Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 149 (2015) 544–549

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Spectrometer calibration protocol for Raman spectra recorded with different excitation wavelengths

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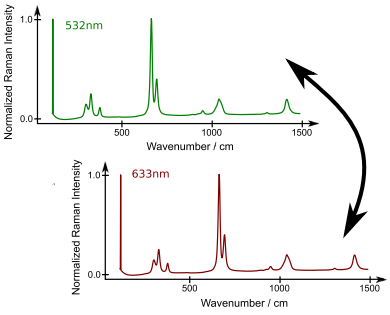
HIGHLIGHTS

- We studied the influence of the excitation wavelength on (non-resonant) Raman spectra.
- Calibration protocol for Raman spectra recorded with different excitation wavelength was developed.
- The impact of the protocol on a classification task of six solvents was investigated.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 22 April 2015
Accepted 25 April 2015
Available online 1 May 2015

Keywords:
Raman spectroscopy
Spectrometer calibration
Chemometric analysis

ABSTRACT

The combination of Raman spectroscopy with chemometrics has gained significant importance within the last years to address a broad variety of biomedical and life science questions. However, the routine application of chemometric models to analyze Raman spectra recorded with Raman devices different from the device used to establish the model is extremely challenging due to Raman device specific influences on the recorded Raman spectra. Here we report on the influence of different non-resonant excitation wavelengths on Raman spectra and propose a calibration routine, which corrects for the main part of the spectral differences between Raman spectra recorded with different (non-resonant) excitation wavelengths. The calibration routine introduced within this contribution is an improvement to the known 'standard' calibration routines and is a starting point for the development of a calibration protocol to generate spectrometer independent Raman spectra. The presented routine ensures that a chemometric model utilizes only Raman information of the sample and not artifacts from small shifts in the excitation wavelength. This is crucial for the application of Raman-spectroscopy in real-world-settings, such as diagnostics of diseases or identification of bacteria.

Introduction

In the last decade Raman spectroscopy coupled with chemometrics has proven its great potential to address a broad range of biomedical and life science problems, like e.g. the differentiation between cancer and normal cells [1,2], the differentiation of different cell types [3], the diagnosis of inflammatory bowel diseases (IBDs) [4,5], and the identification of various bacteria strains [6,7]. This list of examples is by far not complete, but all of these studies reveal, that subtle changes in the Raman spectra not visible by naked eye are responsible for the differentiation between the different groups. These spectral differences are identified by chemometric approaches and a statistical model is build. In order to use such a chemometric model to analyze Raman spectra

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http://dx.doi.org/10.1016/j.saa.2015.04.079
1386-1425/

recorded with a different Raman spectrometer, as the one used to build the spectral database, a calibration routine is required [8–11].

The task of defining an appropriate calibration routine is quite challenging, because it is hard to control all Raman device dependent corrupting effects and to correct for them. Nevertheless, a calibration routine completely removing the spectral influence of the applied Raman spectrometer would significantly enlarge the application possibilities of Raman spectroscopy. This results from the fact, that such a calibration routine would allow merging Raman spectra and databases recorded with different Raman spectroscopic devices and the application of chemometric models with Raman spectra recorded with a different spectrometer. Recent studies showed, that significant improvements in developing calibration routines are necessary, in order to generate spectrometer independent Raman spectra [8,11].

Thus, the present contribution is dedicated to the improvement of Raman spectrometer calibration routines. Here we investigate the differences between Raman spectra, if they were recorded with different non-resonant excitation wavelengths, i.e. the excitation wavelength is away from electronic resonances of the molecules. Such a comparison can be necessary, if a laser source has to be replaced and the new laser has a slightly different wavelength, as it is often the case for diode lasers. The Raman spectra recorded with the new excitation laser have to be calibrated, that they are comparable with the Raman spectra measured before the replacement of the laser source. Another application, where such a calibration routine is required, is the merging of databases recorded with near-by excitation wavelengths.

Within this study a calibration routine is defined and the physical reason for the applied correction is highlighted. This calibration routine should minimize the influence of changing the Raman excitation wavelength. The change of the whole Raman-setup is a more complicated problem and should be subject of further research. The calibration routine is tested for defined samples (six solvents) in a quantitative manner. The spectral differences between spectra recorded with two excitation wavelengths (532 nm and 633 nm) are evaluated and the influence on a classification/identification model is tested. The latter is important for the application of chemometric models for Raman spectra in general. We decided to utilize solvents because they form well separated groups and it is easy to see, if spectra contain contaminations of another solvent. Nevertheless, we applied the analysis workflow, which is normally used in Raman-bio-spectroscopy. In biomedical problems, the spectral differences become tiny and the investigator has to trust the chemometric method chosen for the analysis. Therefore, our experiment indicates problems, which occur in Raman-bio-spectroscopy, and the proposed routine solves the problem connected with an exchange of the excitation wavelength.

Material and methods

Experimental

A LabRam HR 800 micro-spectrometer (Jobin Yvon) was utilized for all experiments. The excitation light of a frequency-doubled Nd:YAG laser @ 532 nm (Coherent Compass) and a He–Ne–Laser @ 633 nm (Coherent) were coupled into the microscope and focused on the sample with an objective (Olympus LMPlanFl 50× N.A. 0.5). The spectra were recorded in back scattering geometry. The back scattered Raman light was dispersed by a 300 lines/mm grating and the spectra were recorded with a Peltier-cooled charge coupled device (CCD) camera. In addition an external fiber optic spectrometer (Avantes AVASPEC) was used to determine the excitation wavelength at the beginning of a measurement session.

As standards a Neon–Argon-lamp (Ocean Optics), 4-acetamidophenol (Sigma–Aldrich) and a calibrated white-light source (Ocean Optics) were utilized as wavelength-, wavenumber- and white-light-standard, respectively. As test substances the solvents acetonitrile, cyclohexane, dimethyl sulfoxide (DMSO), ethanol, propanol and carbon tetrachloride were measured. In order to check the reproducibility of a spectrum, 50 spectra were recorded for each substance and each excitation wavelength. The test substances were of spectroscopic grade and purchased from Sigma–Aldrich.

Computational

All calculation were carried out in R [12] using implemented or in-house written functions. The packages MASS [13], KernSmooth [14] and Peaks [15] were utilized. The spectral pre-treatment consisted of an interpolation to an unique wavenumber grid (400–1540 cm^{-1}) and spectral pre-processing. The pre-processing is composed of spike correction, the SNIP background correction [16] and a maximum normalization. The quality of the calibration was evaluated by the Root-Mean-Squared-Error (RMSE) between two Raman spectra $\vec{S}^1$ and $\vec{S}^2$, which can be calculated by:

$$\mathrm{RMSE}(\vec{S}^1, \vec{S}^2) = \sqrt{\sum_{i=1}^{N} (S_i^1 - S_i^2)^2}. \quad (1)$$

In this equation i is summation index referring to the wavenumber channels and N is the absolute number of these channels. In addition, the Raman spectra were used to build a classification model utilizing a principal component analysis (PCA) and a linear-discriminant-analysis (LDA) [17]. This LDA method constructs, based on the mean Raman spectra of the groups and the covariance matrix, a model, which can be used to distinguish between these groups. As the variance at the border of the Raman spectra was zero a PCA was applied. All non-zero scores were used to build the classification model, in order to incorporate all spectral information. Here, the model performance was evaluated by applying the model to an independent data set. We trained a PCA–LDA model for the Raman spectra of six solvents measured with 532 nm excitation wavelength and tested the prediction of this model for a data set measured with 633 nm excitation wavelength.

Results and discussion

In this contribution we aim to design a calibration routine, which minimizes the differences between Raman spectra recorded with different excitation wavelengths. For different Raman excitation wavelengths it is important to consider the wavelength region where the Raman spectra are recorded, because when Raman spectra are shifted in the wavelength domain, the spectrometer response function which depends on the absolute wavelength is altered. In the following the proposed calibration routine to account for wavelength dependent spectrometer response functions is outlined.

Definition of the calibration routine

The workflow of the proposed calibration routine (*Correction + Pre-processing*, CP-routine) is summarized in Fig. 1 on the right side. It starts with measuring and fitting of the spectrum of the excitation laser source (Fig. 1 step 1). The measurement was carried out with an second spectrometer (see 'Material and methods' section) and the excitation wavelength profile was fitted with a Gaussian profile. Thereafter, Raman-spectra of a wavelength standard (He–Ne–Ar-lamp) and a wavenumber