Short communication

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How to draw the line – Raman spectroscopy as a tool for the assessment of biomedicines

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Abstract: Biomedicines are complex biochemical formulations with multiple components that require extensive quality control during manufacturing and in subsequent batch testing. A proof-of-concept study has shown that an application of Raman spectroscopy can be beneficial for a classification of vaccines. However, the complexity of biomedicines introduces new challenges to spectroscopic methodology that require advanced experimental protocols. We further show the impact of analytical protocols on vaccine classification using R as an Open Source data analysis platform. In conclusion, we advocate for standardized and transparent experimental and analytical procedures and discuss current findings and open challenges.

Keywords: machine learning; pre-processing; quality control; Raman spectroscopy; standardisation; vaccine.

Biomedicines, such as vaccines or therapeutic allergens, are complex biochemical formulations containing multiple components. Biotechnological production or extraction from biological sources results in inherent variability and thus requires high levels of standardisation in production and quality control to ensure drug safety and efficacy. For many products, in particular vaccines, manufacturers’ process controls are complemented by batch release testing through governmental medicines control laboratories. Batch release testing of vaccines and allergen products performed at the Paul-Ehrlich-Institut ranges from biochemical and immunological assays to in vivo testing, which is expensive and time-consuming.

Use of animals further raises ethical concerns. Vibrational spectroscopy (infrared- and Raman-spectroscopy) has already proven to be a valuable tool to address quality control for pharmaceutical products, largely specific, solid chemical compounds (Bunaciu and Aboul-Enein 2017; Ewing and Kazarian 2018; Pivonka et al. 2007). Applications are, on one side the identification of compounds and on the other the detection of counterfeit products. Spectral measurements can be calibrated through reference samples allowing for quantitative measurements (Bonnier and Byrne 2012; Bonnier et al. 2017; Byrne et al. 2020; Makki et al. 2019; Parachalil et al. 2020). Identification and quantification capabilities can be used for the detection of sub-potent formulations, contaminations and other failures to meet quality criteria. Optical methods are non-destructive, do not require specific labelling and are rapid and cost-efficient.

Our initial proof-of-concept studies have shown that an analogous application of vibrational spectroscopy can be equally beneficial in the quality control of vaccines (Silge et al. 2018). The approach can be extended to other biomedicines (Butler et al. 2016) and matches the need for rapid and reliable measurement techniques in quality control of these products. However, the complex nature of biomedicines introduces challenges to spectroscopic methods that need to be addressed by basic research (Baker et al. 2018). Complex, liquid formulations result in strong spectral signals from water and excipients that need to be differentiated from the often weaker signal of the pharmacologically relevant active substances (Bonnier et al. 2017; Parachalil et al. 2020; Zhao et al. 2015). Vaccines are often inhomogeneous and turbid formulations that may show highly localized signals with temporal changes due to sedimentation. Their essential compounds are immunogenic proteins, polysaccharides, and attenuated pathogens and immune enhancers (adjuvants). A workaround to reduce signals from water and excipients and to stabilize measurement conditions is to study dried products. This however, comes with its own challenges in standardisation, in particular with respect to drying protocols and crystallization patterns. No calibrations have as yet been established to link the findings in dried products...
with information about the native product and its active components.

Aside from these probe-specific complications Raman spectroscopy is further challenging because it provides very detailed information about the sample and measurement conditions alike. Signals of interest are typically superimposed by interfering signals such as variable background signals and noise from measurement devices, sample fluorescence, substrate specific signals or sporadic peaks from cosmic radiation. Furthermore, changes that may occur in the wavenumber (x-axis) and intensity (y-axis) of spectra require calibration (Dörfer et al. 2011). Extraction of the signal of interest through pre-processing of raw spectral data is generally considered as mandatory for a subsequent robust and accurate classification (Lasch 2012).

For our study, we followed the experimental protocol for vaccines dried on CaF2 slides as developed and described in Silge et al. (2018) for the vaccines listed in Table 1. For each dried vaccine spot (replicate) we have measured a grid of 100 spectra, each with an acquisition time of 30 s at a laser excitation wavelength of 785 nm and laser power of 100 mW (BioRam, CellTool GmbH). The choice of an excitation wavelength in the near infrared wavelength regime – as compared to the earlier study which used an excitation wavelength of 514 nm – can reduce fluorescence background but requires longer acquisition times. Measurements were started after an initial drying time of 30 min when vaccine dots were visibly dry on CaF2 slides and six dots were measured consecutively (one per hour). In line with earlier findings (Silge et al. 2018), the first two to three measurements showed larger variability before equilibration of the spectra seen in dried samples, which was however minor as compared to between vaccine variation (cf. Supplementary Figure S2). In cases of availability of more than one manufacturing batch per vaccine we found negligible between batch variation as compared to between vaccine variation (cf. Supplementary Figures S2 and S3).

The focus of the current study was to explore the impact of pre-processing schemes on subsequent classification of spectral representations – or fingerprints. To ensure transparent and reproducible pre-processing of raw spectra we used the Open Source environment of the statistical programming language R with the package hyperSpec (Beleites and Sergo) for the pre-processing and analysis of spectral data and the EMSC package for extended multiplicative scatter correction (Liland et al. 2016). The R Stats core function `prcomp()` was used for principal component analysis, `lda()` for linear discriminant analysis. All spectra were calibrated for potential shifts in the wavenumber axis using a Paracetamol reference measurement for each measurement day prior to further pre-processing (Dörfer et al. 2011). To assess the impact of different pre-processing procedures, we followed three variant procedures:

**Procedure 1:** Spectra with values more than four standard deviations from the mean spectrum were skipped as outliers or as contaminated by cosmic spikes. Subsequently, linear baselines were removed and spectra smoothed at a resolution of four wavenumbers in the range 400–3200 cm⁻¹ (without down-sampling). This is followed by an intensity normalisation in which each spectrum is divided by its mean value over the considered wave number range (area normalisation).

**Procedure 2:** Procedure 1 is followed with an additional constraint to the wavenumber range of 400–1500 cm⁻¹ which was chosen due to strong background signals in our setting above 1500 cm⁻¹.

**Procedure 3:** Procedure 2 is followed with additional rescaling within each measurement lot of 100 spectra applying extended multiplicative scatter correction using the mean spectrum of each replicate measurement of 100 spectra as a reference (EMSC [Liland et al. 2016]).

We analyzed spectra of five vaccines as listed in Table 1 and background spectra measured without vaccine as a control largely representing the background signal from the CaF2 slide and measurement device. All spectra were subjected to principal component analysis and linear discriminant analysis as follows:

### Table 1: Vaccine products used in this study.

| Vaccine product (antigen composition) | Type           | Number of manufacturer’s batches | Total number of replicates |
|--------------------------------------|----------------|----------------------------------|----------------------------|
| DTaP-IPV-HepB                         | For primary vaccination | 3                                | 18                         |
| DTaP                                 | For booster vaccination | 3                                | 24                         |
| DTaP-IPV                             | For booster vaccination | 3                                | 18                         |
| Pneu1                                | For primary vaccination | 1                                | 6                          |
| Pneu2                                | For primary vaccination | 1                                | 6                          |

Vaccine antigens: T, tetanus; d; D, diphtheria (low and high antigen content); aP, acellular pertussis antigen; IPV, inactivated poliovirus; HepB, hepatitis B; Pneu, pneumococcal capsular polysaccharides.
to pre-processing procedures 1–3 and major variability in
the resulting spectral data was subsequently assessed
through principal component analysis. The reduction of
spectral space to dimensions of highest variability in the
data allows for an exploratory overview of (dis-)similarities
between vaccine spectra. Mean spectra of each vaccine
type together with the first four principal components are
shown in Figure 1. The latter represent directions in spec-
tral space showing highest variance in data (in consecutive
order). This technique of unsupervised learning allows to
explore between vaccine (and background) variation
without prior knowledge, i.e. spectral measurements were
colored in Figure 1 according to vaccine type for identifi-
cation but this knowledge is not considered in the deter-
mination of principal axes.

The results show that vaccines are separated in spec-
tral space but that their arrangement is affected by the
chosen pre-processing procedures. This means that sub-
sequent classification based on supervised learning is
feasible, but its outcome will be influenced by the chosen
pre-processing procedures.

This is not surprising given that the differences in
considered spectral ranges between procedures 1 and 2
correspond to a selection of features that might corre-
spond to a focus on or neglect of features that are
particularly relevant to distinguish certain vaccines. Feature selection is often necessary to allow for reliable
classification of high dimensional data in which their
complexity cannot be matched by the available data
(Hastie et al. 2017). Extended multiplicative scatter
correction used in pre-processing procedure 3 reduces
fluctuations in spectral intensity among replicate mea-
surements through model-based alignment with a
reference spectrum, in this case a rescaling towards the
mean spectrum of each replicate measurement of 100
spectra (Liland et al. 2016). This reduces the effective
sample size of measured spectra but also variability in
spectral intensity that may arise through inhomoge-
neous drying and crystallization as can be seen in the
right column of Figure 1. Linear discriminant analysis
(LDA) was applied as a classification model for the dif-
fences between vaccine products (Hastie et al. 2017).
The results are summarized in a confusion table (Table 2)
showing overall high percentages of correctly classified
vaccines. Yet, differences in the model predictions
for pre-processing procedures are evident illustrating
the impact of spectral pre-processing on subsequent
classification.

Given the complexity and variety of measurement
setups and devices as well as measurement samples we
have to acknowledge that there is no globally optimal or
preferable pre-processing procedure. Naturally, pre-
processing is guided by the aim to selectively extract fea-
tures relevant for the specific question addressed by the
spectroscopic method (Gerretzen et al. 2015). Distinctive
features may be expected within in specific spectral ranges
or require a certain spectral resolution (Larkin 2011;
McCreery 2000). However, chemometric prediction models
are affected by the pre-processing of spectral data that are
used for their training and validation, this holds particu-
larly for samples with overlapping spectral characteristics.
Therefore, the choice of pre-processing procedures is part
of the design of experiment (Gerretzen et al. 2015) which
should be made transparent as part of the chemometric
model: knowledge about prior information guiding feature
selection and the choice of algorithms and their parameters
used in pre-processing are relevant to define the range of
application and limitations of a chemometric model. A
reproducible implementation of the analysis pipeline
within an OpenSource framework is particularly favour-
able as its full transparency leaves little space for ambi-
guities and can support community efforts towards
standards and best practices in spectral pre-processing.

Overall, we consider reproducibility and stand-
ardisation as one of the major challenges that need to be
met at various levels to exploit the full potential of Raman
spectroscopy for pharmaceutical applications in the
context of biomedicines. While these challenges have
already been met for many pharmaceutical applications,
our case study on vaccines is a good showcase for the
challenges encountered in the spectral characterization of
biomedicines including instrumentation, sample prepara-
tion and chemometric modelling. Drying of vaccines in-
creases the concentration of components of interest but
can result in product- and preparation-specific, inhomoge-
neous crystallization patterns, which may not be stable
over time and subject to environmental conditions (Silge
et al. 2018). As the assessment of the native product is the
eventual goal experimental protocols for measurements of
native products or calibration procedures linking to sub-
stitute formulations (e.g. dried products) need to be
developed (Byrne et al. 2020). The former involves on the
one hand to capture low concentrations of medically
relevant compounds such as proteins or polysaccharides in
liquid solution and on the other hand to deal with complex,
turbid formulations showing sedimentation. This requires
sophisticated sample preparation and instrumentation –
again, calling for standardisation.

A recent proficiency study (Guo et al. 2020) has made
valuable contributions in analyzing the impact of various
spectroscopic platforms and devices on the primary acquisi-
tion of raw spectral data. Similar care is mandatory for
Figure 1: Presentation of vaccine spectra along four principal component axes (PC1–PC4) for pre-processing procedures 1–3. Principal component axes represent highest variance seen in the data (percent explained variance in brackets). Vaccine spectra show distinct clustering in spectral space, however, their particular arrangement is influenced by the chosen pre-processing procedures. Corresponding mean spectra are shown in the bottom panel with bands indicating the 16th and 84th percentile (corresponding to ± one standard deviation for normally distributed data).
sample preparation of biomedicines, i.e. heterogeneous and often liquid formulations, for which reproducible and stable measurement conditions have to be defined. Finally, a well-defined and transparent integration of pre-processing and chemometric modelling will help to develop a reliable and reproducible data- and knowledge-base of spectral information. This will ultimately allow to define spectral fingerprints of biomedical products and to draw the line at falsified products or products of low quality.

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### Table 2: Confusion table for vaccine products used in this study.

| Procedure | Vaccine            | Sensitivity | Specificity |
|-----------|--------------------|-------------|-------------|
| 1         | dTaP               | 96%         | 99%         |
|           | dTaP-IPV           | 94%         | 100%        |
|           | DTaP-IPV-HepB      | 98%         | 99%         |
|           | Pneu               | 96%         | 98%         |
|           | Pneu2              | 98%         | 100%        |
| 2         | dTaP               | 97%         | 98%         |
|           | dTaP-IPV           | 94%         | 99%         |
|           | DTaP-IPV-HepB      | 96%         | 99%         |
|           | Pneu               | 96%         | 98%         |
|           | Pneu2              | 98%         | 99%         |
| 3         | dTaP               | 97%         | 99%         |
|           | dTaP-IPV           | 94%         | 100%        |
|           | DTaP-IPV-HepB      | 96%         | 99%         |
|           | Pneu               | 100%        | 98%         |
|           | Pneu2              | 100%        | 100%        |

Classifications were comparatively tabulated following a 6-fold cross validation: models are trained on five vaccine spots (each with a lot of 100 spectra, as used in EMSC of pre-processing procedure 3) and predictions were made on the remaining vaccine spot based on spectra that were treated with pre-processing procedures 1–3 (number of spectra, percentages of sensitivity and specificity are rounded to whole percentages). Overall there is a high percentage of correctly classified spectra. However, changes in pre-processing procedures introduce shifts in misclassified spectra.

**Conflict of interest statement:** The authors declare no conflicts of interest regarding this article.

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