Diagnostic potential of the oral fluid for the observation of people with multiple dental caries by means of FTIR.

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Abstract. The FTIR-spectra of the oral fluid, as well as the calculated mineral-organic, carbon-phosphate, Amide II/Amide I and protein/thiocyanate ratios were compared between subjects with and without multiple caries. The complex analysis of the experimental IR-data showed that the organic-mineral balance in the oral fluid of those with multiple caries shifted towards a reduction in the mineral complexes, accompanied by an increase in the organic component. The most indicative changes in the composition of the oral fluid of those with multiple caries occurred in relation to the number of $-N=C=S$ groups associated with the presence of thiocyanate observed in the IR-spectrum at 2150–1950 cm$^{-1}$, which increased two-fold. The complex data analysis presented has the potential for application as both tissue markers and as a diagnostic approach for the estimation of cariogenesis in mixed saliva samples.

1. Introduction
IR-spectroscopy has been successfully applied for the detection of tissue marker pathologies [1,2]. With the development of spectroscopic express-methods of human saliva analysis, screening of diseases at the molecular level at any early stage is possible [3]. By comparing the changes in the molecular composition of saliva obtained by FTIR at the different stages of pathology in the oral cavity (caries), it is possible to obtain novel data concerning the course of this disease [2]. This information can help not only to specify the mechanisms responsible for caries development, but also to reveal their relationships with the processes of de-mineralisation/mineralisation of the hard dental tissues, as well as to specify saliva proteomics of caries development to elucidate potentially-significant tissue markers. The complex analysis related to the quantitative and qualitative data on the changes in the molecular composition of the oral fluid by FTIR, as presented in this report, has the potential to increase the accuracy of the detection of future carious processes.

2. Materials and methods
Twenty humans participated in this study: 10 men and 10 women (between 22 and 28 years of age). All participants did not take any medicines or drugs, were non-smokers and did not drink spirits. The first group of participants (5 men and 5 women) was physically healthy with caries-free teeth and without gum disorders. The second group (5 men and 5 women) were conditionally healthy but regularly snacking on easily digestible carbohydrates between meals. On examination, each participant...
in this group had teeth with lesion focuses related with primary and secondary caries at the stage corresponding to the 3rd degree according to the ICDAS scale [4] (Figure 1a,b). Participants abstained from food and did not drink for at least 2 hours before sampling of their oral fluid.

Non-stimulated mixed saliva was sampled during daylight, to minimise circadian rhythm, 5 minutes after the preliminary rinsing of the oral cavity with pure water (Figure 1c). The saliva was placed into 15 ml sterile test tubes for subsequent laboratory investigation according to the standard technique [3] as shown in the insert c in Figure 1. After sampling, the test tubes were cooled down to 4°C, then centrifuged before drying in the oven at 36°C to remove excess moisture (Figure 1d). Analysis of the mixed saliva samples was performed with the Vertex-70 spectrometer (Bruker, Germany) using an attachment for attenuated total reflection provided with a diamond prism according to the technique described in [2,5]. In addition, samples were subjected to Infrared Microspectroscopy (IRM) beamline at the Australian Synchrotron, Victoria, Australia using a Hyperion 3000 IR microscope (Bruker, Germany) and high-pressure diamond cell for the analysis of microsamples. IR-spectra were recorded within the range of 4000–500 cm⁻¹. In order to provide the quantitative estimations with the use of FTIR data and to find the difference in the molecular composition of the oral fluid between the group of healthy patients and the group of patients with multiple caries, an approach was applied that was certified in a number of our previous works [2,6]. Spectral data processing (background subtraction, correction for the atmosphere effect, averaging of the spectra and data integration) and analysis were performed using the professional software suite OPUS (version 7.5).

3. Results
The use of FTIR for the analysis qualitatively demonstrated that the molecular composition of the mixed saliva was characterised by a specific set of vibration modes in the IR-spectra, in agreement
with published data on biological fluids [1–3,7–9]. The analysis of the vibrations intensity in the IR-spectrum of the oral fluid obtained from the patients with multiple caries (Figure 2a) showed that it is considerably more intensive than for the patients from the first group. Attention should be focused to six ranges (Figure 2b): 2150–1950 cm\(^{-1}\), 1765 – 1725 cm\(^{-1}\), 1720-1480 cm\(^{-1}\), 1185 – 1140 cm\(^{-1}\), 1150-900 cm\(^{-1}\) and 870-700 cm\(^{-1}\).

**Figure 2(a, b).** (a) the full range FTIR-spectra of saliva sound and multiple caries:, (b) 1,2 - sound enamel, 3 -sound dentin, 4,5 - carious enamel.

The most indicative changes in the composition of the oral fluid of those with multiple caries occurred in relation to the number of \(-\text{N} = \text{C}=\text{S}\) groups associated with the presence of thiocyanate [3] observed in the IR-spectrum at 2150–1950 cm\(^{-1}\) (Figure 1b, area 1). Vibrations in the IR-spectra in the three ranges: 1765–1725 cm\(^{-1}\) - the vibration of >C=O and is associated with the carboxylic group of ester (ester carbonyl); 1185–1140 cm\(^{-1}\) is related to carbohydrates present in the oral fluid and 870–700 cm\(^{-1}\) - C-H, P-O bonds of phosphodiester, esters, as well as other lipids and carbohydrates of saliva were observed only in the samples of those with multiple caries. For all the samples the mathematical estimation of the changes in the molecular composition of saliva can be given on the basis of the calculations and analysis of different ratios between organic and mineral components in the oral fluid sample [2,6].

4. Discussion

The detected features in the IR spectra of the oral fluid of group members with multiple caries confirmed the known data about the change of the molecular composition of pathological processes in the oral cavity.

The significant changes in the region 2150–1950 cm\(^{-1}\) are attributed to the number of \(-\text{N} = \text{C}=\text{S}\) groups associated with the presence of thiocyanate in saliva (Figure 2b, inserts 1). The level of thiocyanates in saliva having an anti-bacterial effect on the bacterial vital products can be enhanced during pathological processes in the human organism [3]. The ratio of protein/thiocyanate, that was calculated from the ratio of the integral intensities of the amide bands (Amide I and Amide II) in the range of 1700-1500 cm\(^{-1}\) to the integral intensity of \(-\text{N} = \text{C}=\text{S}\) vibration bands, arranged at 2150 – 1950 cm\(^{-1}\), associated with thiocyanate demonstrated in fact, a two-fold decrease. It follows from the fact that molecular composition of the mixed saliva taken from the volunteers of the second group the share of chemical bonds inherent to thiocyanate increased in relation to that of proteins.
The vibrations modes that arise only in IR spectra of people with multiple caries of the mode are also related to pathological processes (Figure 2b, area 2,4,6). The IR-band in the range of 1765 – 1725 cm$^{-1}$ (Figure 2b, area 2), according to the data of [1,10,11] corresponds to the vibration of $>$C=O and is associated with the carboxylic group of ester (ester carbonyl). The presence of esters in a hard dental tissue of humans suffering from dental caries was shown in a number of works [1,11,12]. Their authors noted the fact that esters are more often observed in the carious tissue than in the intact one [13]. Keeping it in mind the experimental proof for the possibility to use this kind of information for the analysis of the oral fluid is yet absent. Also, analysis of literature showed that vibration band in the range of 1185 - 1140 cm$^{-1}$ is related to carbohydrates that are present in the oral fluid. Carbohydrates are involved in the composition of saliva mucins covering and lubricating mucous tunic surface in the oral cavity [14], prevent adherence of anaerobic bacteria and their colonization, protect tissues from their physical damage.

Increasing the proportion between organic components in the composition of the oral fluid of people with multiple cavities alters the ratio of Amide II / Amide I. The ratio of integral intensity for the band of Amid II (CN stretching, NH bending vibrations) in the range of 1590 - 1505 cm$^{-1}$ to the integral intensity of the band of Amid I (C=O stretching) in the interval of 1723-1590 cm$^{-1}$ two times as higher for people with multiple caries. This fact is confirmed by calculations of the mineral-organic ratio (the ratio of the integral intensity of the phosphate bands in the IR spectrum (spectral ranges of 1078-900 cm$^{-1}$), to the integral intensity of vibration band 1700 – 1590 cm$^{-1}$ associated with Amide I) and carbon-phosphate ratio - the relation of the integral intensity for vibration bands of C=O and CH$_2$/CH$_3$ bonds localized in the range of 1430 - 1360 cm$^{-1}$ to the integral intensity of phosphate bands in the IR-spectrum within the region of 1078-900 cm$^{-1}$. In all the cases it can be easily seen that in the case of multiple caries in the group of patients a decrease of mineral-organic ratio can be observed meaning a reduce of the share of mineral groups and complexes in saliva composition and/or increase of organic component share in case of the presence of cariogenic bacteria in the mixed saliva [15,16].

Vibrations with the frequencies of 870 cm$^{-1}$, 827 cm$^{-1}$, 772 cm$^{-1}$, 740 cm$^{-1}$ and 700 cm$^{-1}$ in the IR-spectrum of the second group of the patients participating in the examination (patients with multiple caries) are characterized by a high relative intensity as compared with similar bands in the IR-spectrum of participants from the first group. From the analysis of literature data it is clear that these vibration modes are concerned with C-H, P-O bonds of phosphodiesters, esters and as with other lipids as with carbohydrates of saliva [17]. The reason for an increase of intensity in this group of vibrations in the spectrum of the second group of the examination’s participants is an increase of lipids concentration and esters of saliva related to the same group of the components due to the caries development as it was shown in [18,19]. It should be noted that the content of these substances and the correlated molecular complexes relative to the content of proteins in saliva is quite low. Therefore, the change in intensity of vibrations in the IR-spectra within the range of 870 - 700 cm$^{-1}$ as in regard to the changes in the range of vibrations related to proteins is also insignificant.

5. Conclusion

All features of the IR-spectra of the oral fluid suggest that the organic-mineral balance in the oral fluid of subjects with multiple caries is shifted towards a reduction in the content of the mineral groups and complexes as well as an increase in the organic component. The ratio of Amide II/Amide I was integral to these changes in molecular composition, increasing two times as high for the group with multiple caries compared with those without. By means of the calculation it was proved that the ratio of protein/thiocyanate was, in fact, decreased two-fold, indicating that the chemical bonds inherent to thiocyanate increased in relation to the share of proteins in mixed saliva from participants with multiple caries. The complex data analysis in this work shows the potential of FTIR for application as a diagnostic approach for the estimation of cariogenesis in mixed saliva samples.

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