Supplementary Information

Advancing Cerumen Analysis: exploring innovative vibrational spectroscopy techniques with respect their potential as new point-of-care diagnostic tool

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Raman (RS) and SERS

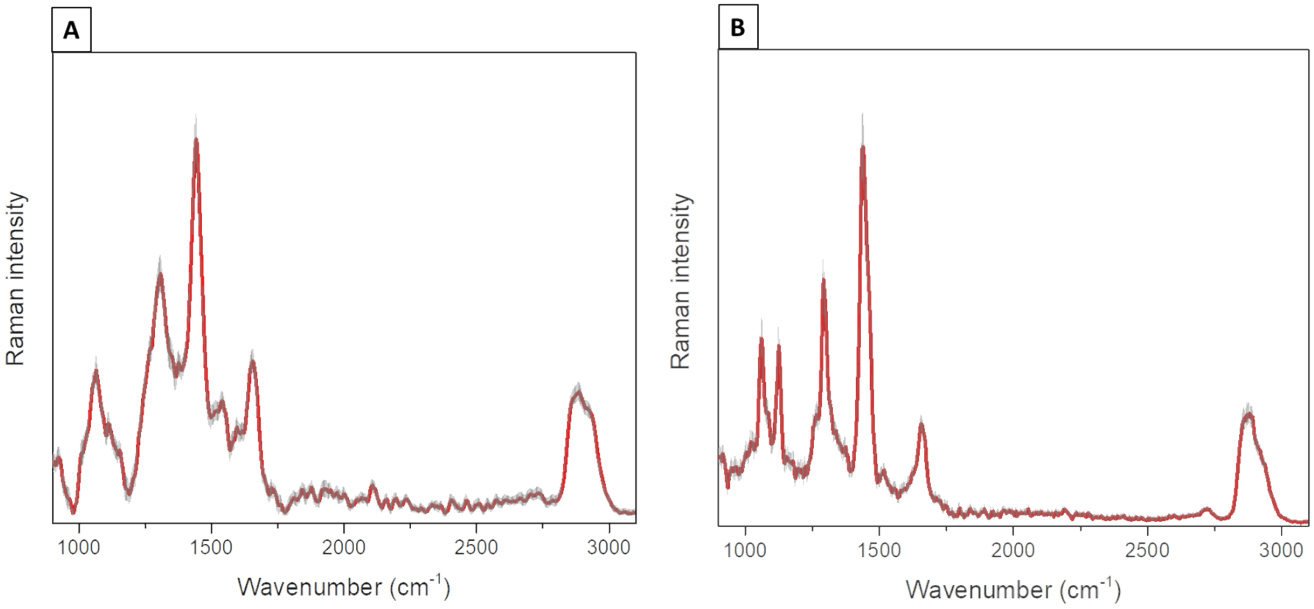

Figure S1. Comparison between semi-solid (A) and liquid (B) background-corrected Raman spectra of cerumen from healthy volunteers. For each sample 50 spectra were acquired, elaborated (as reported in Methods section) and the average spectrum (red line) was plotted, together with the standard deviation of the collected spectra (gray shading).

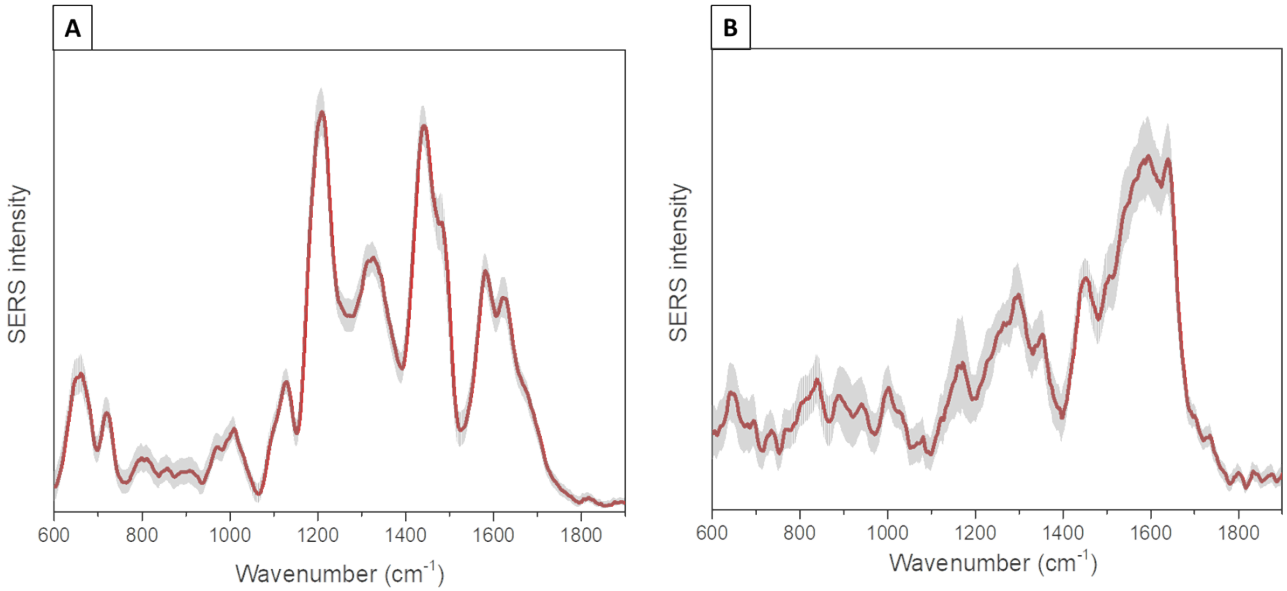

Figure S2. Comparison between semi-solid (A) and liquid (B) background-corrected SERS spectra of cerumen from healthy volunteers. For each sample 150 spectra were acquired, elaborated (as reported in Methods section) and the plots show the mean spectrum of the collected cluster (red line) and the standard deviation of the acquired spectra (gray shading).

Broadband CARS (BCARS)

A home-built broadband CARS spectroscopy platform was used to acquire spectral information on cerumen samples by exploiting coherent anti-Stokes Raman scattering (CARS). The setup, shown in Figure S3, employs a multiplex CARS approach in which a narrowband pump pulse and a broadband Stokes pulse are superimposed on the sample to generate a broadband anti-Stokes signal, which encodes Raman-like spectral information.

Setup description

A femtosecond chirped pulse-amplifying (CPA) ytterbium fiber laser (Active Fiber Systems GmbH) was used as laser source. It can deliver laser pulses of up to 10 µJ pulse energy with a minimum duration of about 360 fs at about 1032 nm. The maximum repetition rate is 19 MHz and the maximum average output power of the air-cooled laser is 10 W. A pulse generator is used to activate the laser's acousto-optic modulator (AOM) at the output for extraction of single or bursts of pulses. By another internal AOM the repetition rate can be varied from 50 kHz to 19 MHz during operation.

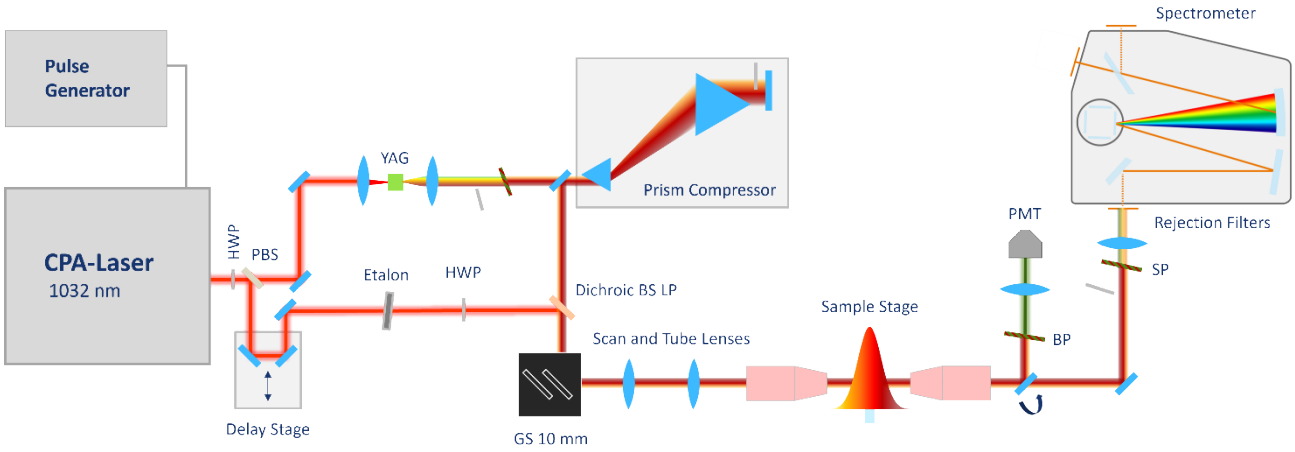

Figure S3. BCARS setup. HWP – half-wave plate, PBS – polarization beam splitter, BS – beam splitter, LP – long pass, SP – short pass, BP – band pass, PMT – photomultiplier tube.

The laser output is split by a polarization beam splitter (PBS) and a half wave plate into two branches for the pump and Stokes pulse, respectively. In the pump path, about 30 % of the original laser power is directed to an etalon (SLS Optics Ltd) and spectrally narrowed to about 0.4 nm corresponding to ~3.8 cm^{-1}. Therefore, the pulse duration of the pump pulse amounts to ~3 ps. The Stokes pulse is generated by focusing the femtosecond pulses on a 10 mm YAG crystal with a 125 mm focal length lens. A 50 mm achromatic lens is collimating the broad stokes pulse after the YAG crystal. A 1050 nm long pass filter filters out the visible components of the supercontinuum. In order to further compress the pulse in the time domain a prism compressor is employed in the stokes path. The spectral bandwidth of the broadband stokes can be set using a mask after the second prism, as depicted in the schematic Figure S3. For the measurements the two-color CARS process was utilized with a set bandwidth of the broadband Stokes pulse of ~ 1100 nm – 1550 nm. This results in a spectral coverage of ~700 cm^{-1} – 3200 cm^{-1} of the CARS spectrum. The temporal overlap is set with a delay stage in the pump path and the beams are recombined in space at a dichroic long pass beam splitter (edge at 1064 nm, Semrock, Di02-R1064-25x36). A second half-wave plate is placed before the beam combiner to return the pump polarization to the same state as the Stokes.

In the BCARS configuration, the galvo scanner (GPS011, Thorlabs) is used as a mirror pair since the scanning is done with a motorized stage (Märzhäuser Wetzlar GmbH). A telescope (magnification of about 0.45) is placed after the galvo scanner to reduce the size of the beam to fit to the pupil of the excitation objective. Two objectives (Mitutoyo 20x NIRAPO/0.4NA) are employed as excitation and collection objective.

The generated BCARS signal is collimated by the collection objective. A 75 mm focal length achromatic lens focuses it on the spectrometer slit. The spectrograph (Kymera 328i Andor, Oxford Instruments) is equipped with a CCD camera (Andor iVAC 316, Oxford Instruments), which is used for detection. The effective spectral resolution that can be achieved in this configuration is <10 cm^{-1}. Before the slit, two 1025nm short pass filters and a 1000 nm short pass filter are used to reject the pump and Stokes wavelengths.

For a fast single channel overview option the galvo scanners are used in combination with a PMT. In order to achieve good contrast in biomedical samples 800 nm bandpass filter with 40 nm bandwidth is placed before the PMT, targeting the intense CH-region of the Raman-active spectrum (2700 – 3100 cm^{-1}) in combination with 950 nm short pass rejection filter. This overview option achieves pixel dwell times of ~3µs and thus frame rates of 1-2 frames per second and is therefore suitable for focusing and field of view selection.

Sample measurements

In preparation for the BCARS measurement, the focus and the selection of the FOV are set with imaging by the Galvo scanners in combination with the PMT. For this purpose, the beam is deflected by a 950 nm dichroic beam splitter onto the PMT directly after the collimation objective subsequent to the sample. The dichroic beam splitter is removed again for the subsequent BCARS measurement.

Broadband CARS spectra of the cerumen droplet, human bulk cerumen and artificial bulk cerumen were acquired with the setup above using an average power of 15 mW for the pump and 2 mW for the Stokes at the sample plane, at a repetition rate of 1 MHz.

To prevent damage on the cerumen samples, the laser focus was scanned on the sample and an image was acquired. An external signal from a DAQ board (NI 6356, National Instruments) synchronized with the stage motion was used to trigger the camera and acquire a spectrum for each pixel in the field of view. The pixel exposure time was set to 16 ms, corresponding to a total dwell time of 20 ms due to the camera readout speed, and the BCARS spectral image consists of 50x50 pixels with a FOV of 50 µm. This results in a measurement time of 50 seconds per frame.

Broadband CARS is not a background free technique: the Raman-like information is encoded in the imaginary part of the resonant susceptibility $X_{3,\,res}$. However, the BCARS signal is proportional to $|\,X_{3,\,res} + X_{3,\,non\ res}\,|^2$, where the $X_{3,\,non\ res}$ term is the non-resonant contribution that arises from the non-resonant four wave mixing process [https://doi.org/10.1002/lpor.201800020]. To retrieve the Raman-like information, most of the computational methods require an a-priori knowledge of the non-resonant background (NRB). Since an exact measurement is not possible, an estimation of the NRB can be obtained by measuring the BCARS spectrum from the glass microscope slide. Therefore, for each sample a glass reference NRB was measured by acquiring 500 spectra from a single point in the glass, with 16 ms exposure time.

Data processing

To remove the NRB, several methods have been developed. Among them, the Kramers-Kronig (KK) phase retrieval algorithm [https://doi.org/10.1364/OL.34.001363], the maximum entropy method (MEM) [https://doi.org/10.1364/OE.14.003622] and deep learning methods [https://doi.org/10.1063/5.0007821] are the most widely used. To process our spectra, we chose to use an algorithm based on the KK phase retrieval method [https://doi.org/10.1002/jrs.4824].

Before processing, the acquired spectral images of the three samples were clustered using K-means clustering to group and average only the spectra of higher intensity.

The KK algorithm was then applied to the average spectrum of each sample using the average BCARS spectrum of the slide as the reference NRB.

The wavenumber calibration was performed by measuring the BCARS spectrum of 4-acetamidophenol and comparing it with the Raman spectrum of the same substance available online (https://www.chem.ualberta.ca/~mccreery/ramanmaterials.html). A third order polynomial fit was applied to the BCARS data for calibration.

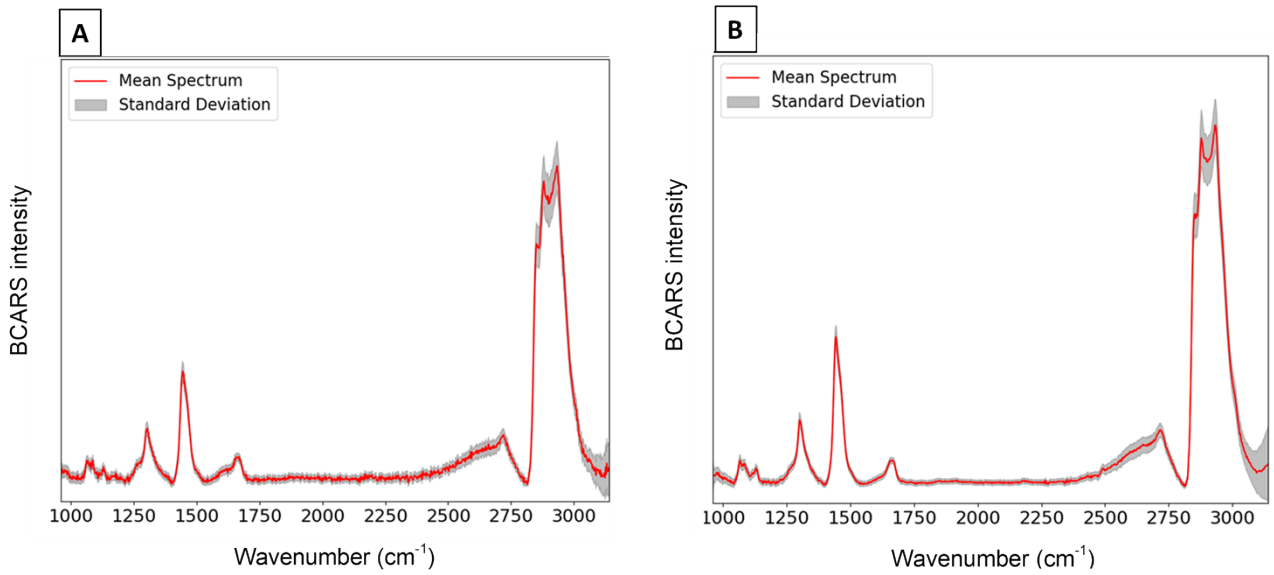

Figure S4. Comparison between semi-solid (A) and liquid (B) BCARS reconstructed spectra of cerumen from healthy volunteers. For each sample a 50x50 pixels image was acquired and K-means clustering was used to divide the image in two clusters. The cluster with the highest intensity was selected. The plots show the mean spectrum of the selected cluster (red line) and the standard deviation of the selected spectra (gray shading).

Stimulated Raman Scattering (SRS)

Stimulated Raman Scattering (SRS) spectra of the three samples were acquired using a commercial multimodal microscopy platform (Stellaris 8, Leica Microsystems GmbH).

Setup description

The microscope is equipped with an SRS detection scheme and enables the acquisition of broadband SRS spectra in a hyperspectral configuration, i.e. the Stokes wavelength is fixed, and the pump wavelength is sequentially scanned. The system features a CARS and SRS excitation laser (picoEmerald, A.P.E. GmbH) that provides a Stokes pulse at about 1031 nm and a tunable pump pulse in the 700-990 nm range at a repetition frequency of 80 MHz. For SRS acquisition, the Stokes pulse is modulated at 20 MHz and a lock-in detection scheme is employed.

Sample measurements

The three samples were measured by acquiring images over a field of view of 1.55 mm x 1.55 mm, with 512 x 512 pixels, 3.16 µs pixel dwell time and using a 10x/0.40NA objective (HC PL APO CS2 DRY) for excitation. Four spectral windows were acquired with widths of 300 cm^{-1} to cover the fingerprint region and the CH stretching region. Within the windows, the pump wavelength was tuned with steps of 0.5 nm. In the 900-1800 cm^{-1} range, the power was set to about 12 mW for the pump and 29 mW for the Stokes. In the CH stretching region, due to high signal intensity, the power was reduced to around 4 mW and 8.5 mW for the pump and Stokes, respectively.

Data processing

K-means clustering was performed on the SRS images to group and average only the spectra with the highest intensity. Being a background-free technique, no further processing, apart for fifth-order polynomial fit baseline, was required to retrieve spectral information from the SRS signal.

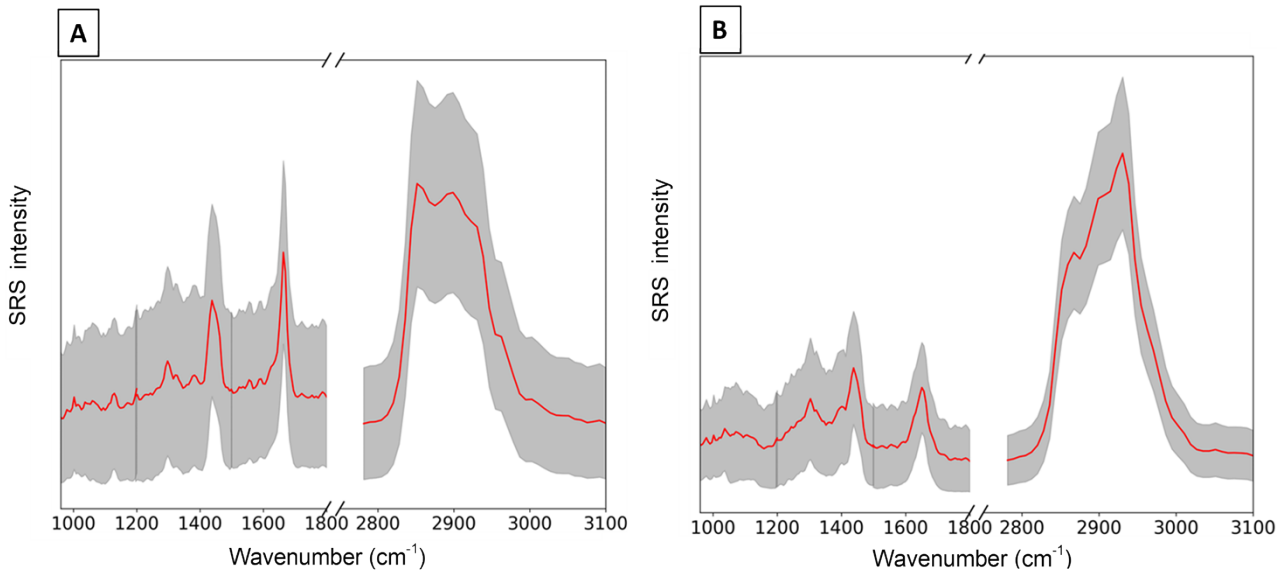

Figure S5. Comparison between semi-solid (A) and liquid (B) SRS spectra of cerumen from healthy volunteers. For each sample a 512x512 pixels image was acquired and K-means clustering was used to divide the image in two clusters. The cluster with the highest intensity was selected. The plots show the mean spectrum of the selected cluster (red line) and the standard deviation of the selected spectra (gray shading). The spectra were acquired consecutively in 4 spectral windows, and K-means clustering was applied individually to each spectral window to optimize the signal-to-noise ratio in all wavenumber intervals.

The higher variability of SRS spectra can be justified by considering the difference in acquisition parameters compared with CARS. Specifically, the field of view of the acquired images is much larger with respect to CARS (1.55x1.55 mm^2 vs. 50x50 μm^2). This results in SRS images being affected by 'low spatial frequency noise,' which is due to the high heterogeneity of the sample surface (especially bulk cerumen) and leads to greater intensity variation over the larger field of view. The selection of the highest intensity spectra via K-means clustering is one method to reduce data variability, as it restricts the analysis to only a subset of pixels, i.e., a smaller region of the sample. However, for such large samples, the variation remains significant. A solution to this problem would be the acquisition of a smaller field of view.
Moreover, the SRS images are also affected by a 'high spatial frequency noise', which increases pixel independence and contributes to data variability. This noise can be attributed to various factors, such as laser power fluctuations within the spatial and spectral scan and low signal detection due to the sample's large thickness and short pixel dwell time. In a transmission technique like SRS, where the detected signal is a $10^{-4/5}$ change in the transmitted beam, using thick samples decreases considerably the signal, reducing the signal-to-noise ratio. Indeed, the standard deviation is observed to be higher in the bulk cerumen sample. In CARS, this effect was mitigated by carefully selecting a region of the sample where the signal intensity was higher, indicating a thinner sample. Moreover, the pixel dwell time was much shorter than CARS, contributing to the lower signal intensity. A higher noise can be also attributed to the low spatial resolution with which we acquired the image. Having a pixel size of 3 µm, which is comparable to the laser spot size, the pixel-to-pixel variability increases, as the pixel signal is not averaged with neighboring pixels. In CARS, the pixel is only 1 µm, so the effect of averaging is higher and helps in the reduction of noise. Due to the high spatial frequency noise, simple intensity normalization does not effectively reduce the standard deviation unless binning of the image is performed to decrease pixel-to-pixel variability.

OPTIR

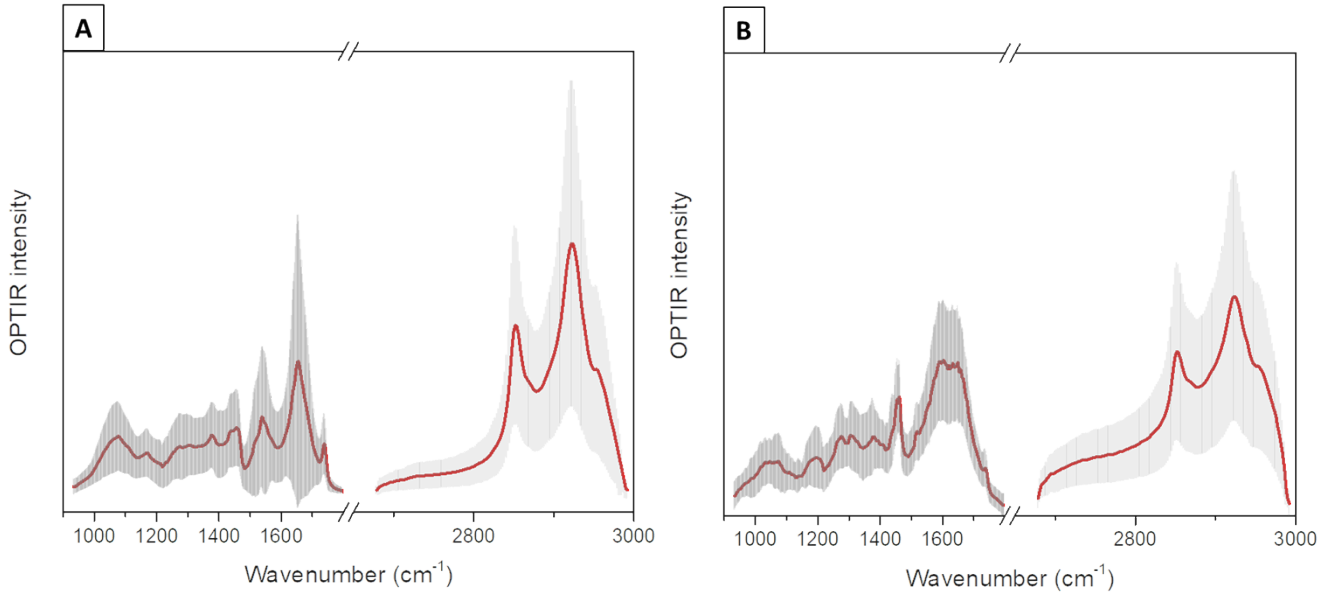

Figure S6. Comparison between semi-solid (A) and liquid (B) OPTIR spectra of cerumen from healthy volunteers. The spectra were acquired in 10 different positions on the sample, elaborated (as reported in Methods section) and the average spectrum (red line) was plotted,together with the standard deviation of the collected spectra (gray shading).