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Characterization of resting *versus* stimulated saliva fingerprints using Middle-Infrared Spectroscopy assisted by Principal Component Analysis

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Abstract

Fourier Transform Mid-Infrared spectroscopy with Attenuated Reflection combined with multivariate data Principal Component Analysis have been applied for the discrimination of 12 couples of resting and stimulated saliva obtained from healthy volunteers. The resting saliva samples were collected before eating, while stimulated saliva, after chewing stimulation with parafin and preserved in Natrium azide 1%, deproteinized by methanol, before analysis. The spectra were compared by identifying the main absorption regions and quantitatively, by measuring the area peaks for each group of saliva samples. The Principal Component Analysis was performed discriminate differences among samples, either based on raw FTIR or second derivative spectra. The region 1000-1700 cm^{-1} corresponded to specific absorptions of saccharides and aminoacids/peptides/proteins where higher intensities for stimulated saliva against resting saliva (samples 1-8) while samples 9-12 showed similar or even decreased intensities after stimulation. In the region 2000 to 2200 cm^{-1}, corresponding to specific absorptions of azides, thiocyanates and lipids, there were noticed higher intensities for stimulated saliva (1-4 and 7) against resting saliva samples, while samples 5,6,8, 9-12 showed similar or even decreased intensities after stimulation.

The stimulated saliva were grouped similarly and clustered in an intermediate region, between positive PC-1 and neutral PC-2 region, being more represented by a stronger PC-2 component, while resting saliva was represented by a positive PC-1.

Therefore, FTMIR spectra are able to discriminate between the composition differences between resting and stimulated saliva samples. Further studies are needed to discriminate better among the characteristics of physiological and pathological saliva samples.

Keywords: *Metabolomics,* Fourier Transform Mid-Infrared-ATR Spectroscopy, stimulated versus nonstimulated saliva, Principal Component Analysis.

1. Introduction

Saliva is a complex biological fluid, "a mirror of the body health" [1,2] easy to collect and of considerable interest as a source of biomarkers, which reflect some blood biomarkers and many specific molecules secreted from the three pairs of major salivary glands (parotid, submandibular and sublingual) as well from minor salivary glands located in the oral cavity.

Saliva originates from crevicular or gingival fluids, derived from the gingival sulcus, desquamated oral epithelial cells and microorganisms, i.e. viruses, fungi, bacteria and

endotoxins [3,4] as well a large number of inorganic electrolytes and organic components [5]. The salivary glands produce 90% of slight acidic (pH 6–7) secretions and 10% fluids from labial, buccal or palatal salivary glands [5,6]. Saliva components may reflect also a pathological condition, by its modified qualitative or quantitative composition [1,2,7,8].

Saliva proteins bind up to 80% of carbohydrates (i.e.,MUC5B mucins), mainly sialic acid, but also aminosugars (N-acetylgalactosamine, N-acetylglucosamin), galactose, mannose, glycolipids (i.e., neutral and sulphated glyceroglucolipids), neutral lipids (i.e. free fatty acids, cholesteryl esters, triglycerides and cholesterol), as well phospholipids (i.e. phosphatidylethanolamine, phosphatidylcholine) [9]. Saliva includes as well proteins like amylase, mucin, lysozyme, IgA, lactoferrin, peroxidase, metalloproteases, glycoproteins and lipoproteins [10]. The main fatty acids, e.g. oleic, stearic and palmitic acids, some organic acids and amino acids, arising from the degradation of proline-rich proteins, were found to be over-represented in stimulated saliva, whereas taurine, and propionate were over-represented in the resting, non-stimulated saliva [9]. Serum free nonpeptide hormones (cortisol, testosterone, estriol, estradiol, progesterone, aldosterone, androstendiol, dihydroandrostendiol, and insulin) can be also found in saliva, as well amino acids like glycine, alanine, serine and proline, representing a good diagnosis tool [11,12].

Recently, the salivary biomolecules were identified by omics' technologies, including genomics, transcriptomics, proteomics and metabolomics [13-15]. The "omics" technology applied to saliva may reflect a complete set of small metabolites found in, from carbohydrate, lipid, protein metabolites (peptides and aminoacids), to free organic acids, amino acids and hormones or signaling molecules. The use of high throughput techniques, such as liquid- or gas- chromatography coupled with mass spectrometry (LC-MS, GC-MS) capillary electrophoresis or NMR allow the fingerprinting of saliva metabolome to be used in translational and clinical applications, including personalized dentistry and medicine, monitoring of oral cancers [16-19]. Saliva is an appropriate diagnostic fluid with interesting perspectives for personalized therapy [20,21]. The metabolic profiling of saliva in patients with primary Sjögren's syndrome was recently reported [22]. By metabolites fingerprint analysis, saliva proved to be an adequate biofluid for chronic periodontitis signature [23]. However, the utility of saliva for diagnostics remains controversial since its quantity and composition is influenced by many factors such as circadian rhythms, gland stimulation, diet, age, gender, and physiological condition [24].

Saliva can be collected without exogenous stimulation (resting saliva) or by stimulation, which is influenced by olfactory stimulus, exposure to light, diurnal and seasonal factors [25,26], as well related to collection and sample processing, use of specific inhibitors or additives before and after collection or storage [25]. Recently, the metabolic profiling of human saliva before and after induced physiological stress by ultra-high performance liquid chromatography-ion mobility-mass spectrometry was performed in saliva of smokers and nonsmokers [27] and by GC-TOF-MS technique [28] there were identified 13 altered metabolites in smokers, such as tyramine, adenosine, and glucose-6-phosphate. Meanwhile such methods are expensive and time consuming and needs extremely sensitive equipments and protocols.[1].

In recent years, much attention was dedicated to the identification protocols of appropriate saliva biomarkers by non-distructive methods, cheaper and easy to perform a specific fingerprint for diagnosis and disease monitoring [10]. Spectroscopic techniques, based on molecular vibrations, especially IR- and Raman- based techniques, applied directly to samples, contain useful information about the functional groups, without preliminary extraction and separation and, even are less are less selective, are cheaper and easy to use. The

mid-infrared (MIR) spectroscopy combined with multivariate data analysis proved to be the most promising technique, due to its good reproducibility, especially when combined with attenuated total reflection (ATR) system, for the determination of clinically relevant constituents of body fluids (glucose, lactate, immunoglobulins) or thiocyanate [29-36].

Recently, also diabetes-related molecular signatures were obtained by IR spectra of human saliva [37]. Our previous investigations proved the utility of such technique, in combination with chemometrics, as useful metabolomics tools to identify biomarkers, as well as for the determination of food and beverages authenticity [38].

The aim of this study was to apply Fourier Transform Mid-Infrared-ATR spectroscopy to evaluate comparatively the fingerprint of patients' saliva, in order to identify possible biomarkers, which discriminate among resting, non-stimulated saliva *versus* stimulated saliva (as a stress indicator). As reported previously [39], this kind of biochemical monitoring for physiological stress in saliva spectroscopy is a valuable diagnosis tool for pathological conditions. One key-condition to identify biomarkers by relevant discriminations among groups was the use of an appropriate biostatistical analysis [40]. Such studies represent a preliminary step for the identification of pathological biomarkers revealed by saliva fingerprinting, such as Sjogren syndrome or parodonthosis.

2. Material and Methods

2.1. Collection of saliva

A Number of 12 volunteers were randomly selected from patients (8 females/4 males) coming to Prevention Department of the University of Medicine and Pharmacy „Iuliu Hatieganu" in Cluj-Napoca (from April to May 2013). The study was approved by the university Ethics Committe, the subjects being informed about the aims of the study and gave their written consent. The inclusion criterions were respected, the mean age of patients was 23.1 years. The exclusion criteria was chronic deseases, periodontic desease and use of medication. Resting, non-stimulated (NSS) saliva samples were collected from each patient, avoiding any chemical (i.e., acids), physical (i.e., pressure, warm, cold), biologic (i.e., taste, chewing), and psychologic (i.e., imagination of a meal) stimulation. The sample collection was made in the morning, before eating, within 60 minutes prior to sample collection. For saliva recovery, alcohol, caffeine, and dairy products were not allowed. The stimulated saliva (SS) was collected after chewing stimulation with parafin. The whole saliva was collected by drooling it into a vial, allowing the saliva to accumulate in the mouth and then expectorate it into a special cup used for saliva testing. Volumes of 1 ml saliva were introduced into Eppendorf vials containing 1 ml Natrium azide 1% , in order to avoid microbial contamination. Both collected NSS and SS samples were homogenized by a vortex mixer, 1 min. and stored at -20ºC before FT-MIR(ATR) analysis.

2.2. Fourier Transform Middle Infrared, FT-MIR (ATR) Spectroscopy

The FT-MIR (ATR) spectra were obtained with a Shimadzu IR Prestige-21 spectrometer including a HATR (Horizontal Attenuated Total Reflection) device and an internal reflection accessory made of Diamond crystals. The saliva samples were placed on the crystal surface and evaporated under Nitrogen, for 2 min, directly on the ZnSe-ATR (Attenuated Total Reflection) crystal. The FT-MIR (ATR) spectra were recorded using 64 scans/sample and the data were processed using Shimadzu IR solution 1.30 software (2005). The first and second derivatives of spectra were also made using the same software.

2.3. Multivariate Data Analysis. Advanced chemometrics was applied to discriminate between different saliva samples, related to the absorption regions, using Unscrambler X 10.1 Software, version 10.1 (CAMO Software AS, Norway).

3. Results and Discussions

3.1 FT-MIR (ATR) spectra fingerprinting of saliva samples and identification of specific functional groups.

Fig. 1 represents comparatively the FT-MIR (ATR) spectra of resting, unstimulated saliva (1-12A) and stimulated saliva (1-12B) collected from the 12 patients. It was selected the interval of 1000 to 2250 cm^{-1}, being more relevant for compare between the two sets of samples.

Table 1. represents the main peaks identified in samples, their characteristic wavenumbers (cm^{-1}) corresponding to streching and bending IR vibrations, as well as the assignement of functional groups which absorb at the specific wavenumbers.

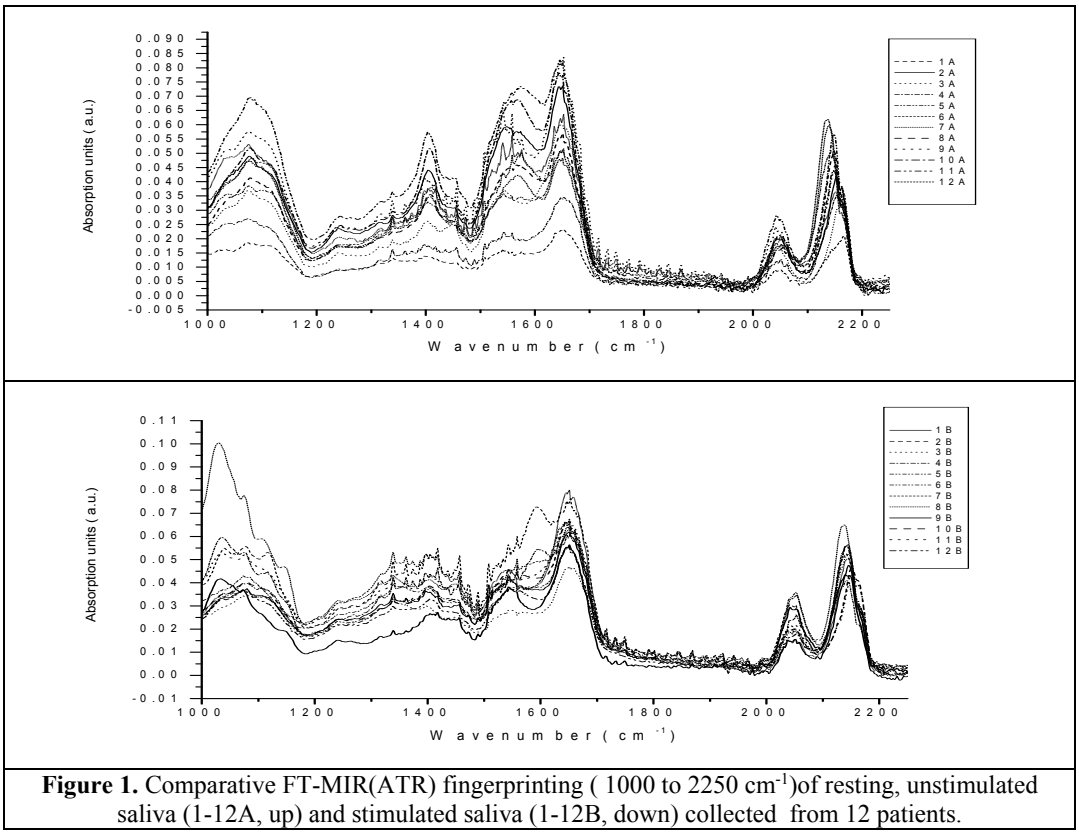


Figure 1. Comparative FT-MIR(ATR) fingerprinting (1000 to 2250 cm^{-1})of resting, unstimulated saliva (1-12A, up) and stimulated saliva (1-12B, down) collected from 12 patients.

Table 1. Main peaks (wavenumbers, **cm^{-1}**) identified in FTMIR spectra of saliva samples, corresponding to streching and/or bending IR vibrations, with the assignement of functional groups which absorb at these wavenumbers. For details see Fig.1.

Absorption peak (cm^{-1})	**Assignment of functional groups**
1029	Mono and oligosaccharides
1075-1078	Oligo, polysaccharides, glycosilated proteins and phosphorus derivatives
1240-1244	Stretching vibrations of C=O groups (Amide I)
1332	Stretching vibration of carboxyl (COO-) group (Amide III)
1397-1410,1452	Stretching vibrations of methyl- (CH_3) and methylene (CH_2) groups
1570,1553-1598	Amide II (N-H) bending vibrations (aminoacids, peptides, proteins)
1645-1650	Amide I (C=O) and III (COO-) stretching vibrations
2043-2049	The azide (NaN_3) bound to the air-oxidized form of citocrom c oxidase, ubiquinol oxidase exhibits an infrared band, characteristic to the binuclear centers
2137-2140	Stretching vibrations of SCN^- , unique to saliva, converted by salivary peroxidases to hypothiocyanate, a local antibacterial agent
2280-2380	Stretching vibrations of CH- links of lipid molecules
3290-3293	Stretching N-H vibrations of primary and secondary amines (NH2, NHR) and O-H vibrations

According to our data presented in Fig.1, we noticed a large distribution of peaks in a first region, from 1025 to 1078 cm^{-1}, corresponding to oligo- and polysaccharides, in both NSS and SS groups, without significant differences. From 1240 to 1450 cm^{-1}, and 1645-1650 cm^{-1}, differences between NSS and SS were noticed, mainly related to stretching and bending vibrations of carboxyl groups found in aminoacids and proteins, as well CH_2 groups inside polymers (bands nominated as amide I and III), with a larger distribution of intensities observed in the SS group (1-12B). From 1553 - 1598 cm^{-1}, the bending vibrations corresponding to band amide II were identified bending vibrations corresponding to amino-derivatives, in larger ranges for NSS samples. The peaks found in the region 2043-2049 2280-2380 showed no significant differences, attributed to azide (NaN_3) bound to the air-oxidized form of citocrom c oxidase, ubiquinol oxidase since this azide derivative was added to saliva samples. The peaks found in the region 2137-2140 cm^{-1}, related to SCN^- , specifically related to saliva showed also, no significant differences between NSS and SS samples. Non-significant minor peaks, for a few NSS samples, were identified between 2280 and 2380 cm^{-1}, representing stretching vibrations of CH- bonds inside lipids. Finally, stretching N-H vibrations of primary and secondary amines (NH2, NHR) and O-H vibrations were identified between 3290 - 3293 cm^{-1}, more intense in SS samples.

As reported by other authors [37] the amide I region is visible in the spectrum of diabetic saliva and it is related to the decreased intensity of intermolecular antiparallel protein β-sheets (1670 cm^{-1}), and altered vibrations of the tyrosine-rich proteins (1517 cm^{-1}). The amide II band at 1550 cm^{-1}, representing N-H bending, became less prominent in diabetic saliva than normal saliva, while the lipid ester band at 1735 cm^{-1} was more intense. The bands located at 1400 and 1582 cm^{-1} are the symmetric and asymmetric carboxyl radical stretching vibrations, originating from lactic acid or protein side chains.

Meanwhile, the 1452 cm^{-1} band corresponds to amino acid CH_2 protein group bending vibration, while the spectral range 950-1180 cm^{-1} originates from sugar moieties, underlying that 1020 cm^{-1} band is attributed to stretch vibration of glycogen while the bands between 1070 -1169 cm^{-1} can be assigned to sugar moieties and phospholipids. To mention also, the SCN- band intensity is associated with glucose concentration in the saliva of the diabetes subjects.

Our results are in good agreement with the later ones, as well to other findings of authors working specifically on physiological samples of saliva [39].

3.2. Multivariate Data Analysis by Principal Component Analysis (PCA)

The raw data using direct FT_MIR (ATR) spectra or first or second derivative spectra were processed by multivariate data analysis (chemometry), mainly using PCA analysis, in order to identify the significant differences among the NSS and SS samples. Fig. 2 shows the specific similarities for samples 1-12A vs 1-12B considering principal components, corresponding to the IR regions, from 650 to 2200 cm^{-1}. Both direct and second-derivative spectra shows significant differences between NSS and SS saliva samples in this region, explained by their different composition in saccharides and aminoacid/peptides/proteins, Lipids are not involved in the discrimination.

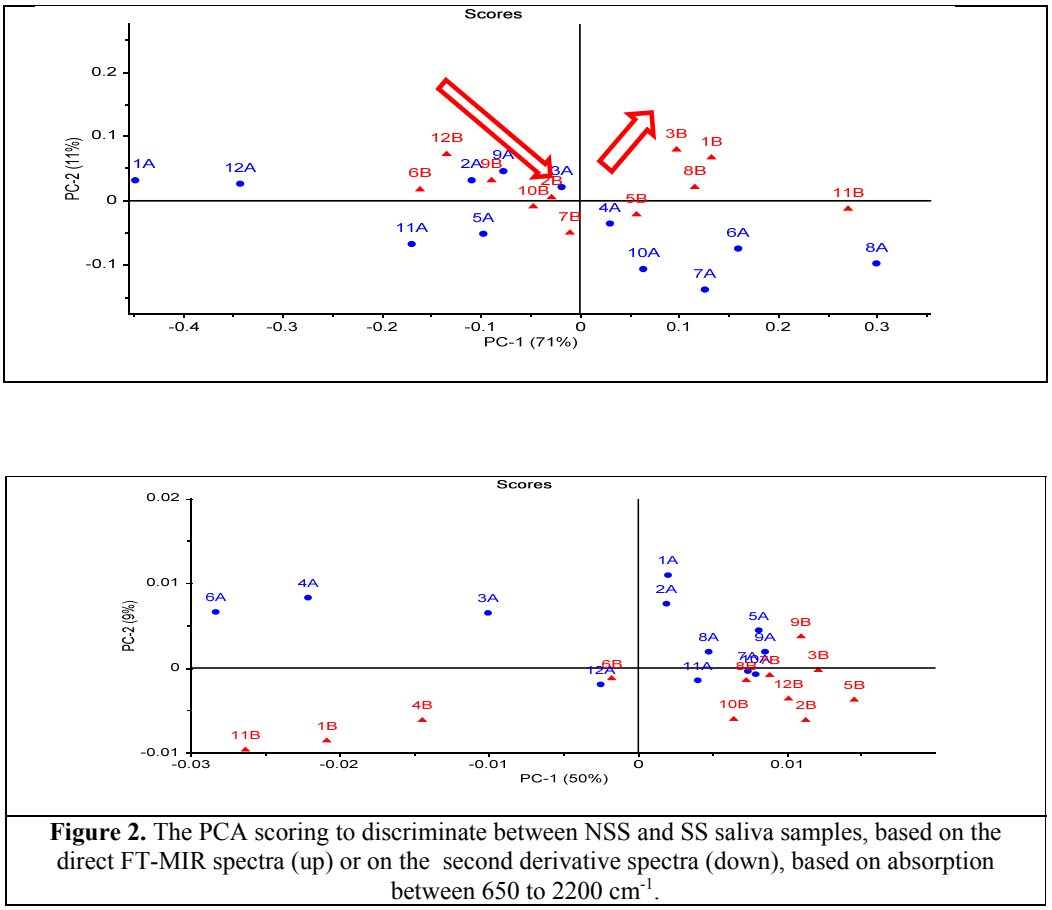


Figure 2. The PCA scoring to discriminate between NSS and SS saliva samples, based on the direct FT-MIR spectra (up) or on the second derivative spectra (down), based on absorption between 650 to 2200 cm^{-1}.

Fig. 3 shows by two different PCA graphics, which discriminate NSS and SS samples, according to their absorptions in the regions 1000-1700 cm^{-1} and 2000 - 2200 cm^{-1}, respectively.

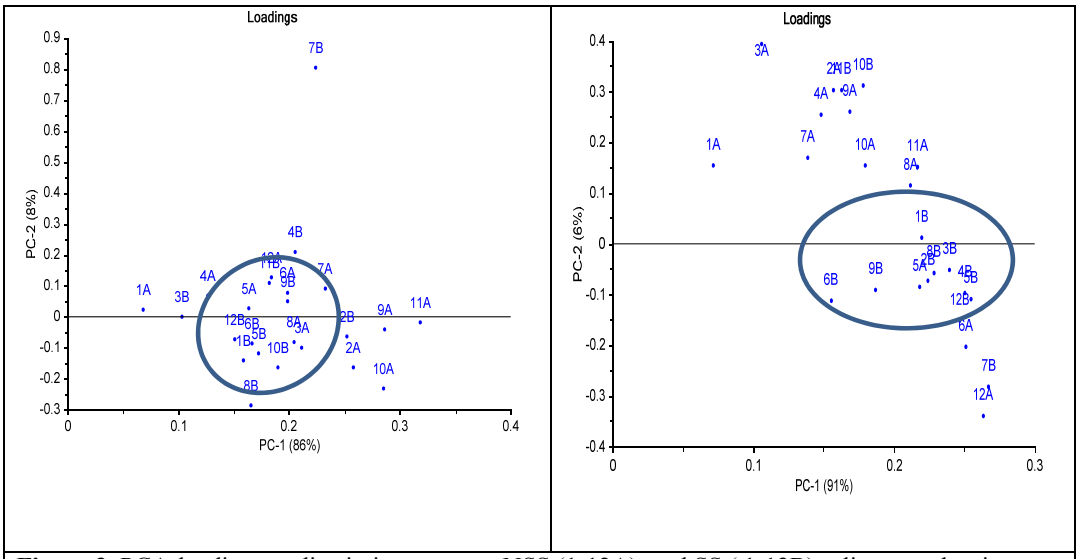
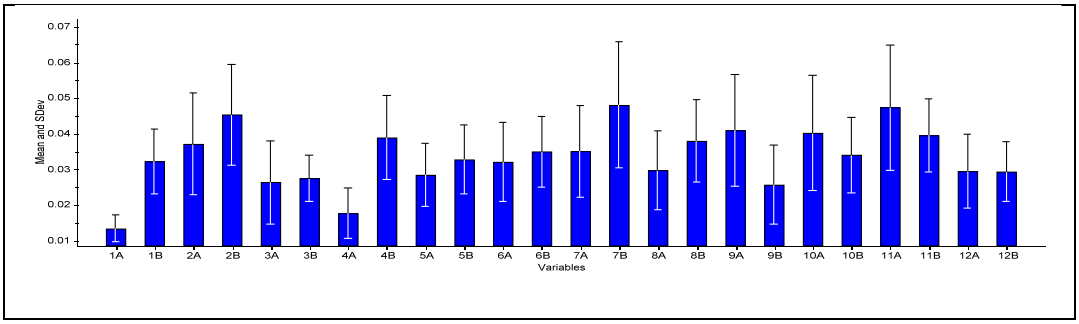


Figure 3. PCA-loadings to discriminate among NSS (1-12A) and SS (1-12B) saliva samples, in two different FT-MIR regions, from left to right (1000-1700 cm^{-1} and 2000-2200 cm^{-1})

According to these data, the SS samples were similar and clustered generally in an intermediate region, between a positive PC-1 (0.15 to 0.25%) and neutral PC-2 region (0.1 to – 0.2%) . The significance of this position suggests that SS samples are more represented by PC-2 component, namely by the minor biomarker of PCA, differently from NSS which are mainly represented by the positive PC-1. Therefore, such spectra, either direct ones or second derivative ones, can discriminate compositional differences between resting and stimulated saliva samples.

Finally, to see the individual differences between the twelve NSS and SS samples in the same regions (1000 - 1700 cm^{-1} and 2000 - 2200 cm^{-1}), we represented their mean and standard deviation (SDev) values of IR absorption units. **Figure 4.**



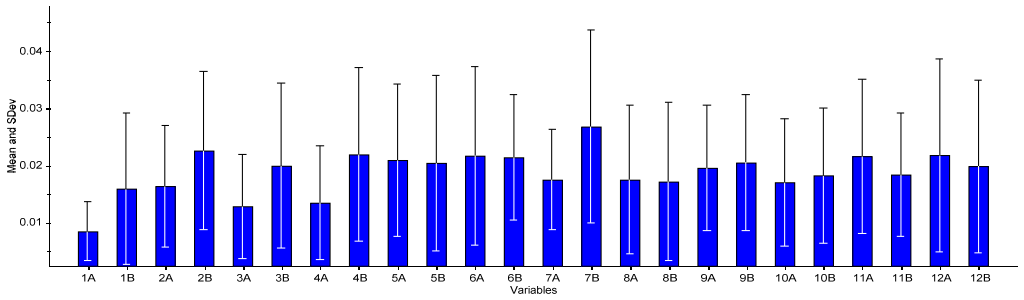


Figure 4. Comparative mean and standard deviation (SDev) values of MIR absorption units, in the regions 1000 to 1700 cm^{-1} (up) and 2000 to 2200 cm^{-1} (down) for resting (1-12A) *vs* stimulated (1-12B) saliva.

The region 1000-1700 cm^{-1} corresponded to specific absorptions of saccharides and aminoacids/peptides/proteins where higher intensities for SS samples (1-8) against NSS samples were noticed, while samples 9-12 showed similar or even decreased intensities after stimulation. Looking to the region 2000 to 2200 cm^{-1} , corresponding to specific absorptions of azides, thiocyanates and lipids, we noticed higher intensities for SS samples (1-4, 7) against NSS samples, while samples 5,6,8, 9-12 showed similar or even decreased intensities after stimulation. Finally, we can conclude that the samples analysed showed high heterogenity and further studies are needed to clasify better the former characteristics of each analysed sample. These results are in good agreement with similar investigations on saliva from professional athlets submitted to professional stress [39].

4. Conclusion

The FT-MIR (ATR) Spectroscopy was applied to characterize and compare 12 different resting and stimulated saliva samples from healthy patients, in order to identify specific biomarkers of discrimination. Considering the whole MIR region, from 650 to 4000 cm^{-1} we were able to identify specifically the main regions and wavelengths ranges of specific vibrational absorptions, corresponding to functional groups and mixtures of molecules found in these samples, e.g. oligo- and polysaccharides, aminoacids/peptides/proteins or other non-proteic derivatives, such as thiocyanates.

By qualitative (absorption in a specific wavelengh region) and quantitative evaluation (intensity of the peak in a specific region) of each molecular category, it was possible, by biostatistics- assisted techniques (PCA) applied on direct FTMIR spectra or second–derivative spectra to discriminate among the molecules which are determinant for the resting *versus* stimulated saliva discrimination.

Therefore, these preliminary investigations on ATR-FT-MIR analysis to supervise the saliva fingerprints proved to be a fast, cheap and efficient, non-destructive tool for screening and identifying specific biomarkers, which are responsible of stress condition. Further studies will investigate the FT-MIR (ATR) fingerprints in normal and pathological conditions.

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