Saliva has garnered a lot of interest as a non-invasive, easy to collect, and biochemical rich sample for attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) based disease diagnosis. Although a large number of studies have explored its potential, the preparation methods used differ greatly. For large scale clinical studies to aid translation into clinics, the collection/processing methodology needs to be standardized. Therefore, in this study, we explored different saliva collection (spitting, method A/cotton soaking, method B) and processing protocols (unprepared, TS; supernatant from the centrifugation, CS; and drying, C) to find which gives the best ATR-FTIR signals. Analysis showed highest proteins, carbohydrates, amino acids, and nucleic acid + proteins/lipids in BTS, BCS, ACS, and BC, respectively. Notably, only BC shows a 1377 cm⁻¹ nucleic acid band that is also uniquely identified in multivariate analysis. We conclude that the collection-processing protocol should be based on a biochemical component that best gives a differential diagnosis.

Keywords ATR-FTIR, vibrational spectroscopy, saliva, disease diagnosis, preparation methods, best protocol

(Received January 30, 2020; Accepted March 25, 2020; Advance Publication Released Online by J-STAGE April 3, 2020)
Table 1: Experimental groups description according to the method of saliva collection and saliva preparation (N = 40, 20 volunteers who donated 2 samples each, 20 for each form of the collection).

| Saliva obtaining method | Type of saliva preparation | Abbreviation |
|-------------------------|---------------------------|--------------|
| A: Collection of saliva by passive drool, directly into sterile vial (20 samples) | Total saliva: no preparation | ATS |
|                         | Centrifuged: supernatant  | ACS          |
|                         | Concentrate by drying     | AC           |
| B: Collection of saliva by means of the cotton roller (20 samples) | Total saliva: no preparation | BTS          |
|                         | Centrifuged: supernatant  | BCS          |
|                         | Concentrate by drying     | BC           |

Declarations of 1975, as revised in 2000, and its protocol was independently reviewed and approved by the Ethics and Human Research Committee of the Universidade do Vale do Paraíba (UNIVAP; protocol number 2.747.437). The experiments were undertaken with the understanding and written consent of each subject and in full accordance with ethical principles, where all volunteers were previously informed and guided concerning the research and procedures, confirming their free and full acceptance by the informed consent form signature.

The study population was composed of 20 healthy volunteers of both genders: 14 females (20 – 36 years; average, 23.0 ± 4.98 years) and 6 males (20 – 33 years; average, 22.5 ± 5.02 years). The average age considering both genders was 22.65 ± 4.88 years.

This age group was chosen to avoid systemic changes peculiar to the senility process alter the spectra and thus the results. The participants were advised to wash their mouth with water prior to saliva collection in order to remove excess residues that may be contained into oral cavity. Saliva samples were then collected directly and non-invasively. Exclusion criteria included the existence of any oral disease or a systemic pathology, alcohol consumption, smokers or systemic medication usage.

Saliva collection and processing methods

Saliva samples were collected in an environment with controlled temperature, without the volunteer having performed oral hygiene prior to the collection and after at least 2 h of a meal. Saliva was collected from each volunteer by the following two methods (Table 1):

Method A (spitting): The volunteers were instructed to let the saliva accumulate in the oral cavity and to deposit it directly, by passive drool, into a sterile container (Eppendorf tubes, 3 mL, Axygen do Brasil, São Paulo, SP, Brazil).

Method B (cotton soaking): The volunteers received a kit containing a sterile cotton roller, a sterile 10-mL syringe and a sterile container (Eppendorf). The participants were instructed to deposit the cotton roller under the tongue, on the floor of the mouth, for 1 min to soak the cotton; subsequently, the cotton roller was placed inside the sterile syringe and 3 mL of its contents deposited into the Eppendorf tubes.

Each collected sample was stored in a refrigerator (2°C) and analyzed within a maximum period of 72 h after collection.

The samples were each sub-divided into three processing groups:

Total saliva (TS): 10 μL of unprocessed saliva was deposited over the germanium crystal by a micro-pipette (Eppendorf Research Plus pipette, Eppendorf AG).

Centrifugation supernatant (CS): 1 mL saliva was centrifuged (1400 rpm, 240 g for 10 min at 40°C) in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany). While being careful not to disturb the precipitate, 10 μL of the supernatant was deposited over the germanium crystal by a micro-pipette (Eppendorf Research Plus pipette, Eppendorf AG).

Concentrated (C): 1 mL of saliva was dried and concentrated (1400 rpm, 240 g for 4 h at 40°C) in a vacuum concentrator (Concentrator 5301, Eppendorf AG). The samples were dried for 4 h based on experience from our standardization studies, which results in a well-dehydrated saliva mass. The dried mass was transferred on to the germanium crystal using a spatula. The amount used was enough to cover the germanium crystal.

**ATR-FTIR spectroscopy analysis**

For TS and CS, 10 μL saliva was passively dried on the germanium crystal of the Spectrum Spotlight 400 FT-IR ATR (Perkin Elmer) for 11 min before recording the spectrum. Based on our previous standardization experiments, 11 min of passive drying gave the best spectrum with minimal interference from water and carbon dioxide bands. For C, the solid mass was deposited on the germanium crystal.

Spectra were recorded in the 450-4000 cm⁻¹ range, at a resolution of 4 cm⁻¹, by averaging 32 scans.

After each analysis, the sample was removed from the crystal with an absorbent paper and cleaned with 70% alcohol for the total removal of the crystal sample, avoiding contamination between samples. Background scans of the equipment were performed to remove possible interferences.

**Data processing and statistical analysis**

The obtained spectra were vector normalized in OPUS software (Bruker Optik GmbH, Ettlingen, Germany), and 900 - 1750 cm⁻¹ was chosen for further analysis. Several different spectral ranges were analyzed, and this range showed the most differences. The spectra in the mentioned range were baseline corrected by subtracting a fifth order. The areas under the curve for different peaks were calculated using a Gaussian fit (Origin 5.0, Microcal Software, Inc., Northampton, MA, USA).

For multivariate analysis, the spectra were pre-processed—second derivatization, spectral range selection (900 - 1750 cm⁻¹), and area normalization, and subjected to principal component analysis (PCA) and principal component-linear discriminant analysis (PC-LDA) followed by leave one out cross validation (LOOVC).

**Results and Discussion**

Figure 1 (a, b) shows the mean spectra of human saliva in the different sample preparation groups. All samples that were collected directly (spit method) are referred to as A (Fig. 1a), while those collected using cotton swab are labelled B (Fig. 1b). An unprocessed saliva sample, that is a total sample, is labelled TS, while the supernatant after centrifugation and concentrated samples (under vacuum) are labelled CS and C, respectively. Thus, a sample labelled AC means a saliva sample collected directly (A) and concentrated by drying (C), whereas ACS refers to as saliva sample collected directly (A) and supernatant after centrifugation (CS).

Figure 1a shows the mean spectra of human saliva for samples processed by different forms after collection by method A. Table 2 shows the respective band assignments. Group C has a higher intensity at bands 1033, 1076 (nuclear material), 1240, 1405, and 1551 cm⁻¹. The CS and TS groups also display all these different bands, though with a low intensity and have a
higher intensity at 1648 cm⁻¹ (proteins). Figure 1b reveals that the mean spectra for saliva samples collected by method B, where group C shows an extra band at 1377 cm⁻¹ (nuclear material), and have a higher intensity of 1033, 1405, and 1450 cm⁻¹ (methylene deformation) bands compared to CS and TS groups.

The analysis by the difference spectra showed similar results (Fig. 2). As can be seen, in method A (Figs. 2a – 2c), group C has an intensity difference when compared to CS (Fig. 2a) and TS groups (Fig. 2b) at 1033 and 1551 cm⁻¹ (protein). In method B (Fig. 2d – 2f), the group C has higher intensities at 1033, 1377 cm⁻¹ (nuclear material), and 1551 cm⁻¹ (protein) than in the CS group (Fig. 2d). Compared to TS, C group has higher intensity at 1033 and 1377 cm⁻¹ (nuclear material), but lower intensities at 1551 and 1650 cm⁻¹ (proteins) (Fig. 2e). The group CS follows the same behavior when compared to the TS group (Fig. 2f). When the collection methods A and B are compared (Figs. 2g – 2i), keeping the processing constant, and little difference is seen in the case of C (Fig. 2g) and CS groups (Fig. 2h). However, the TS group showed a major difference between collection methods A and B where the BTS group has higher intensities at 1400, 1550, and 1650 cm⁻¹ (proteins) (Fig. 2i).

To explore in further details the difference between the methods and processing, the spectra were fitted with Gaussian curves and the concerning areas under specific bands were calculated. The areas were converted into percentages by dividing the area under the curves for all bands for a group and multiplying by 100. Then, wavenumbers were grouped according to the biomolecule assignment (Table 3). The results of fitting suggest the percentage of protein to be highest in BTS, percent carbohydrate highest in BCS, and DNA/phosphate and protein/lipid highest in BC. Methylene deformation is highest and amino acid assignment present only in ACS. A high presence of carbohydrate in ACS and BCS suggest that they separate into the supernatant more than other groups, increasing its percent content in the same. It also appears that ACS has a higher percent of peaks assigned to amino acid than protein.

A multivariate analysis PCA plot of loading factors 2 and 3 (Fig. 3) showed partial separation between clusters of ATS and BTS. BCS cluster more in the region of BTS. ACS spectra are scattered evenly in ATS and BTS space. AC and BC overlap greatly, and both groups are present in space between the ATS and BTS clusters.

We also grouped all spectra irrespective of the collection/processing method, as obtained from males and females separately and performed a PCA. We found no clustering between male and female spectra, suggesting that there may not be any difference in spectra based on gender.

PC-LDA LOOCV (Table 4) shows that all BC spectra are correctly classified. Eighty percent of the AC misclassify as BC. Approximately 50% ACS misclassify with the BCS and BTS. ATS spectra misclassify with all groups, majorly with ACS and BCS. Sixty per cent of BCS spectra misclassify with ACS. BTS spectra misclassify with ACS, ATS and ACS.

Taken together, these results highlight the importance of saliva collection and processing methods for ATR-FTIR analysis. As mentioned earlier, saliva as samples for diagnostic purposes have several advantages, including cost-effectiveness, reproducibility, easy storage and transport, and patient comfort. When combined with ATR-FTIR, diagnosis can be achieved real time, with great accuracy, and based on multi-marker analysis. The last property is very important for cancer diagnosis, as disease heterogeneity means that no single marker
can be correctly used to predict/detect cancer effectively. However, all of these advantages are subject to the ability to acquire high-quality spectra, which may be affected by the sample collection and processing methodologies.

Several studies have used saliva as samples for the diagnosis of diseases; however, the collection and processing methods differ greatly between these reports. Some of the protocols used are: 1. Collection of saliva (3 mL) by drooling and used unprocessed.22 2. Expelling saliva from mouth (1 mL) followed by centrifugation at 10000 rpm for 10 min to collect the supernatant.23 3. Gargle with physiological saline (15 mL) which is collected and processed by repeated centrifugation, discarding supernatent, and adding smaller volumes of water.24 4. Passive drool (10 mL) followed by 2 min centrifugation.

### Table 3

| Absorption peak/cm⁻¹ | Biomolecule/assignment | AC    | ACS   | ATS   | BC    | BCS   | BTS   |
|----------------------|------------------------|-------|-------|-------|-------|-------|-------|
| 1077                 | Phosphate/DNA          | 0.61  | 0.31  | 0.42  | 0.92  | 0.24  | 0.92  |
| 1108                 | Carbohydrates          | 1.03  | 1.26  | 1.01  | 0.78  | 1.47  | 1.10  |
| 1148                 | Carbohydrates          | 0.44  | 0.38  | 0.37  | 0.44  | 0.23  | 0.19  |
| 1240                 | Protein                | 0.36  | 0.11  | 0.06  | 0.12  | 0.07  | 0.21  |
| 1315                 | Protein                | 0.06  | 0.07  | 0.03  | 0.03  | 0.03  | 0.03  |
| 1377                 | Protein                | 0.05  | 0.15  | 0.05  | 0.15  | 0.05  | 0.15  |
| 1405                 | Protein/lipid          | 0.43  | 0.40  | 0.36  | 0.65  | 0.52  | 0.47  |
| 1450                 | Methylene deformation  | 0.32  | 0.38  | 0.37  | 0.37  | 0.26  | 0.24  |
| 1549                 | Protein                | 1.75  | 1.98  | 1.98  | 1.98  | 1.98  | 1.98  |
| 1569                 | Protein                | 1.93  | 1.83  | 1.83  | 1.83  | 1.83  | 1.83  |
| 1589                 | Amino acid             | 2.44  | 2.44  | 2.44  | 2.44  | 2.44  | 2.44  |
| 1638 - 1653          | Protein                | 3.39  | 3.16  | 4.05  | 3.31  | 3.68  | 5.01  |

![Fig. 2](image) Analysis of the difference spectrum: AC-ACS (a), AC-ATS (b), ACS-ATS (c), BC-BCS (d), BC-BTS (e), BCS-BTS (f), AC-BC (g), ACS-BCS (h), ATS-BTS (i).

![Fig. 3](image) PCA of spectra from saliva collected by different methods and processed in different ways.
followed by a. using directly, b. 10000g centrifugation for 10 min, c. saliva soaked in cotton and cotton centrifuged for 10 min at 10000g. 5. Collected by spit method, centrifuged at 4000 rpm for 20 min, and supernatant used. 6. Gargling with water for 15 min, secreted into container, and used directly. 26

From the above, the basic collection methods are broadly collection by spit, although one study mentions the use of cotton. Moreover, most studies use centrifugation as a processing step, followed by use of the supernatant for FTIR. Our previous studies have shown the effectiveness of using completely dehydrated samples for bio-fluid based study. 27 Therefore, we used two collection and three processing methods, as mentioned in the Materials and Methods section. The protocols do not represent every saliva preparation method reported, only the common ones. Our study indicates that based on the collection and processing method used, the spectra may be dominated by proteins, amino acids, carbohydrates, or nucleic acids.

A previous FT-IR study of saliva exosome from oral cancer patients showed a difference in nucleic acids (1072 cm–1), proteins (1543 cm–1), and lipids (2924 and 2854 cm–1) compared to healthy subjects. 28 Raman spectroscopy, a technique complementary to FT-IR, showed a major difference in proteins (specifically Amide III), and to less extent in carbohydrates and amino acids, compared to healthy. 23,26 Raman spectroscopy of saliva from premalignant oral lesions differs from healthy in protein, nucleic acid, and amino acid groups. 22,26 FTIR of saliva from periodontal disease, another major focus of saliva-based diagnostic applications, showed a difference in phosphate bands in 1000 - 1300 cm–1 range, N-H and C-N vibrations in 1500 - 1600 cm–1 range, and C=O vibration in 1600 - 1700 cm–1 range. 29 This underlines the importance of choosing the correct protocol for getting the best results.

It is interesting to note that saliva collected using cotton and completely dehydrated (BC) has a unique 1377 cm–1 DNA band. To verify it is not due to the influence of one of two spectra in the group on the mean spectrum, we performed PCA between the spectra of samples collected by spit and dehydrated (AC) and BC, and found the 1377 cm–1 band in the loading factor. In PCA between samples collected by cotton, and unprocessed (BTS), supernatant (BCS), and BC, again the loading factor had the 1377 cm–1 band. It is possible that this is a contamination in cotton. This suggests that most spectra in the BC group had the band, and it is the reason behind the PCA based separation of groups. More studies are required to exclude the possibility that the band is due to contamination of the cotton used.

Apart from the 1377 cm–1 DNA band, which has not been reported to be different in disease to date, the unprocessed sample shows all bands. An unprocessed sample collected using cotton in the mouth also has a higher intensity and area under the curves for protein peaks. This indicates that the unprocessed sample may be used for saliva diagnostics using FT-IR. It is important to note that unprocessed saliva still has to be passively dried for a few minutes on the FT-IR machine window; itself, and is not truly unprocessed, but has not undergone additional/active processing.

Finally, it is important to discuss the behavior of these spectra when subjected to multivariate analysis (Fig. 1C), since the same is used in the majority of studies to show the efficiency of separating healthy from abnormal based on the spectral pattern, and thus the clinical applicability of the methodology. Such an analysis also considers all spectra, as opposed to the spectral analysis discussed above, which uses average spectra. The multivariate analysis primarily informs on the similarity and dissimilarity between the spectra of groups. In our study, we found that the dried sample collected by the cotton in mouth method shows a 100% correct classification (Table 4). This means that all spectra are distinct and different from all other groups. Dried samples, but collected using spit method, misclassifies 80% with the above, which means there is little difference between dried samples obtained, irrespective of the collection method. Centrifuged samples collected by either method are also distinct and misclassify less (20 and 5% for spit and cotton collection methods, respectively) with unprocessed and not at all with dried samples. This suggests that centrifuged samples share some characteristics with unprocessed ones. Unprocessed samples misclassify greatly (35 and 45% for spit and cotton collection methods, respectively) with centrifuged and less (10 and 0% for spit and cotton collection methods, respectively) with dried samples, suggesting that they share features with centrifuged samples, and dried samples (in the case of the spit method). Thus, the multivariate analysis clearly shows that dried samples have a unique spectral signature, and this may be important for disease classification.

### Conclusions

In this study, we asked two questions: 1. Which method of saliva collection is better—spitting into a tube or placing cotton under tongue? 2. Which processing method gives the most spectral bands—supernatant after centrifugation (10 min) + passive drying (11 min) or complete dehydration (4 h centrifugation under vacuum) compared to minimal processing (11 min passive drying). The results suggest that 1. Both methods give similar spectral bands, except that there is a dominance of nucleic acid band 1377 cm–1 in complete dehydrated cotton collected samples. 2. Complete dehydration shows more bands in both methods, supernatant have dominance of carbohydrates (cotton method) and amino acids ( spit method). Minimally prepared samples show most spectral bands combined with dominance of protein bands.

Therefore, unless there is a specific requirement for sensitive nucleic acid measurement for disease diagnosis, the faster method may be adopted. For specifically exploring carbohydrate-based classification, the supernatant after centrifugation of saliva collected by the cotton method may give better answers, as carbohydrate bands were found to be elevated in spectra of this group. Similarly, the supernatant of samples collected by the spit method after centrifugation may be best for amino acids-based classification. Multivariate analysis suggests a spectral pattern to be most unique in dried saliva samples, irrespective of the collection method. The study thus provides a

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**Table 4** PC-LDA LOOCV (in percentage) of spectra from saliva collected by different methods and processed in different ways

| PC-LDA LOOCV, % | AC | ACS | ATS | BC | BCS | BTS |
|-----------------|----|-----|-----|----|-----|-----|
| AC              | 20 | 0   | 0   | 80 | 0   | 0   |
| ACS             | 0  | 55  | 0   | 25 | 0   | 0   |
| ATS             | 5  | 15  | 45  | 5  | 20  | 10  |
| BC              | 0  | 0   | 100 | 0  | 0   | 0   |
| BCS             | 0  | 60  | 0   | 35 | 5   |
| BTS             | 0  | 15  | 15  | 0  | 30  | 40  |

AC, ACS, ATS collected directly into the sterile vial (spit), and BC, BCS, BTS collected by means of the cotton roller. TS, Total saliva; CS, centrifuged supernatant; C, concentrate by drying.
guide to future studies with respect to saliva-based vibrational spectroscopy. Based on important bands identified in this study, irrespective of processing method used, researchers can identify the best method suited for their analysis, concerning band comparison, spectral curve fitting, or multivariate analysis.

Acknowledgements

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the Breno Pupin scholarship (CAPES PNPD 20132078-330510110009P0), Daniele Riêra Paschotto (CAPES, PROSUC, Financing Code 001) and Tanmoy T. Bhattacharjee (PNPD/CAPES/2016) scholarship.

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