently, spectrum "before" and "after" exercise are characterized by the increasing ratio between the area of the absorption band at 1648 cm^{-1} and the area of the peak at 1553 cm^{-1}. Significant changes in the spectra of saliva are also observed in the region of 1450–1412 cm^{-1} (Fig. 6).

The analysis of the first and the second derivatives of saliva spectra discover the characteristic spectral regions, which most clearly reflect the changes in saliva composition occurring during the ramp test (Table 3).

Total protein

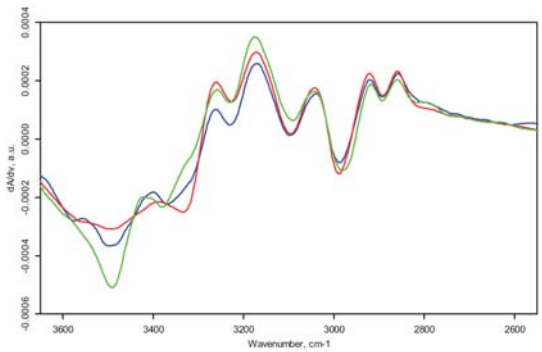
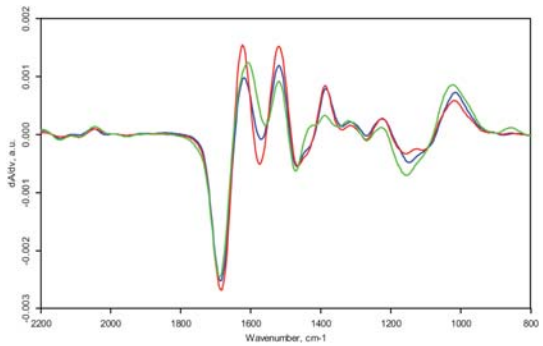
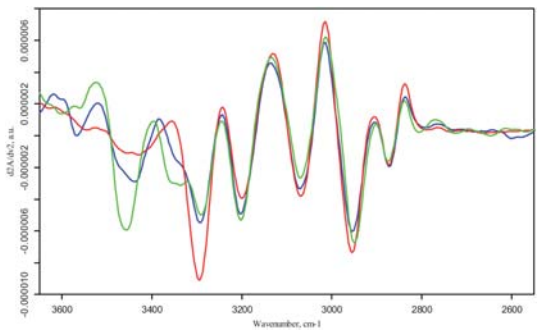
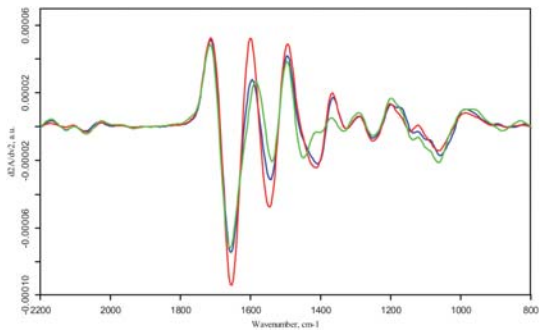
The total protein level in saliva increased after the ramp test from 0.48 ± 0.21 g l^{-1} to 1.31 ± 0.37 g l^{-1} for all athletes. After recovery for 30 min the total protein content in saliva seems to have a tendency to return to its basal level (0.75 ± 0.28 g l^{-1}), that agrees with the previously published results.[4,13,14,19] Saliva secretion occurs mainly due to the impact of adrenergic mediators to acinar cells of salivary glands. So, a rise in sympathetic activity during a physical activity causes the increase in saliva protein level. In addition, a high protein level after the physical stress may be promoted by the increased β-sympathetic activity in the salivary glands.[3,4,14]

sIgA

SIgA plays an important role in the first line of organism defense against pathogens entered the oral cavity.[6,9,12] The basal concentration of sIgA for all athletes was 214 ± 93 mg l^{-1} thus being within the normal limits.

We observed no changes in sIgA concentration after ramp test, that is consistent with the results obtained in other types of stress testing.[7] However, it is known that the concentration of sIgA is affected by the rate of saliva secretion, which decreases under the physical activity.[10] In order to accommodate the change in the rate of saliva secretion caused by stress, as well as water evaporation due to breathing,[42] the concentration of sIgA was normalized to the saliva total protein level, similar so that

Table 3 Pre-processed FTIR saliva spectra: 1st and 2nd derivatives. Spectral details of the two regions indicate different changes in saliva obtaining before (red line) and after (green line) the physical stress, and after the recovery (blue line)

	3650–2550 cm^{-1}	2200–800 cm^{-1}
1st derivative, Savitzky-Golay smoothing filter (9 points)		
2nd derivative, Savitzky-Golay smoothing filter (17 points)		

described in Ref. 11 and 43. Ramp test leads to a significant decrease the sIgA/total protein ratio for all athletes (Fig. 7) that is consistent with the results obtained earlier for other types of stress testing.[6,10,13] This biomarker response to the exercise testing, as noted in the literature,[10] does not depend on functional training of the subject. The two groups of sportsmen do not differ by the changes sIgA/protein ratio (Table 4).

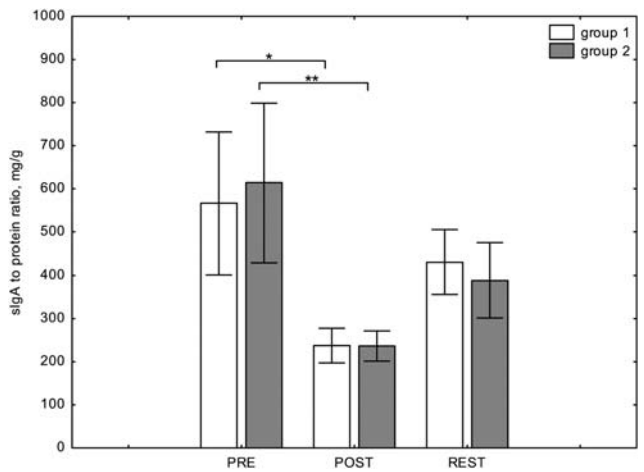


Fig. 7 sIgA/protein ratio in two groups of athletes before (PRE) and after (POST) exercise testing, after the rest (REST) (mean ± SD, * $-p <$ 0.05, significantly different from PRE, ** $-p <$ 0.005, significantly different from PRE).

It should be noted that among 48 participants there are athletes (about 20%), in the saliva of which the sIgA/protein ratio at the 30 min of recovery after ramp test was significantly lower than the basal concentration (3.3 times). The rate and the way of sIgA recovery depend on the type and duration of exercise.[9] In our case the ramp test not only caused a decrease of sIgA/protein ratio, but also helped to identify a group of athletes with slow recovery of sIgA to basal level, correlating with increased risk of respiratory diseases.[6,12]

Cortisol

The exercise test used in this work also had an impact on the level of the other critical saliva biomarker—cortisol. It is known that cortisol in saliva exists in the free form[24] and its concentration does not depend on the saliva secretion rate,[27,28] so its level correlates with that in serum.[22,28] Ramp test leads to a significant increase in saliva cortisol concentration, similarly to that described in[21,23,26,44] for other types of stress testing (Table 4). Physical activity also leads to an increased activity of the PNS and HPA axis, which is responsible for the maintenance of the homeostatic balance in the body.[45] The HPA axis activation leads to an increase in the synthesis cortisol releasing hormone and arginine vasopressin in hypothalamus, which then stimulate the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH causes the adrenal synthesis of cortisol.[27] As a result of ramp test, the observed cortisol level changes in saliva are different for higher and less fit groups of athletes (Table 4, Fig. 8), while the basal cortisol levels are similar for two groups,

Table 4 Concentrations of salivary total protein, sIgA, cortisol, α-amylase, urea and phosphate, and sIgA/protein ratio[e]

	Group 1	Group 2
Total protein/g l^{-1}		
PRE	0.51 ± 0.21	0.45 ± 0.20
POST	1.33 ± 0.49[a]	1.29 ± 0.25[a]
REST	0.72 ± 0.25	0.78 ± 0.31
sIgA absolute concentration/mg l^{-1}		
PRE	227 ± 96	202 ± 89
POST	315 ± 111	309 ± 120
REST	290 ± 105	284 ± 109
sIgA to protein ratio/mg g^{-1}		
PRE	491 ± 242	614 ± 189
POST	237 ± 62[a]	236 ± 50[b]
REST	430 ± 74	388 ± 91
Cortisol/mmol l^{-1}		
PRE	16.8 ± 2.3	14.6 ± 1.9
POST	22.8 ± 4.1[a]	16.7 ± 2.1[c]
REST	32.6 ± 5.0[b]	17.1 ± 2.9[d]
α-amylase/U ml^{-1}		
PRE	141 ± 27	82 ± 25[c]
POST	514 ± 105[a]	270 ± 96[ac]
REST	294 ± 70	126 ± 49[c]
Urea/mmol l^{-1}		
PRE	4.7 ± 1.1	4.5 ± 1.0
POST	6.3 ± 1.9[a]	6.9 ± 2.1[a]
REST	6.5 ± 1.7	6.2 ± 2.2
Phosphate/mmol l^{-1}		
PRE	1.9 ± 0.4	1.5 ± 0.6
POST	2.2 ± 1.3	2.0 ± 1.1
REST	2.3 ± 0.8[a]	2.3 ± 0.9[a]

[a] – $p < 0.05$, significantly different from basal level [b] $-p < 0.005$, significantly different from basal level. [c] $-p < 0.05$, significantly different from group 1. [d] $-p < 0.005$, significantly different from group 1. [e] Values are mean ± SD.

and varies from 12.7 to 19.1 mmol l^{-1}. After the ramp test the saliva cortisol level in the group of more enduring athletes is significantly higher than that for the less fit athletes (Fig. 8). After 30 min of recovery concentration of cortisol continues to increase, as well as difference between two groups (Fig. 8).

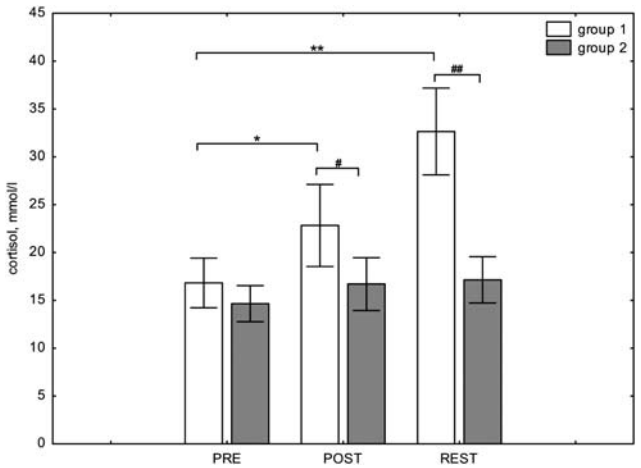


Fig. 8 Cortisol levels in two groups of athletes before (PRE) and after (POST) exercise testing, after the rest (REST) (mean ± SD, * $-p < 0.05$, significantly different from basal level, ** $-p < 0.005$, significantly different from basal level, # $-p < 0.05$, significantly different from group 1, ## $-p < 0.005$, significantly different from group 1).

Previously it was noted that the amount of free active cortisol and its changes may be related to adaptive changes[21,46] and the degree of the athlete's fitness.[21]

α-amylase

The basal activity of α-amylase of 111 ± 26 U ml^{-1} lies within the physiological norm. Ramp test causes an increase in α-amylase activity (Table 4), which is consistent with the data described in the literature[14,16–18,20] obtained during the influence of other types of physical activity. The change in α-amylase activity does not depend on the saliva secretion rate[15] and may characterize the SNS-status of the athletes at stress.[15,16,18,21,24]

It should be noted that the dynamics of α-amylase activity for two groups of athletes are different (Table 4). In the group of more enduring athletes the basal level and changes in α-amylase activity during physical exertion is high that indicates a greater activation of SNS. It should be emphasized that in the literature is little information about the specificity of α-amylase activity change in response to physical stress and recent studies are aimed the identification of individual differences in α-amylase activity response to stressful conditions related to health status and behavior.[45] The nature of α-amylase activity changes is concerned with the individual properties of the human organism, age, gender, and functional status.[16,17,20] The ramp test identified the different individual α-amylase response for two groups of sportsmen, that is consistent with the data obtained in,[20] where the response of α-amylase was monitored in the mode of competition.

We have found also a significant change of urea level in saliva after the ramp test. The phosphate concentration in saliva immediately after the exercise increased insignificantly or did not change whereas after 30 min of recovery its level exceeded the basal concentration with a high degree of reliability (Table 4).

The composition of saliva and its secretion rate are governed by several transmitters and biological messengers in the secretory cells of salivary glands.[19] The change of saliva biochemical profile (protein, electrolyte, hormonal composition) in response to short-term high-intensity exercise, which causes the changes of concentrations of saliva total protein, urea, cortisol, α-amylase, and sIgA/protein ratio, can result from a complex and integrated interaction between different control systems.

Correlations

In recent years there are an increased number of studies on the relationships between the dynamics of change in cortisol, α-amylase and sIgA levels in saliva under different stress conditions.[47–53] Most of these studies are devoted to the effect of only psychological stressors.[16,47,48] The integrated analysis of three biomarkers of major regulatory system in the athletes' saliva in short-term high-intensity exercise was performed for the first time.

Both increased or decreased levels of α-amylase, as well as a significant increase of enzyme activity after exposure to stressful conditions may be associated with health problems, including increased risk of respiratory diseases, fatigue and an

increase in incidence,[48] and the activity of α-amylase is related to the concentration of sIgA in saliva. On the other hand, the ANS has an impact on immune functions, including SNS, the activation of which leads to a rise of the immune cell count (B cells, natural killer cells) and their activity.[49,50] Short-term stress of high intensity (ramp test) leads to significant changes in the concentrations of α-amylase and sIgA, however, we found no correlation between the concentrations of α-amylase and sIgA in saliva before and after stress.

Also, physiological stress leads to increased activity of the HPA axis, resulting in increased levels of glucocorticoids, which can inhibit the function of lymphocytes, macrophages and monocytes.[51] Glucocorticoids can affect the production of cytokines and expression of their receptors; in addition, cytokines such as IL-1, IL-6 are potential activators of HPA axis.[52] The immune response and, especially, the changes in the concentration of sIgA in saliva during physical exertion are influenced by neuronal and endocrine systems,[53] while increasing levels of glucocorticoids are necessary but not sufficient to suppress the immune response to physiological stressors. Ramp test leads to a significant decrease of the sIgA level, while the variation is not correlated with variations of free cortisol in saliva. The observed effect is consistent with the data obtained for other types of stress testing.[7,8]

HPA axis and SNS are physiologically linked,[16,45] basal level and nature of response of α-amylase in saliva may relate[16,20,47,48] or do not relate[16,18,48] to the nature of cortisol response to stress.

Here the relationship between levels of cortisol and α-amylase during physical ramp test was identified. Correlation coefficients between basal levels of cortisol and α-amylase in the group of the less trained athletes, as well as the correlations coefficients between basal activity of α-amylase and the concentration of cortisol after the stress are high ($r = 0.90$ and $r = 0.92$, respectively, $p < 0.05$) (Table 5).

The nature of co-ordinated action of SNS and PNS is the matter of discussions,[47,48] so the relationship between changes in α-amylase activity and cortisol concentration in saliva is of considerable interest.[16,39,47,48]

Type of changes in α-amylase and cortisol levels caused by ramp test differs. Both saliva analyte levels increased after exposure to stress, however, α-amylase reaches the peak of activity and recovery to the baseline faster than cortisol. Kinetic differences may be explained by the fact that α-amylase activity is controlled by SNS, which responds quicker to the stress than PNS which controls the cortisol level. Moreover, the differences in the ways of α-amylase and cortisol appearance in saliva play an important role; α-amylase is released from the salivary glands directly into the oral cavity,[48] whereas cortisol level in serum continue to increase even 10–15 min after the end of the stress stimulation, and only then its level started to increases in the saliva. In addition, α-amylase is a more sensitive biomarker to stress and its activity increases in response to stress more often than the concentration of cortisol.[20,47]

It could be noted, that differences in synthesis, kinetic profile and sensitivity to physical stress, the correlation between salivary cortisol level and α-amylase activity after stress and after recovery in the group of less-trained athletes may be explained by the concerted action of PNS and SNS.

Table 5 Correlation coefficients between cortisol and α-amylase levels in saliva for two groups of athletes before (PRE) and after (POST) exercise, after the recovery (REST)

	Group 1			Group 2		
	α-amylase PRE	α-amylase POST	α-amylase REST	α-amylase PRE	α-amylase POST	α-amylase REST
cortisol PRE	0.30	0.34	0.29	0.90[a]	0.69[a]	0.60
cortisol POST	0.07	−0.09	−0.25	0.92[a]	0.75[a]	0.66[a]
cortisol REST	−0.08	−0.01	−0.08	−0.83[a]	−0.78[a]	−0.56

[a] $-p < 0.05$.

Conclusions

Here, we presented a new, rational and convenient approach to saliva biochemical profile analysis using ATR FTIR spectroscopy. This mid-infrared FTIR spectroscopy-based method opens a new level of non-invasive out of the laboratory diagnostics. The method makes it possible to determine the concentrations of the most important saliva substances without special sample preparation and reagents, from the minima sample volume and (almost) immediately after the sample collection. Saliva total protein, cortisol, α-amylase, sIgA, urea and phosphate concentration changes allow us to characterize the organism state regardless to age, physical fitness and stress level. The patterns of cortisol, α-amylase and secretory immunoglobulin A level changes during short-term high-intensity exercises could be used for the real-time checking of response to stress. This will be the next step in the understanding of the regulatory systems working and adaptive reorganization.

Acknowledgements

We warmly thank all the athletes for their patience and participation in this study. We also acknowledge Akimov E.B. for help with exercise testing; Maltseva D.V., Sakharov D.A. for time and commitment given to this study, and Davydov I.I. for taking part in data analysis. The financial support from the Federal Ministry of Education and Research, Germany (project DLR number RUS 08/A13) and the Federal target Program "Investigation and Development in Priority Fields of Scientific and Technological Complex Development of Russia in 2007–2012" (Government contract No. 02.522.11.2015) is gratefully acknowledged.

References

1 S. P. Humphrey and R. T. Williamson, *J. Prosthet. Dent.*, 2001, **85**, 162–169.
2 L. H. Schneyer, J. A. Young and C. A. Schneyer, *Physiol. Rev.*, 1972, **52**, 720–777.
3 N. Emmelin, *J. Dent. Res.*, 1987, **66**, 509–517.
4 J. L. Chicharro, A. Lucia, M. Perez, A. F. Vaquero and R. Urena, *Sports Med.*, 1998, **26**, 17–27.
5 C. F. Streckfus and L. R. Bigler, *Oral Dis.*, 2002, **8**, 69–76.
6 M. Gleeson, W. A. McDonald, D. B. Pyne, R. L. Clancy, A. W. Cripps, J. L. Francis and P. A. Fricker, *Int. J. Sports Med.*, 2000, **21**, 302–307.
7 G. D. Tharp, *Eur. J. Appl. Physiol.*, 1991, **63**, 312–314.

8 S. L. McDowell, K. Chaloa, T. J. Housh, G. D. Tharp and G. O. Johnson, *Eur. J. Appl. Physiol.*, 1991, **63**, 108–111.
9 M. Gleeson, W. A. McDonald, A. W. Cripps, D. B. Pyne, R. L. Clancy, P. A. Fricker and J. H. Wlodarczyk, *Adv. Exp. Med. Biol.*, 1995, **371A**, 571–574.
10 L. T. MacKinnon and D. G. Jenkins, *Med. Sci. Sports Exerc.*, 1993, **25**, 678–683.
11 D. C. Nieman, D. A. Henson, O. R. Fagoaga, A. C. Utter, D. M. Vinci, J. M. Davis and S. L. Nehlsen-Cannarella, *Int. J. Sports Med.*, 2002, **23**, 69–75.
12 A. M. Novas, D. G. Rowbottom and D. G. Jenkins, *Int. J. Sports Med.*, 2003, **24**, 223–229.
13 A. K. Blannin, P. J. Robson, N. P. Walsh, A. M. Clark, L. Glennon and M. Gleeson, *Int. J. Sports Med.*, 1998, **19**, 547–552.
14 N. P. Walsh, A. K. Blannin, A. M. Clark, L. Cook, P. J. Robson and M. Gleeson, *J. Sports Sci.*, 1999, **17**, 129–134.
15 N. Rohleder, U. M. Nater, J. M. Wolf, U. Ehlert and C. Kirschbaum, *Ann. N. Y. Acad. Sci.*, 2004, **1032**, 258–263.
16 U. M. Nater, R. La Marca, L. Florin, A. Moses, W. Langhans, M. M. Koller and U. Ehlert, *Psychoneuroendocrinology*, 2006, **31**, 49–58.
17 U. M. Nater and N. Rohleder, *Psychoneuroendocrinology*, 2009, **34**, 486–496.
18 R. T. Chatterton, K. M. Vogelsong, Y. C. Lu, A. B. Ellman and G. A. Hudgens, *Clinical Physiology*, 1996, **16**, 433–448.
19 G. Ljungberg, T. Ericson, B. Ekblom and D. Birkhed, *Scand. J. Med. Sci. Sports*, 1997, **7**, 214–219.
20 K. T. Kivlighan and D. A. Granger, *Psychoneuroendocrinology*, 2006, **31**, 703–714.
21 B. T. Crewther, T. Lowe, R. P. Weatherby, N. Gill and J. Keogh, *J. Strength. Cond. Res.*, 2009, **23**, 2046–2053.
22 R. Stupnicki and Z. Obminski, *Eur. J. Appl. Physiol.*, 1992, **65**, 546–549.
23 D. E. Jacks, J. Sowash, J. Anning, T. McGloughlin and F. Andres, *The Journal of Strength and Conditioning Research*, 2002, **16**, 286–289.
24 D. H. Hellhammer, S. Wüst and B. M. Kudielka, *Psychoneuroendocrinology*, 2009, **34**, 163–171.
25 B. M. Kudielka, D. H. Hellhammer and S. Wüst, *Psychoneuroendocrinology*, 2009, **34**, 2–18.
26 P. Passelergue and G. Lac, *Int. J. Sports Med.*, 1999, **20**, 109–113.
27 S. L. King and K. M. Hegadoren, *Biol. Res. Nursing*, 2002, **4**, 92–103.
28 R. F. Vining, R. A. McGinley, J. J. Maksvytis and K. Y. Ho, *Ann. Clin. Biochem.*, 1983, **20**, 329–335.
29 H. Neubert, J. Gale and D. Muirhead, *Clin. Chem.*, 2010, in press.
30 C. E. Christersson, L. Lindh and T. Arnebrant, *Eur. J. Oral Sci.*, 2000, **108**, 418–425.
31 C. P. Schultz, M. K. Ahmed, C. Dawes and H. H. Mantsch, *Anal. Biochem.*, 1996, **240**, 7–12.
32 C. Petibois, K. Gionnet, M. Goncalves, A. Perromat, M. Moenner and G. Deleris, *Analyst*, 2006, **131**, 640–647.
33 D. I. Ellis and R. Goodacre, *Analyst*, 2006, **131**, 875–885.
34 G. Hosafci, O. Klein, G. Oremek and W. Mantele, *Anal. Bioanal. Chem.*, 2007, **387**, 1815–1822.
35 E. Diessel, P. Kamphaus, K. Grothe, R. Kurte, U. Damm and H. M. Heise, *Appl. Spectrosc.*, 2005, **59**, 442–451.
36 L. Benezzeddine-Boussaidi, G. Cazorla and A. M. Melin, *Clin. Chem. Lab. Med.*, 2009, **47**, 83–90.
37 D. Haaland and M. E. Thomas, *Anal. Chem.*, 1988, **60**, 1193–1202.
38 D. A. Sakharov, M. Thevis and A. G. Tonevitsky, *Bull. Exp. Biol. Med.*, 2008, **146**, 466–469.
39 D. A. Schneider, S. E. Phillips and S. Stoffolano, *Med. Sci. Sports Exerc.*, 1993, **25**, 1180–1184.
40 G. Lippi, F. Schena, G. L. Salvagno, M. Montagnana, M. Gelati, C. Tarperi, G. Banfi and G. C. Guidi, *Scand. J. Clin. Lab. Invest.*, 2008, **68**, 667–672.
41 J. Strahler, C. Berndt, C. Kirschbaum and N. Rohleder, *Biol. Psychol.*, 2010, **84**, 248–256.
42 N. P. Walsh, S. J. Laing, S. J. Oliver, J. C. Montague, R. Walters and J. L. Bilzon, *Med. Sci. Sports Exercise*, 2004, **36**, 1535–1542.
43 M. L. Tsai, M. H. Ko, C. K. Chang, K. M. Chou and S. H. Fang, *Scand. J. Med. Sci. Sports.*, 2010, in press.
44 Z. A. Rahman, N. Abdullah, R. Singh and W. Sosroseno, *J. Oral Sci.*, 2010, **52**, 133–136.
45 C. Tsigos and G. P. Chrousos, *J. Psychosom. Res.*, 2002, **53**, 865–871.
46 J. P. Ahtiainen, A. Pakarinen, M. Alen, W. J. Kraemer and K. Häkkinen, *Eur. J. Appl. Physiol.*, 2003, **89**, 555–563.
47 E. B. Gordis, D. A. Granger, E. J. Susman and P. K. Trickett, *Psychoneuroendocrinology*, 2006, **31**, 976–987.
48 D. A. Granger, K. T. Kivlighan, M. el-Sheikh, E. B. Gordis and L. R. Stroud, *Ann. N. Y. Acad. Sci.*, 2007, **1098**, 122–144.
49 R. M. Landmann, F. B. Müller, C. Perini, M. Wesp, P. Erne and F. R. Bühler, *Clin. Exp. Immunol.*, 1984, **58**, 127–135.
50 R. J. Benschop, R. Jacobs, B. Sommer, T. H. Schürmeyer, J. R. Raab, R. E. Schmidt and M. Schedlowski, *FASEB J.*, 1996, **10**, 517–524.
51 C. T. Ambrose, *Bacteriol. Rev.*, 1966, **30**, 408–417.
52 A. V. Turnbull and C. L. Rivier, *Physiol. Rev.*, 1999, **79**, 1–71.
53 M. Fleshner, *Int. J. Sports Med.*, 2000, **21**, S14–S9.

