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TITLE:

Resolving Water, Proteins, and Lipids from In Vivo Confocal Raman Spectra of Stratum Corneum through a Chemometric Approach

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KEYWORDS:

in vivo confocal Raman, principal component analysis, multivariate curve resolution, chemometrics, preprocessing, outlier removal

SUMMARY:

Here, we present a protocol for collection of confocal Raman spectra from human subjects in clinical studies combined with chemometric approaches for spectral outlier removal and the subsequent extraction of key features.

ABSTRACT:

Development of this in vivo confocal Raman spectroscopic method enables the direct measurement of water, proteins, and lipids with depth resolution in human subjects. This information is very important for skin-related diseases and characterizing skin care product performance. This protocol illustrates a method for confocal Raman spectra collection and the subsequent analysis of the spectral dataset leveraging chemometrics. The goal of this method is to establish a standard protocol for data collection and provide general guidance for data analysis. Preprocessing (e.g., removal of outlier spectra) is a critical step when processing large datasets

from clinical studies. As an example, we provide guidance based on prior knowledge of a dataset to identify the types of outliers and develop specific strategies to remove them. A principal component analysis is performed, and the loading spectra are compared with spectra from reference materials to select the number of components used in the final multivariate curve resolution (MCR) analysis. This approach is successful for extracting meaningful information from a large spectral dataset.

INTRODUCTION:

In clinical studies, in vivo confocal Raman spectroscopy has shown its unique ability for determining stratum corneum thickness and water content[1-4], and tracking the penetration of active materials topically applied to the skin[5,6]. As a noninvasive approach, confocal Raman spectroscopy detects molecular signals based on vibrational modes. Thus, labeling is not needed[7]. In vivo confocal Raman spectroscopy provides chemical information with depth resolution based on the confocal nature of the technique. This depth-dependent information is very useful in studying the effects of skin care products[4,8], aging[9,10], seasonal changes[3], as well as skin barrier function diseases, such as atopic dermatitis[11,12]. There is a lot of information in the high frequency region of confocal Raman spectroscopy (2,500–4,000 cm^{-1}), where water produces distinct peaks in the region between 3,250–3,550 cm^{-1}. However, the Raman peaks of proteins and lipids, which are centered between approximately 2,800–3,000 cm^{-1}, overlap each other because the signals are mainly produced from methylene ($-CH_2-$) and methyl ($-CH_3$) groups[13]. This overlapped information presents a technical challenge when obtaining relative amounts of individual molecular species. Peak fitting[14,15] and selective peak position[12,16] approaches have been used to resolve this challenge. However, it is difficult for these single peak-based methods to extract pure component information because multiple Raman peaks from the same component change simultaneously[17]. In our recent publication[18], an MCR approach was proposed to elucidate the pure component information. Using this approach, three components (water, proteins, and lipids) were extracted from a large in vivo confocal Raman spectroscopic dataset.

The execution of large clinical studies can be demanding on individuals collecting in vivo spectroscopic data. In some cases, spectral acquisition can require operating equipment for many hours in a day and the study can extend up to weeks or months. Under these conditions, spectroscopic data may be generated by equipment operators that lack the technical expertise to identify, exclude, and correct for all sources of spectroscopic artifacts. The resulting data set may contain a small fraction of spectroscopic outliers that need to be identified and excluded from the data prior to analysis. This paper illustrates in detail a chemometric analysis process to "clean up" a clinical Raman dataset before analyzing the data with MCR. To successfully remove the outliers, the types of outliers and the potential cause for the generation of the outlier spectra need to be identified. Then, a specific approach can be developed to remove the targeted outliers. This requires prior knowledge of the dataset, including a detailed understanding about the data generation process and the study design. In this dataset, the majority of outliers are low signal-to-noise spectra and originate primarily from 1) spectra collected above the skin surface (6,208 out of 30,862), and 2) strong contribution to the spectrum from fluorescent room light (67 out of 30,862). Spectra collected above the skin surface produce a weak Raman response, as the laser focal point approaches the skin surface and is mostly in the instrument window below the skin.

Spectra with a strong contribution from fluorescent room light are generated due to either instrument operator error or subject movement, which produces a condition where the confocal Raman collection window is not fully covered by the subject's body site. Although these types of spectral artifacts could be identified and remediated during spectral acquisition by a spectroscopic expert at the time of data acquisition, the trained instrument operators used in this study were instructed to collect all data unless a catastrophic failure was observed. The task of identifying and excluding outliers is incorporated into the data analysis protocol. The protocol presented is developed to resolve this challenge. To address the low signal-to-noise spectra above the skin surface, the location of the skin surface needs to be determined first to allow removal of spectra collected above the skin surface. The location of the skin surface is defined as the depth where the Raman laser focal point is half in the skin and half out of the skin as illustrated in **Supplemental Figure 1**. After removing low signal-to-noise spectra, a principal component analysis (PCA) is implemented to extract the factor dominated by fluorescent room light peaks. These outliers are removed based on the score value of the corresponding factor.

This protocol provides detailed information for how six principal components are determined in the MCR process. This is done through a PCA analysis followed by spectral shape comparison between the loadings for models generated with a different number of principal components. The experimental process for data collection of reference materials as well as the human subjects is also explained in detail.

PROTOCOL:

This study was approved by the institutional review committee of Beijing Children's Hospital in compliance with the ethical guidelines of the 1975 Declaration of Helsinki. It was conducted according to ICH guidelines for Good Clinical Practice. The study took place from May to July 2015.

1. Collection of in vivo confocal Raman spectra from human subjects with atopic dermatitis

1.1. Include subjects in compliance with the following criteria.

1.1.1. Include subjects between the ages of 4–18.

1.1.2. Include subjects with mild to moderate atopic dermatitis (score of 2 or 3 according to the Physician's global assessment) with active disease symptoms on 5%–30% of the body surface, with at least two lesions on the arms.

1.1.3. Include subjects that are in good health, excluding symptoms directly related to the AD disease.

1.1.4. Include subjects that provide written informed consent.

1.1.5. Include subjects that have an individual topology angle (ITA) value higher than 40 at the testing location.

1.2. Exclude subjects that meet any of the following criteria.

1.2.1. Exclude subjects that are either currently participating or have previously participated in a clinical study at any test facility within the previous 4 weeks.

1.2.2. Exclude subjects with cancer or that have been diagnosed or treated for cancer within 5 years prior to the study.

1.2.3. Exclude diabetic subjects.

1.2.4. Exclude subjects who have an immunologic or infectious disease that could place the subject at risk or interfere with the accuracy of the study results (i.e., hepatitis, tuberculosis, HIV, AIDS, lupus, or rheumatoid arthritis).

1.2.5. Exclude subjects that have skin conditions that might interfere with instrumental measurements or will prevent the clear assessment of the skin only to atopic dermatitis. Examples include extremely dry skin, damaged skin, cuts, scratches, sunburn, birthmarks, tattoos, extensive scarring, rashes, excessive hair growth, or acne.

1.2.6. Exclude subjects that use oral immunosuppressive drugs, antibiotics, or other systemic therapies within the past month, except for minor tranquilizers.

1.2.7. Exclude subjects with any other medical conditions that, in the opinion of the investigator, precludes them from study participation.

1.2.8. Exclude subjects with higher pigmentation in the testing area.

1.3. Label the lesion area of the atopic dermatitis study participant and mark with a 3 cm x 4 cm area on or near the lesion site as shown in **Figure 1A**.

1.4. Label the non-lesion area with the same marker in the counterpart body site (e.g., left forearm vs. right forearm) as shown in **Figure 1B**.

1.5. Place the marked body site in close contact with the window of the in vivo confocal Raman instrument as shown in **Figure 2A** and **Figure 2B**. Cover the whole window to avoid the impact of room light on the body site.

1.6. Perform the Raman data collection.

NOTE: The instrument has a spectral resolution of 2 cm^{-1} and 50x microscopy objective (NA = 0.9 oil immersion), using a 671 nm laser with a power of 17 mW. Wavelength is calibrated using the spectrum of a built-in neon-argon lamp. The intensity calibration is done by measuring the spectrum of a NIST (National Institute of Standards) glass calibration standard.

1.6.1. Move the focus until a spectrum as illustrated in **Figure 2C** is observed, then move the focus away from the skin surface 10 µm.

1.6.2. Start the data collection for 26 steps with a 2 µm step size in the 2,510 cm^{-1}–4,000 cm^{-1} frequency region. Use an exposure time of 1 s and measure eight replicates for each area lasting ~10–15 min total.

2. Collection of confocal Raman spectra from reference materials

2.1. Place the reference materials, the major components in human skin stratum corneum[19], on the window of the confocal Raman instrument (see **Table of Materials**: Bovine Serum Albumin (BSA), deionized water (DI water), ceramide, cholesterol, free fatty acid, and squalene).

2.2. Collect the reference materials' Raman spectra consecutively from the outside of the material to the material center using the same collection parameters as described above.

2.3. Integrate the area under each Raman spectra between the range of 2,510–4,000 cm^{-1} and identify the top three maximum value points in these 26 measurements. Average the Raman spectra from those three points to obtain the final reference material spectra.

3. Removal of the outlier spectra through chemometrics analysis

3.1. Determine the skin surface and remove the out-of-skin spectra.

3.1.1. Change the file extension from '.ric' to '.mat' and load the .mat file to the MATLAB software platform.

3.1.2. Correct the baseline using **Baseline(Automatic Weighted Least Squares)** by right-clicking the imported dataset under **Analyze | Other Tools | Preprocessing** in the PLS_Toolbox software with default setting.

3.1.3. Sum up the values between 2,910–2,965 cm^{-1} to obtain the intensity values under each Raman spectrum from the 26 consecutive steps measurement as shown in **Figure 3A** via the **sum** function in MATLAB.

3.1.4. Interpolate the instrument offset value (value in the X-axis in **Figure 3B**) from 26–260 using the **linspace** function in MATLAB.

3.1.5. Interpolate the intensity value from 26 to 260 using the **spline** method in MATLAB, leveraging the newly-generated 260 position values.

3.1.6. Use MATLAB's **polyfit** and **polyval** functions to obtain the new set of intensity values with 260 points. First, use the 260 position and intensity values as X and Y inputs for the **polyfit** function, respectively. Set the degree value to 20. Then, use the output coefficients and the 260

extended position values as the input for **polyval** to obtain the final 260 intensity values.

3.1.7. Use MATLAB's **max** and **min** functions to identify the maximum and minimum points from the newly interpolated 260 intensity values.

3.1.8. Calculate the mean intensity value by dividing the sum of the maximum and minimum intensity value by two.

3.1.9. Identify the intensity value from the 260 intensity values (calculated from step 3.1.6) that is closest to the mean intensity value and set its corresponding position value as skin surface. Set this position value as the zero point in the X-axis as illustrated in **Figure 3B**.

3.1.10. Change all the other position values according to the zero point and the known 2 µm step size.

3.1.11. Remove all the spectra collected above the skin surface according to their position value.

3.1.12. Import the rest of the data to PLS_Toolbox to create a dataset and rename it "RamanData.mat".

3.2. Remove the outlier spectra with the room light effect.

3.2.1. Load the Raman spectra dataset (RamanData.mat) after removal of the out-of-skin spectra and implement the PCA analysis.

3.2.2. Load the dataset into the PLS_Toolbox software under the MATLAB platform and right-click the dataset to choose **Analyze | PCA**.

3.2.3. Select **Mean Center** as the preprocessing approach and choose **None** for the cross validation.

3.2.4. Use the three components for the PCA decomposition analysis as shown in **Supplementary Figure 2**.

3.2.5. Remove the cover on the in vivo Raman instrument's collection window and collect the room light spectra in the high frequency region using the same parameters used for the reference materials data collection.

3.2.6. Identify the room light effect factor through comparison with the room light background spectra as shown in **Supplementary Figure 3**.

3.2.7. Remove the spectra with a significantly higher corresponding score value than normal (More than 99.8% of the score values of the whole dataset, which is 0.16 in this study).

4. Selection of the number of the components in MCR decomposition analysis

4.1. Correct the Raman spectra baseline using the same approach described above (section 3.1.2).

4.2. Perform the PCA analysis on the preprocessed dataset as described above (section 3.2) and plot the eigenvalues in logarithmic scale along with the number of components (20) as the default number in the decomposition analysis by clicking the **Choose Components** button and select **log(eigenvalues)** as the Y value. Choose three to eight as the number of the components used for the MCR analysis.

4.3. Perform MCR analysis.

4.3.1. Load the dataset into the MCR_main software[20] through the **Data Selection** button.

4.3.2. Choose the number of components (three to eight) by clicking the **Determination of the number of components** button.

4.3.3. Click the **Pure** button under the **Initial Estimation** tab, select **Concentration** under the **Direction of the variable selection** tab, and click the **Do** button.

4.3.4. Click the **OK** button and then the **Continue** button to the next page.

4.3.5. Click **Continue** in the next page and then select **fnnls** and **6** under the **Implementation** and **Nr. of species with non-negativity profiles** tabs, respectively. Click the **Continue** button.

4.3.6. Choose the same parameters as section 4.3.5 for this page and click **Continue**.

REPRESENTATIVE RESULTS:

In this clinical study, in vivo confocal Raman spectra were collected from 28 subjects from 4–18 years old. A total of 30,862 Raman spectra were collected with the data collection protocol mentioned above. This large spectral dataset contains 20% spectral outliers as shown in **Figure 4A**. The low signal-to-noise outlier spectra were removed after determining the skin surface, followed by the PCA to identify the spectra with room light features. The third factor in this PCA model is identified room light peaks. This is confirmed by comparison of the loading spectra of factor 3 with a spectrum of fluorescent room light collected separately at the study site using the same confocal Raman instrument (see **Supplementary Figure 3**). **Figure 4B** indicates that most of the outlier spectra were removed after this process.

PCA was performed on the preprocessed confocal Raman dataset and the eigenvalue along with the number of factors used are plotted in **Figure 5**. According to prior studies[12,19], the model should include at least three components: water, protein, and lipid. A significant decrease in eigenvalue was observed for factor 9 as shown in **Figure 5**. This observation suggests investigating models with the number of principal components varying between three and eight factors for

inclusion in the MCR model. MCR loadings that contain spectroscopic features most consistent with protein, water, and lipid are shown in **Figure 6**.

FIGURE AND TABLE LEGENDS:

Figure 1. Illustration of the lesion and non-lesion mark on forearm. (**A**) A 3 cm x 4 cm marked area on a lesion site. (**B**) A 3 cm x 4 cm marked area on a non-lesion site.

Figure 2. Illustration of the confocal Raman data collection. (**A**) Confocal Raman instrument. (**B**) Spectra collection on the forearm of human subject. (**C**) A screen shot of determining the reference position for data collection.

Figure 3. Determining the skin surface. (**A**) Integration of the protein area under each Raman spectrum. (**B**) Setting the skin surface based on the maximum and minimum points.

Figure 4. Raman dataset spectra. (**A**) Confocal Raman spectra before removal of the outlier spectra. (**B**) Confocal Raman spectra after removal of the outlier spectra.

Figure 5. Determining the number of components from PCA analysis. (**A**) Eigenvalue on a logarithmic scale plotted as a function of the number of components used in PCA model. (**B**) Difference in eigenvalues between 'n' and 'n + 1' components

Figure 6. Comparison of the loading shape with the corresponding reference materials' spectra with three to eight components in MCR model. (**A**) Protein, (**B**) water, and (**C**) lipid factors' shape with three to eight components in the MCR model compared with BSA, water, and lipid reference materials' spectra, respectively.

Figure 7. The additional three loadings from the six component MCR model not used in the final model. These three MCR components are dominated by fluorescence and baseline artifacts.

Supplemental Figure 1. Illustration of the determination of the skin surface where the center of the laser focus touches the skin.

Supplemental Figure 2. Illustration of the selection of three components in the PLS_Toolbox software PCA analysis.

Supplemental Figure 3. Identification of the loading factor dominated by room light superimposed on a reference spectrum of room light.

Supplemental Figure 4. Comparison of loadings from the MCR model before and after removal of cosmic rays. (**A**), (**B**), and (**C**) are the factors representing water, protein, and lipid, respectively. The additional loading factors not used in the final MCR model are d, e, and f.

Supplemental Figure 5. Raman spectra of typical lipid materials in stratum corneum. (**A**) Cholesterol 3-sulfate sodium and cholesterol. (**B**) Oleic, palmitic, palmitoleic, and stearic acid. (**C**)

Squalene. (**D**) N-behenoyl-D-erythro-sphingosine, N-Lignoceroyl-D-erythro-sphinganine, and D-Erythro-Dihyrosphingosine.

DISCUSSION:

During the data collection, as described in section 2 and 3 of the protocol, each depth profile was collected in an area with contact between the instrument window and the skin by finding the darker areas from the microscopic images highlighted in the red circles in **Figure 2C**. Once these areas were located, it was critical to start the depth profile above the skin surface to accurately determine the location of the skin surface for the data analysis procedure. The location of the skin surface was subsequently used to determine the relative depth of each spectrum in the corresponding depth profile. As mentioned in section 1 of the protocol, starting the depth profile 10 µm above the skin surface produces five data points outside the skin. This allows for successfully determining the locations of the maximum and minimum signal intensity on both sides of the skin surface. It is also important to avoid measuring locations that contain pen marks and higher pigmented areas such as freckles, because these areas produce a high fluorescence background signal. The selection of the exposure time is a balance between spectral quality and measurement duration. Longer exposure time improves signal-to-noise and significantly increases the overall measurement time. However, many subjects find it challenging to remain motionless for extended periods of time. This is extremely challenging for children, for example. Increasing laser power increases the signal-to-noise. However, too much power can damage the skin due to the absorption of the energy. The maximum permissible exposures, 17 mW laser power as defined by the Chinese national standard (GB 7247.1-2012), and the international laser safety standard (IEC 60285-1:2007; <20 mW for 671 nm and <30 mW for 785 nm), cannot be exceeded. Other safety precautions include ensuring that each subject is wearing eye protection prior to data acquisition, that body sites have an individual topology angle (ITA) value higher than 40, and avoiding areas with high skin pigmentation.

To determine the location of the skin surface, the area under the protein Raman peak (2,910-2,965 cm^{-1}) was integrated to obtain the depth profile of the protein signal. The Raman spectra were first baseline-corrected using the automated weighted least square method from PLS_Toolbox prior to the integration of the peaks. The 26 data points from one depth profile were interpolated to 260 points using the **linspace** method for the instrument offset vaue (X-axis value in **Figure 3A**) and the **spline** method for the corresponding intensity value. The resulting data were interpolated onto a 20^{th} order polynomial using the **polyfit** and **polyval** functions in MATLAB and the maximum and minimum points of the interpolated data were determined. The mean intensity value was calculated by dividing the sum of the maximum and minimum values by 2. The skin surface was defined as the location where the intensity value from the interpolated depth profile was closest to the mean intensity. The exact location of the skin surface does not need to coincide with an experimental data point. This method can only measure a limited depth of the skin due to the absorption and scattering of the beam[21]. Collecting spectroscopic data below ~50 µm under the skin surface may require significant changes to the experimental parameters.

As described in section 3 of the protocol, after removal of outlier spectra with low signal-to-noise

and high contribution from room lights, a small fraction of spectra containing cosmic rays remained in the data set. A comparison of the loading spectra generated before and after cosmic ray removal is shown in **Supplementary Figure 4**. A comparison of loading spectra shown in **Supplementary Figure 4** indicates that the impact of a small number of spectra with cosmic rays was negligible. The three factors representing water, protein, and lipid were identical, and the additional three loadings associated with noise and spectral artifacts were also very similar. This might be attributed to a low occurrence of cosmic rays in the spectra (~0.25%) because the location of cosmic rays in the spectra are random.

The selection of the number of the components used in the MCR analysis is critical, because interpretation of the loadings' shape in terms of the corresponding molecular species responsible for each loading significantly impacts both how the corresponding score values are used and overall method performance. As described in section 4 of the protocol, PCA was performed first to investigate the eigenvalue evolution associated with the increasing of the number of the components. This investigation was used to identify the number of the components that should be used in the following MCR analysis. Plotting the eigenvalue on a logarithmic scale can make this identification process easier than examining the raw eigenvalues, as shown in **Figure 5A**. Each eigenvalue is a representation of the variance that one component can capture. The larger the eigenvalue, the more variance this component can model in the spectra. Eigenvalues with similar size should be selected or eliminated together[22]. Following this guideline, two, five, and eight components were considered for the MCR analysis because components three, four, and five produce eigenvalues similar in size. A similar trend was also observed for components six, seven, and eight. **Figure 5B** is a plot of the difference in eigenvalues between 'n' and 'n+1' components showing local maxima after the second, fifth, and eighth components. Prior knowledge about the molecular composition of skin combined with the study design supports a minimum of three components required to model the high frequency Raman spectra. Therefore, multiple MCR models containing three to eight components were investigated and the loadings were compared to spectra from reference materials to identify the key components required for the final model.

Comparison of the loadings with Raman spectra from reference materials easily allows identifying and assigning two of the final MCR components to protein and water because they dominate the MCR loadings for all models tested and match the corresponding reference spectra, which are BSA and DI water. However, the expected spectroscopic properties of lipid in some of the MCR components was a weaker match to the lipid reference spectrum illustrated in MCR models that contain three and four components. In addition, residual protein peaks (2,840–3,000 cm^{-1}) were observed in the MCR water loadings for all models tested below six components. Based on these observations, a six component MCR model was used in the final MCR analysis. Three of the six components were assigned to water, protein, and lipid by matching their loading spectrum to the corresponding reference spectrum. The interpretation and assignment of the lipid factor is based on comparison of the loading to Raman spectra of three representative ceramide materials, including N-behenoyl-D-erythro-sphingosine, N-Lignoceroyl-D-erythro-sphinganine, and D-Erythro-Dihyrosphingosine. The Raman spectra of other lipid materials in the stratum corneum were also examined. These materials include fatty acids (oleic, palmitic,

palmitoleic, and stearic acid), cholesterol (cholesterol 3-sulfate sodium and cholesterol), and squalene, as shown in **Supplementary Figure 5**. The lipid factor used in the final MCR model was a strong match to ceramide spectra and consistent with other materials that contain long chain hydrocarbons. The other three MCR components were dominated by fluorescence and baseline artifacts and their corresponding score values were not used in any calculations. These three components are shown in **Figure 7**.

The overall analysis approach presented in this manuscript produces a final method with improved specificity and accuracy for measuring the key components in skin compared to other single peak or peak-fitting approaches. This methodology demonstrates that critical components can be extracted from a clinical dataset that contains a relatively small fraction of bad spectra. Future efforts are focused on the automation of this methodology into a software package to improve its efficiency and reduce the amount of technical expertise required for the analysis. Similar methodology is being developed for Raman spectra collected in the fingerprint region (400–1,800 cm^{-1}) using a 785 nm laser source rather than the 671 nm laser incorporated into the same instrument.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

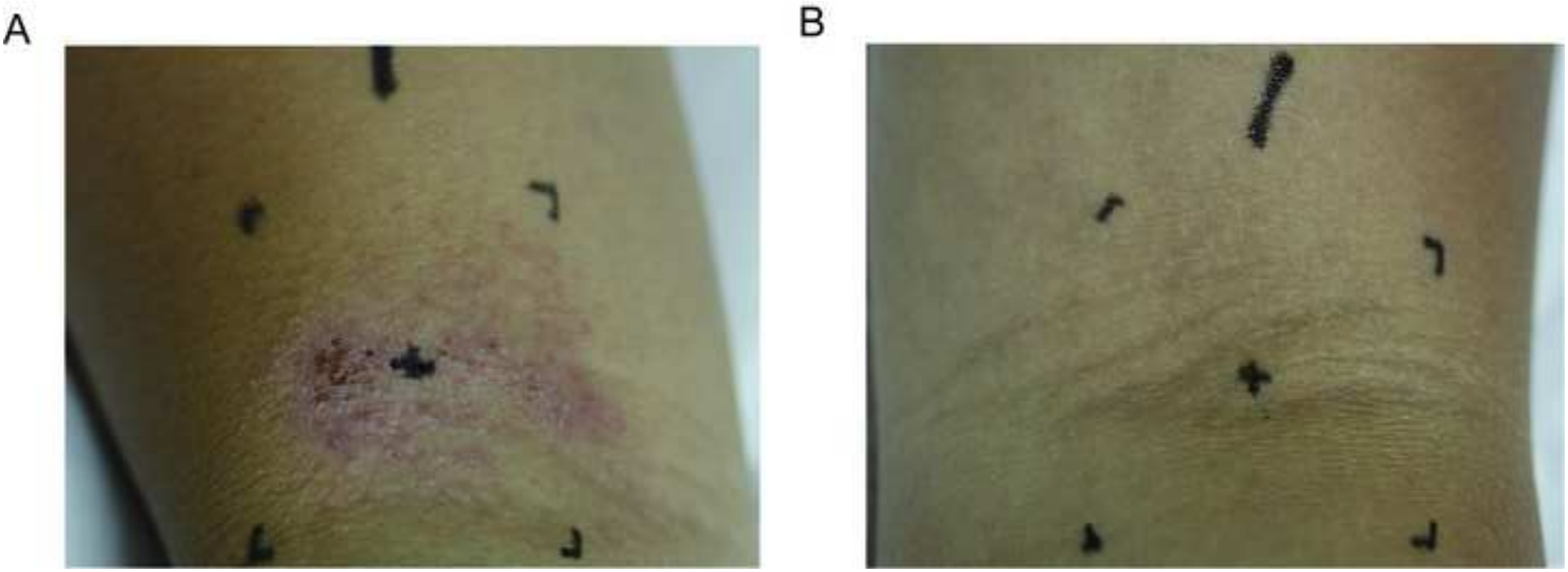

Figure2

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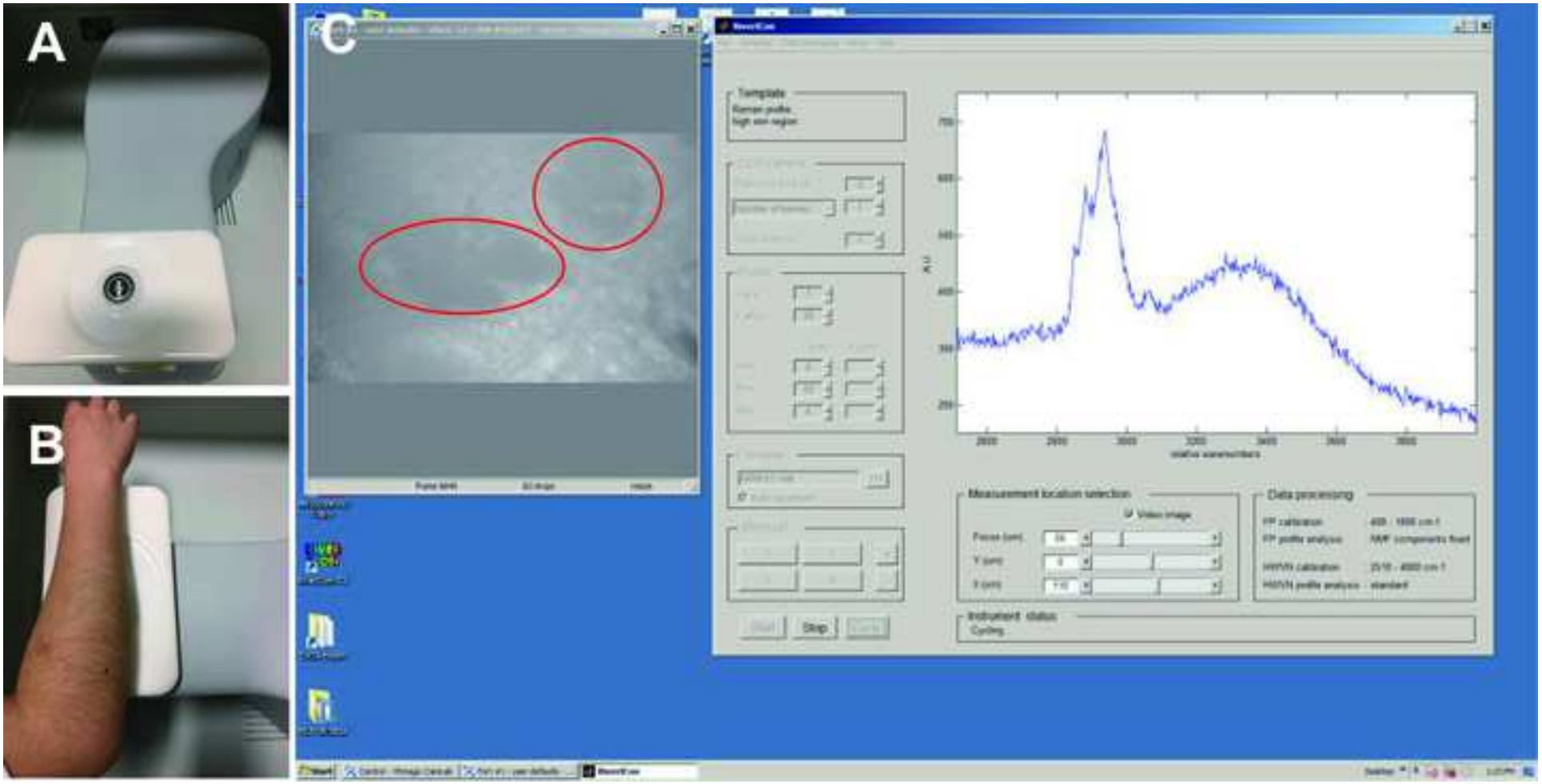

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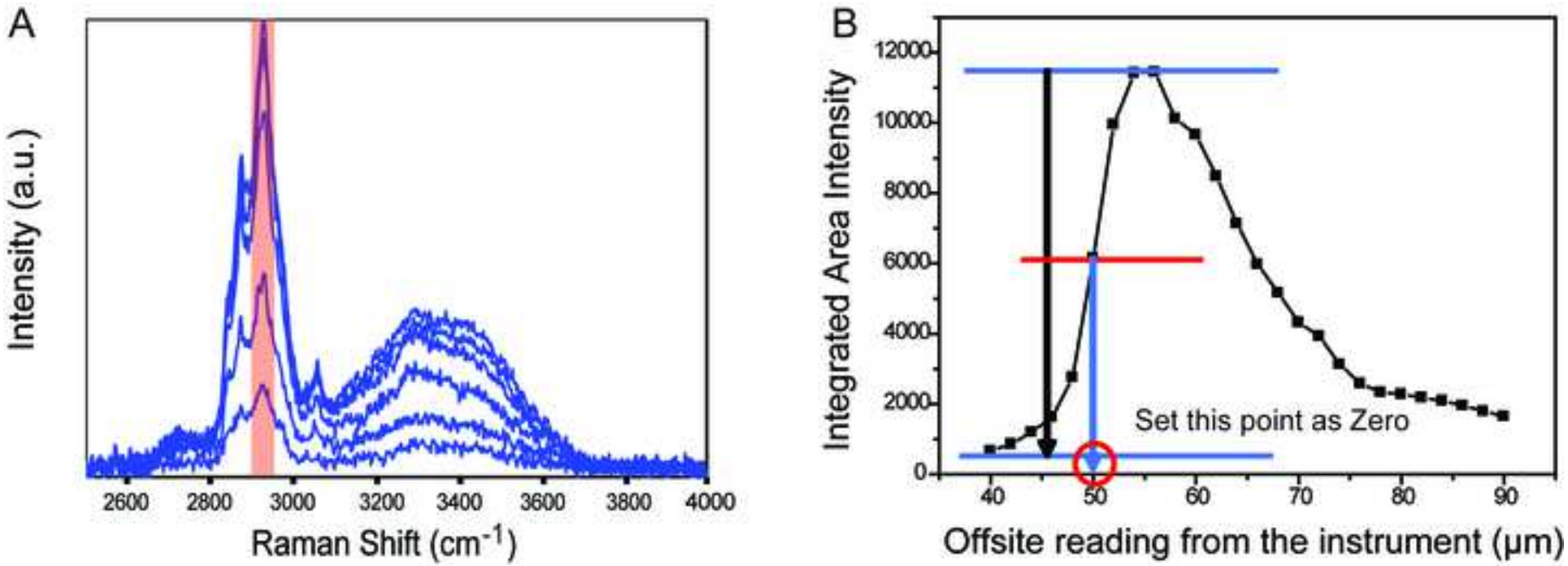

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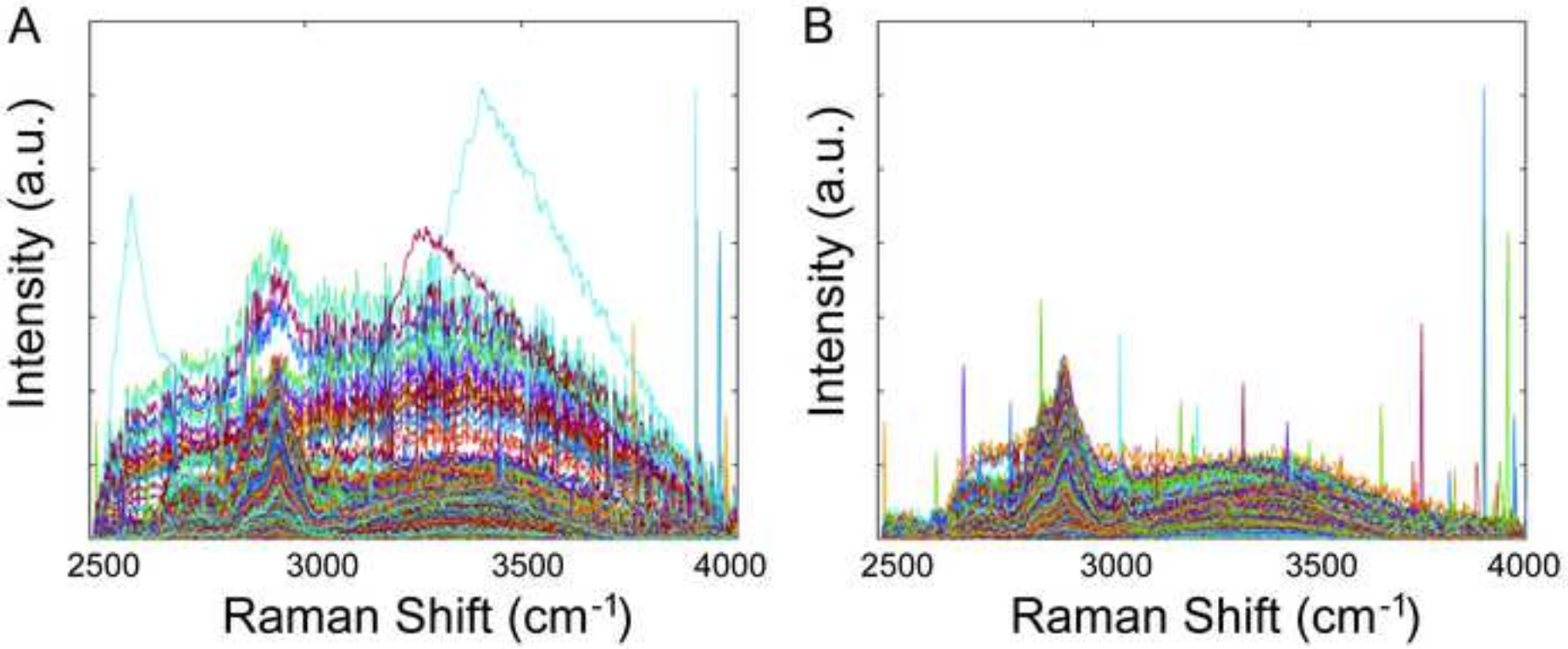

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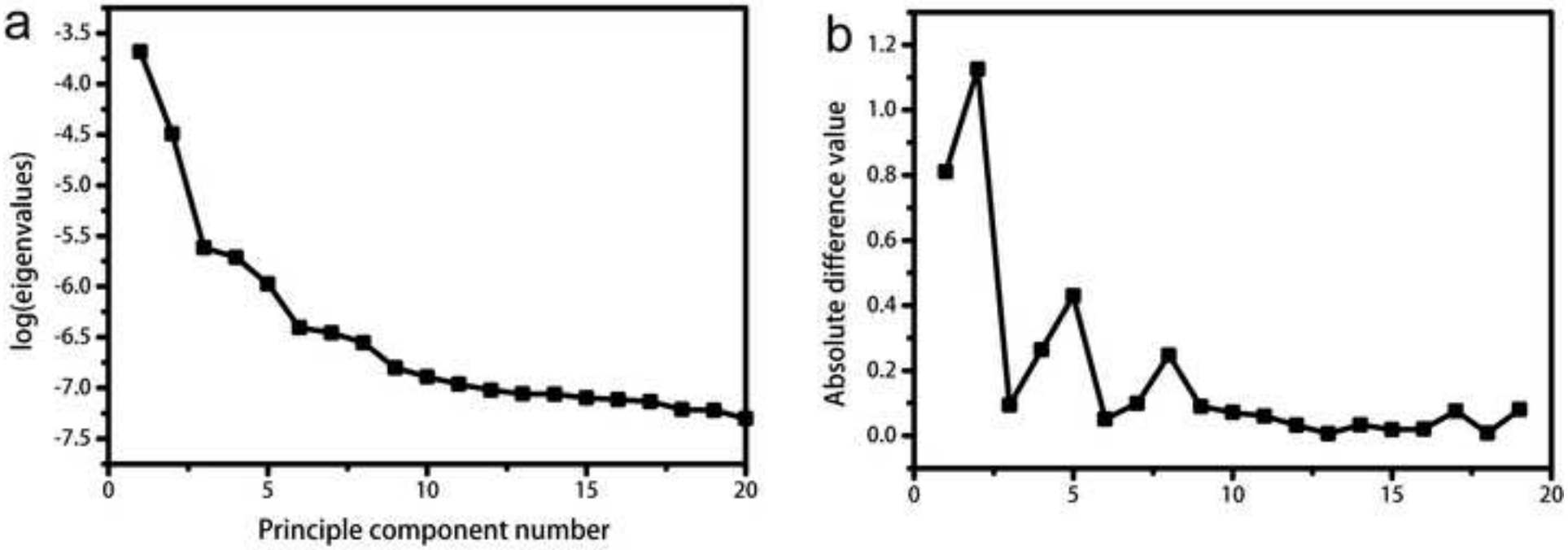

Figure6

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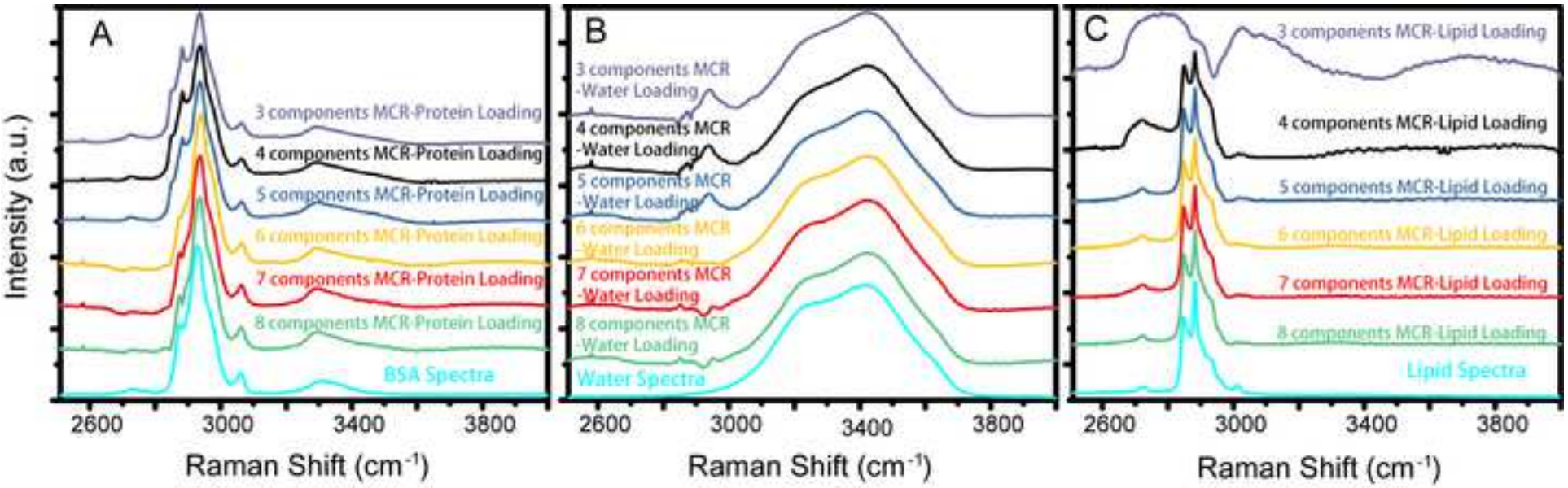

Figure7

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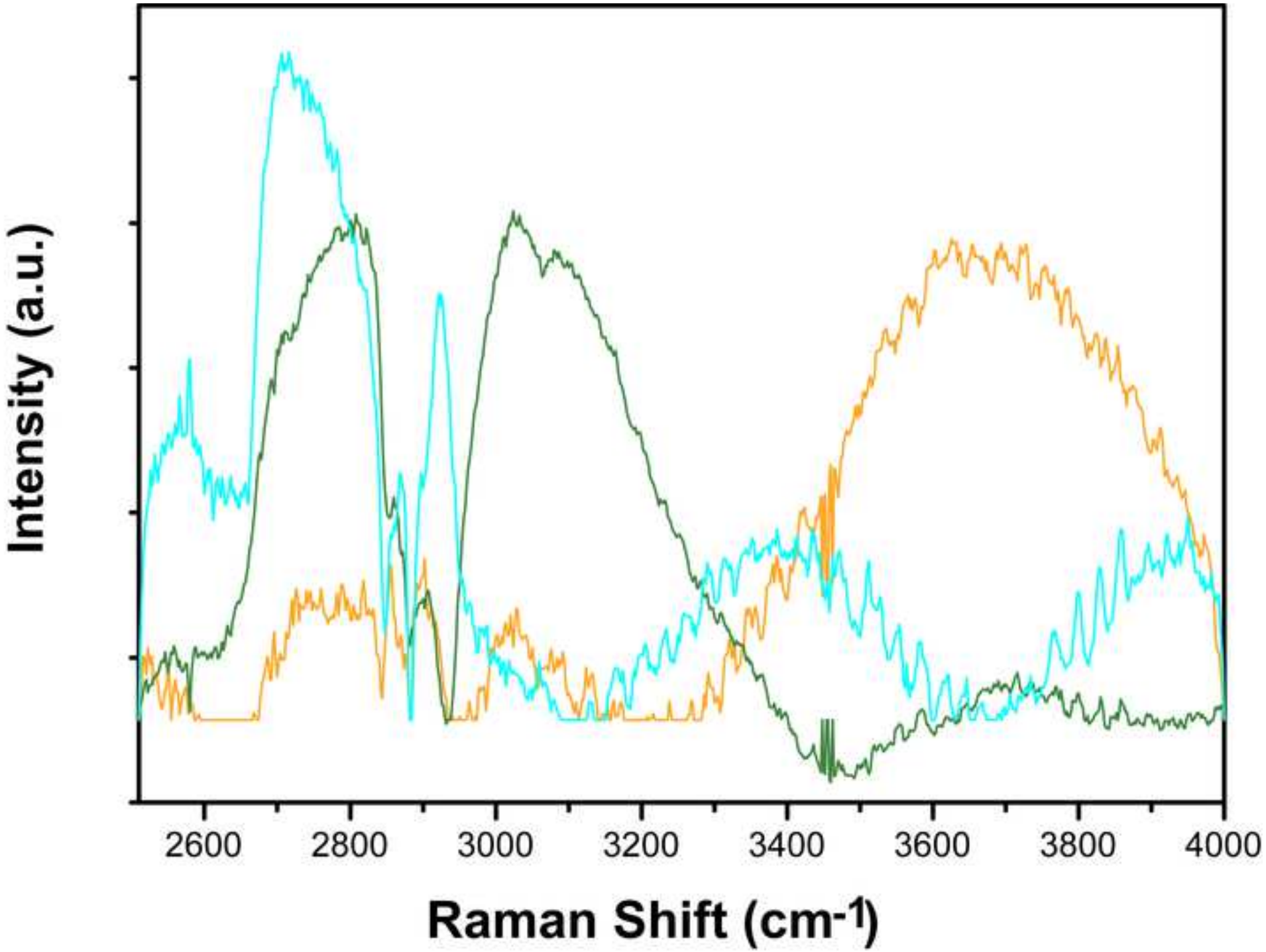

Name of Material/Equipment	Company	Catalog Number
Bovine Serum Albumin	Sigma-Aldrich	
Cholesterol	Sigma-Aldrich	
Cholesterol 3-sulfate sodium	Sigma-Aldrich	
D-Erythro-Dihydrosphingosine	Sigma-Aldrich	
DI water		
Gen2-SCA skin analyzer	River Diagnostics, Rotterdam, The Netherlands	Gen2
Matlab 2018b	Mathwork	2018b
N-behenoyl-D-erythro-sphingosine	Avanti Polar Lipids, Inc.	
N-Lignoceroyl-D-erythro-sphinganine(ceramide)	Avanti Polar Lipids, Inc.	
Oleic Acid	Sigma-Aldrich	
Palmitic Acid	Sigma-Aldrich	
Palmitoleic Acid	Sigma-Aldrich	
PLS_Toolbox version 8.2	Eigenvector Research Inc.	8.2
RiverICon	River Diagnostics, Rotterdam, The Netherlands	version 3.2
Squalene	Sigma-Aldrich	
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Comments/Description

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Author(s): Lesheng Zhang, Tom Cambron, Yueqing Mu, Zigang Xu, Ming Su, Hongyan Zheng, Karl Wei, Paula Ray

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Dear editor:

This letter is in response to editorial comments associated with the submission of the manuscript titled "Resolving water, protein, and lipid from in vivo confocal Raman spectra of stratum corneum through a chemometric approach" by Lesheng Zhang, Tom Cambron, Yueqing Niu, Zigang Xu, Ning Su, Hongyan Zheng, Karl Wei, Paula Ray, to *Journal of Visualized Experiments* through the journal website.

We have read the editorial comments carefully and revised the manuscript in response to his/her suggestions. A detailed list of revisions is listed in this communication.

We believe the revised manuscript addresses the editorial comments, and now meets the requirements for publication.

Sincerely

Le-Sheng Zhang, Ph. D

Editorial comments:

The manuscript has been modified and the updated manuscript, **60186_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the reviewer for this suggestion. The article has been revised thoroughly and multiple spelling and grammar issues has been addressed in the revised manuscript.

2. Step 1.1.1-1.1.5: Please ensure that each step is written in the imperative tense.

Following the reviewer's comments, those steps have been rewritten as:

1.1.1. Include subjects between the ages of 4 and 18.

1.1.2. Include subjects with mild to moderate atopic dermatitis (score of 2 or 3 according to the Physician's Global Assessment) with active disease symptoms on 5% to 30% of the body surface, and with at least two

lesions on arms.

1.1.3. Include subjects that are in good health; excluding symptoms directly related to the AD disease.

1.1.4. Include subjects that provide written informed consent.

1.1.5. Include subjects that have an individual topology angle (ITA) value higher than 40 at the testing location.

3. Step 1.2.1-1.2.8: Please ensure that each step is written in the imperative tense.

Following the reviewer's comments, those steps have been rewritten as:

1.2.1 Exclude subjects either currently participating or have previously participated in a clinical study at any test facility within the previous 4 weeks.

1.2.2 Exclude subjects with cancer or have been diagnosed or treated for cancer within 5 years prior to the study.

1.2.3 Exclude diabetic subjects.

1.2.4 Exclude subjects who have an immunologic or infectious disease such as hepatitis, tuberculosis, HIV, AIDS, lupus, or rheumatoid arthritis which could place the subject at risk or interfere with the accuracy of the study results.

1.2.5 Exclude subjects that have skin conditions that might interfere with instrumental measurements or will prevent the clear assessment of his/her skin only to atopic dermatitis. Examples include extremely dry skin, damaged skin, cuts, scratches, sunburn, birth marks, tattoos, extensive scarring, rashes, excessive hair growth, or acne.

1.2.6 Exclude subjects that use oral immunosuppressive drugs, antibiotics, or other systemic therapies within the past month, except for minor tranquilizers.

1.2.7 Exclude subjects with any other medical conditions that, in the opinion of the investigator, precludes them from study participation.

1.2.8 Exclude subjects with higher pigmentation in the testing area.

4. Figure 4: Please add a short description of the figure in the Figure Legend.

Following the reviewer's comments, a short description has been added to Figure 4 as :

Figure 4. Raman dataset spectra. (A) Confocal Raman spectra before removal of the outlier spectra;(B) Confocal Raman spectra after removal of outlier spectra.

5. Figure 5: Please add a short description of the figure in the Figure Legend.

Following the reviewer's comments, a short description has been added to Figure 5 as :

Figure 5. Determining the number of components from PCA analysis. A) Eigenvalue on a logarithmic scale plotted as a function of the number of components used in PCA model; B) Difference in eigenvalues between 'n' and 'n+1' components

6. Figure 7: Please add a short description of the figure in the Figure Legend.

Following the reviewer's comments, a short description has been added to Figure 7 as :

Figure 7. The additional three loadings from the six component MCR model not used in the final model. These three MCR components are dominated by fluorescence and baseline artifacts.

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Following the reviewer's comments, section 1.5-1.6.2 and 3-4 have been highlighted in yellow as filmable content.

8. Please avoid long notes (more than 4 lines).

Following the reviewer's comments, this has been changed to "Note: The instrument has a spectral resolution of 2 cm^{-1} and 50 × microscopy objective (NA 0.9 oil immersion), using a 671 nm laser with a power of 17 mw. Wavelength is calibrated using the spectrum of a built in Neon-Argon lamp. The intensity calibration is done by measuring the spectrum of a NIST (National Institute of Standards) glass calibration standard."

9. Please do not abbreviate journal titles for references.

Following the reviewer's comments, the full name of reference No. 8 has been added as "*International Journal of Pharmaceutics*". We also checked the other journal titles to avoid any other abbreviation.

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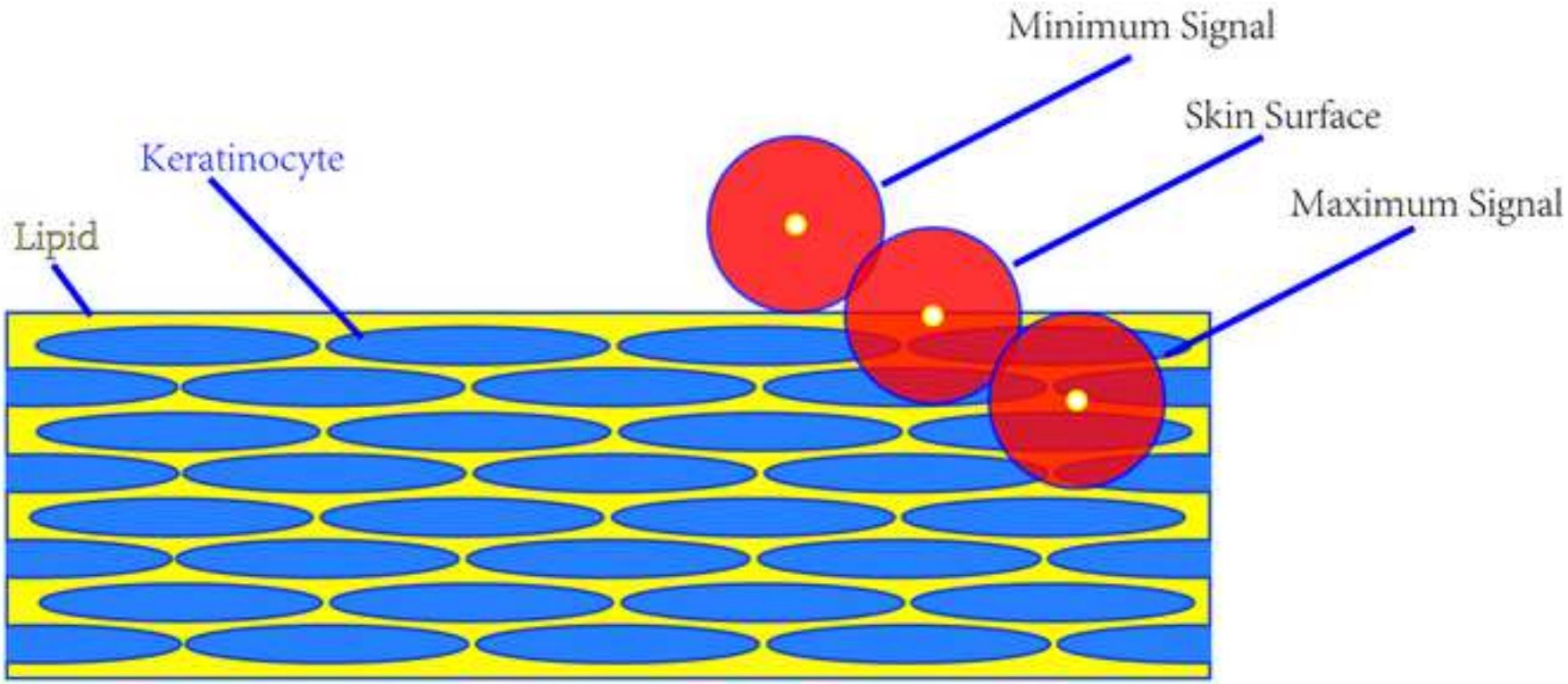

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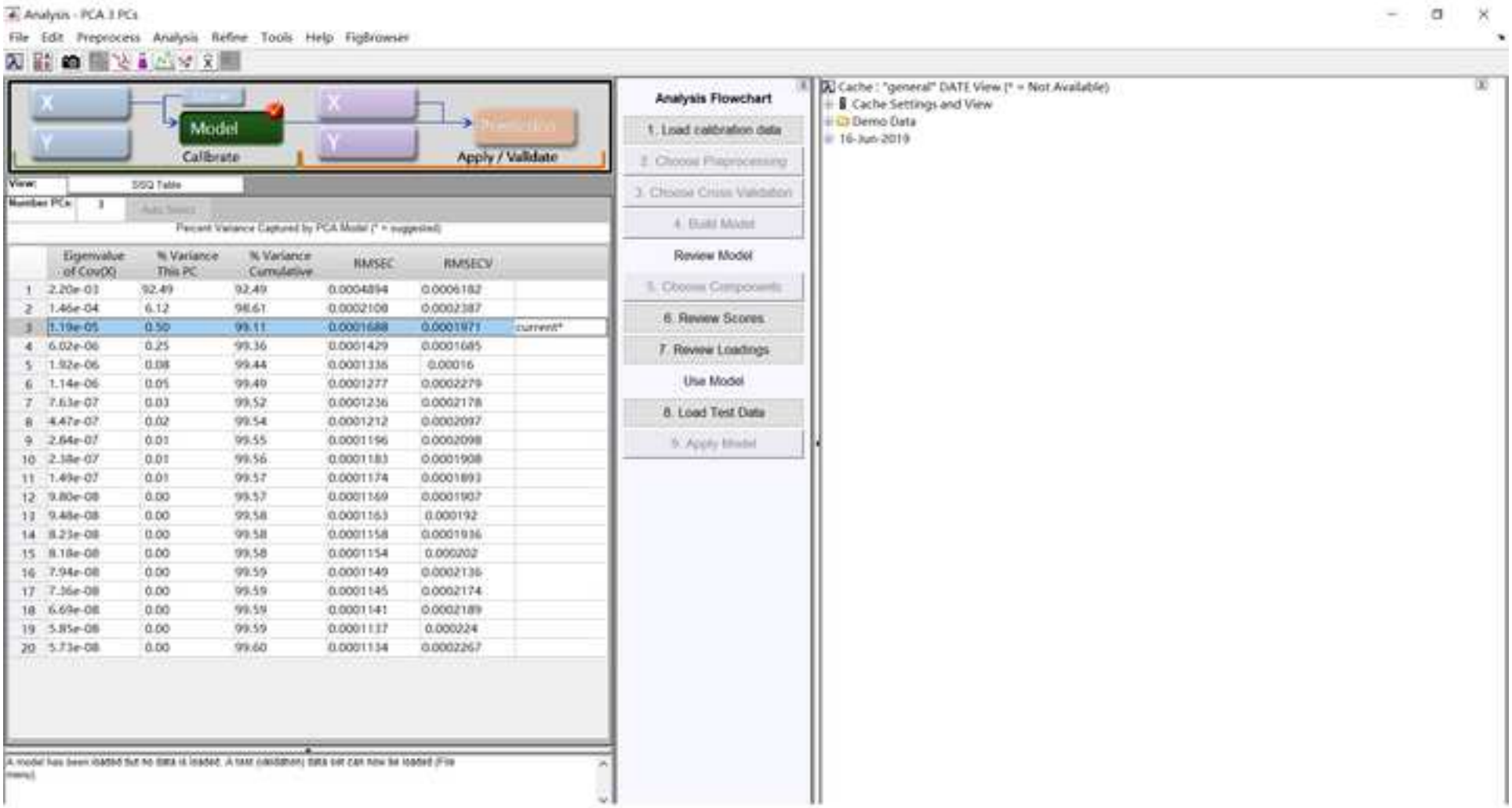

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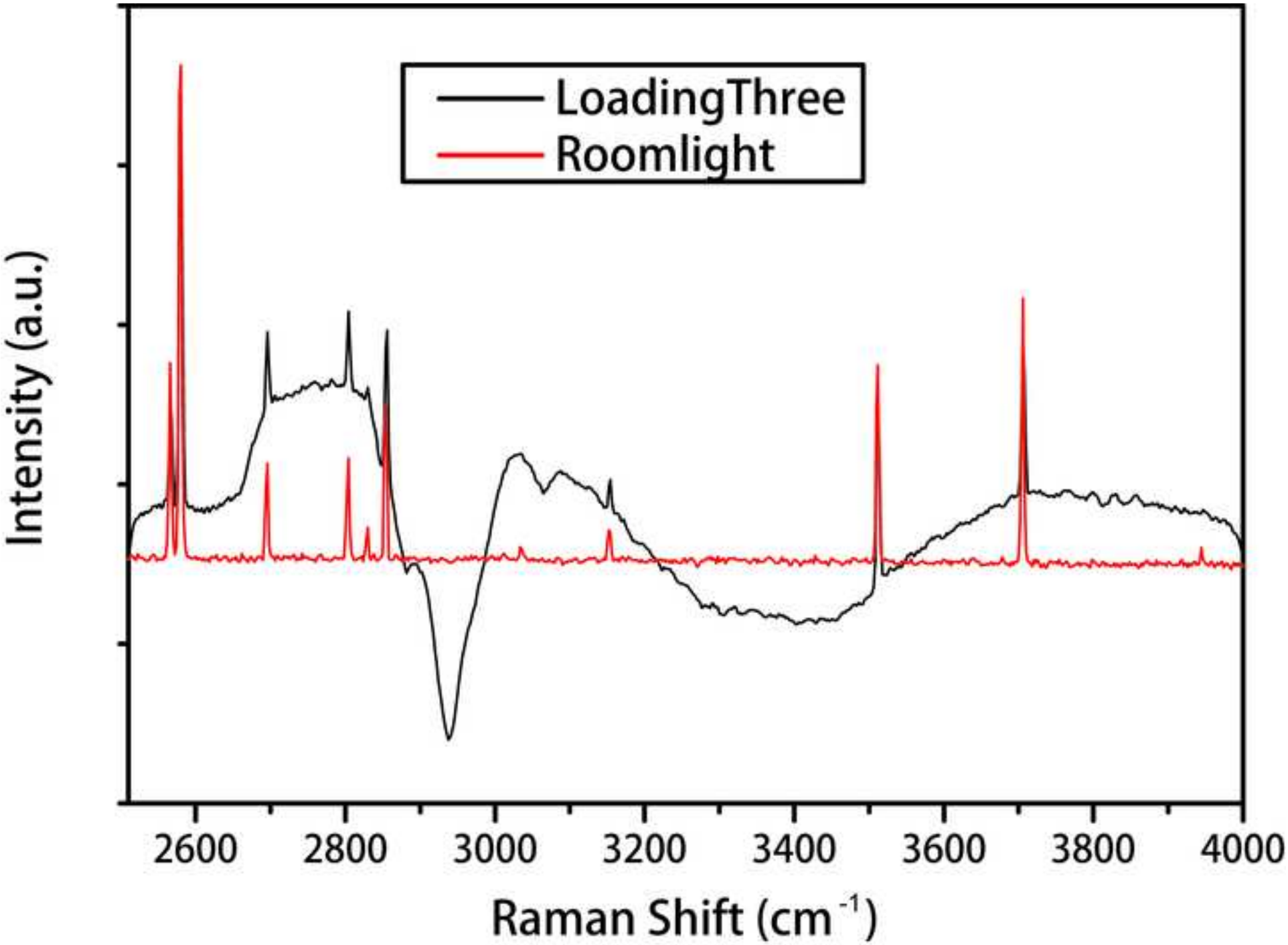

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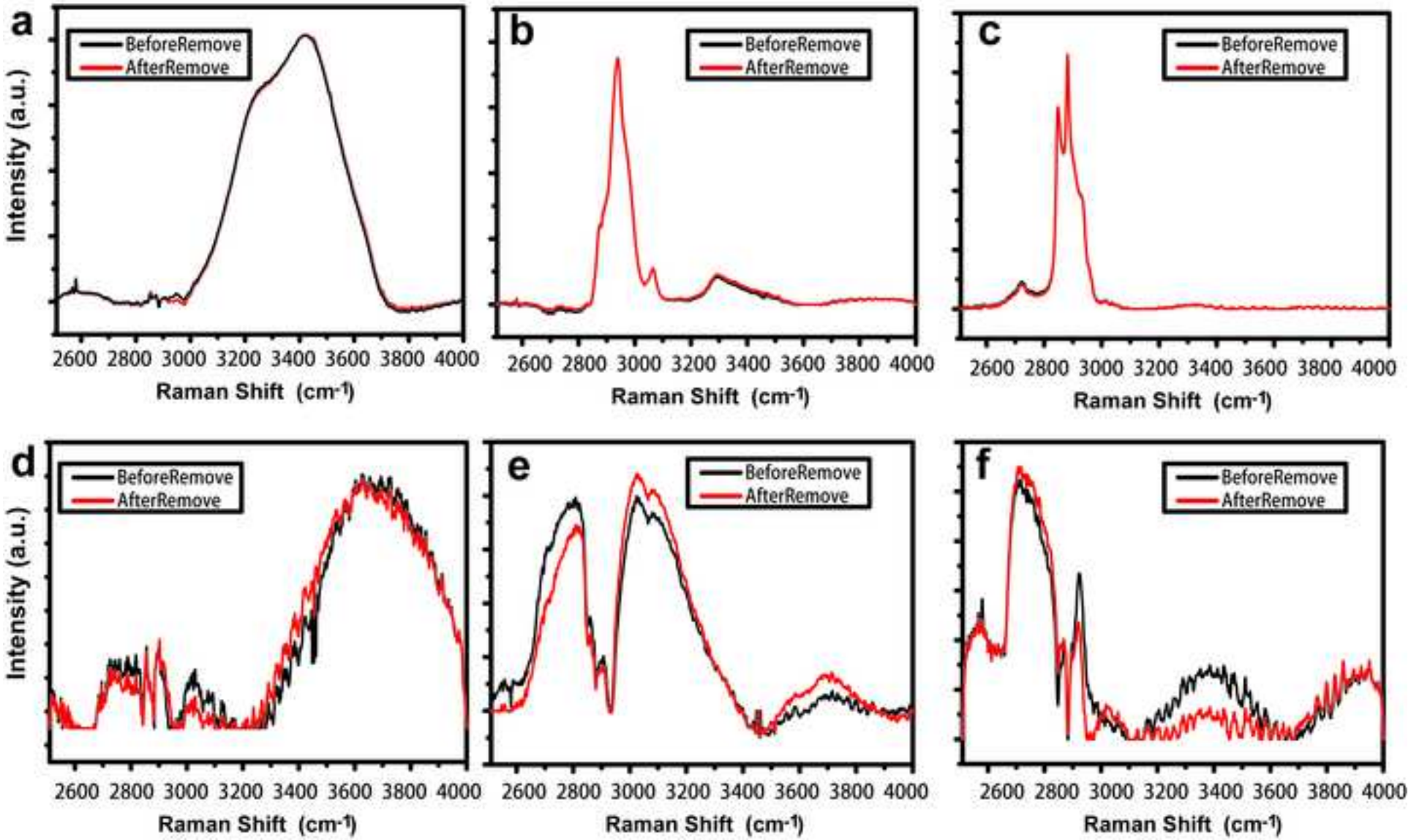

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