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Technical Note

Protein-based medicines analysis by Raman spectroscopy for the detection of counterfeits

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ABSTRACT

Protein-based medicines, or large molecule medicines, are innovative products used to treat various diseases like hepatitis or cancer. This new generation of molecules are usually expensive, and thus represents an attractive target for the counterfeiters. Due to the complexity of their chemical structure, their analysis for counterfeit detection is more difficult than small molecule medicines. The aim of the article is to demonstrate that Raman spectroscopy and microscopy can be used for the fast analysis of counterfeits of protein-based medicines. Twelve types of medicines, under liquid or lyophilized form, have been analyzed by a Raman spectrometer through their glass packaging and ten of them also by a Raman microscope with drop deposition on a gold plate. The optimization of the acquisition parameters has first been described. Then the identification of the studied products has been presented with the attribution of the protein bands observed on the spectra. Finally the methods were successfully applied to seven counterfeits of these products and their chemical composition identified by spectral analysis. Counterfeits can indeed be detected if the excipient profile differs, if no protein is present, or if the genuine sample has been strongly diluted by the counterfeiters. Raman spectroscopy and microscopy have thus proved efficient for the fast analysis of counterfeits of protein-based medicines.

1. Introduction

The counterfeiting of medicines is a serious issue that concerns nowadays all parts of the world and all types of medication. While its long targeted life style medicines principally, lifesaving products are today largely impacted. The chemical composition of the counterfeits is also very variable. The counterfeits can contain another API (active pharmaceutical ingredient), a different amount of API, no API, toxic compounds, or sometimes even only dirty water [1–8]. Independently from the composition of the samples, another risk coming from the counterfeits is the appalling conditions in which they are produced and stored [9–11]. Organized crime has been proved to be behind the trafficking of counterfeits, thus requiring fast investigation of the cases [12–15]. Quick and efficient analytical tools are essential for the authentication of suspect samples and the detection of the compounds present in the confirmed counterfeits [16,17]. So far numerous methods have been presented in the literature for the detection of counterfeits, mostly based on spectroscopic [18–24] and chromatographic [25–28] techniques.

While several solutions have been offered for the analysis of medicines under the form of tablets and capsules, much fewer publications are available for the analysis of counterfeits of large molecules. More and more protein-based products are being launched on the market. These new and often costly medicines constitute an attractive target for the counterfeiters. For their analysis the same technology could be investigated than the one used for solid-state products, with some adaptation concerning especially the sampling. Raman spectroscopy has for instance been increasingly studied for the analysis of protein samples in general, using simple instrumentation [29–34], or more advanced sampling like Surface-Enhanced Raman Spectroscopy (SERS) [35–38] or Tip-Enhanced Raman Spectroscopy (TERS) [39,40]. Raman spectroscopy offers indeed the possibility to detect protein bands, with little sample preparation and measurement lasting from a few seconds to a few minutes. The application to the analysis of counterfeits of protein-based medicines has been approached by Kalyanaraman et al. [41] in 2013 with the identification of one lyophilizate medicine and later by Peters et al. [42] in 2016 with the identification of three proteins by Drop Coat Deposition technique and confocal Raman spectroscopy.

This paper presents the authentication of twelve protein-based medicines under different forms, liquid and lyophilizate, and also the analysis of seven of their counterfeits. The study was

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performed using first Raman spectroscopy through glass packaging and then Raman microscopy with drop deposition on gold plates.

2. Material and methods

2.1. Instruments' characteristics

2.1.1. Instruments and software

The DXR SmartRaman spectrometer from Thermo Fisher Scientific was used for the analysis of the proteins in their packaging. The measurements were made through glass syringes and glass vials. No preparation of the samples was needed.

The DXR Raman microscope (Thermo Fisher Scientific) was then used for the measurements of the proteins on standard gold coated plates (75 mm × 25 mm). A drop of the liquids of about 20 μL was deposed on the plates and then dried using the under vacuum dryer EM SCD050 from Leica for 10 min.

The devices can use either a 532 nm or a 785 high power near infrared excitation laser. The measurements range from 50 cm^{-1} to 3500 cm^{-1} (using the "full range" grating) or from 50 cm^{-1} to 1800 cm^{-1} (using the "high resolution" grating).

The OMNIC™ software is used to set parameters, perform the measurements, exploit the spectra and register them into databases.

2.1.2. Studied parameters

2.1.2.1. Raman spectrometer. The quality of the Raman spectra highly depends on the parameters chosen for the acquisition, especially for liquid samples. Moreover taking Raman spectra through the glass might be quite challenging. That is why tests have been made prior to the analyses in order to optimize the acquisition parameters for each type of analyzed sample – liquid in syringe, liquid in vial, and lyophilizate in vial.

Two lasers can be used to analyze the samples, 532 nm or 785 nm, which can be set at different powers: up to 10 mW for the 532 nm laser and up to 150 mW for the 785 nm laser. Moreover for each laser two grating types are available, providing either a wider range or a better resolution. The grating "Full Range" enables measurements from 50 to 3500 cm^{-1} with a 5 cm^{-1} nominal resolution, while with the "High Resolution" grating, measurements can be performed between 50 and 1800 cm^{-1} with a 2 cm^{-1} nominal resolution.

Different possibilities of sampling have also been tested. While the syringes can only be measured on the side, the vials can be measured either on the bottom or on the side.

The exposure time and the number of scans per measurement have an influence on the quality of the spectra and were therefore also investigated. The "autoexposure" function proposed by the software was also tested, which automatically optimizes the parameters of exposure needed for each sample.

Raman spectroscopy can be subject to fluorescence. This phenomenon tends to decrease the quality of the spectra by masking the bands of the components. A fluorescence correction was tested in order to reduce the influence of this phenomenon on the spectra.

The height of the focalization point is regulated by the focus function. Because samples were measured through the glass, it had to be checked that the focalization point was on the sample and not on the glass. Different values of focus were then tested for each sample type.

The aperture controls how much signal passes into the spectrometer and the detector. The "pinhole" apertures are confocal holes, which increase the spectral resolution and decrease the field depth. The "slit" apertures improve the optic resolution while decreasing the field depth on one axis. Both types of aperture were tested, together with two sizes of aperture: 25 μm and 50 μm.

2.1.2.2. Raman microscope. Such as for the Raman spectrometer, several parameters were studied that can influence the quality of the spectra. Most are common to Raman spectroscopy. The additional parameter to study was the objective of the microscope. Four objectives were available: ×10, ×20, ×50 objectives with standard working distance and ×50LM objective with long working distance. For each measurement, the focus had to be made manually on each sample. Contrary to the analyses made with the spectrometer, only one sampling mode was tested: the measurement of the dried residues on the gold plates.

2.1.3. Pretreatments

The spectra generated by both the Raman spectrometer and microscope might need the use of pretreatments for further exploitation, for instance when spectral match correlation or supervised or unsupervised classification using chemometric tools are applied for the identification of the protein. While the fluorescence was corrected by the previously described fluorescence correction provided by the software, the quality of the spectra might be altered by other phenomenon, like laser fluctuations [43]. Smoothing filters like Savitzky–Golay filtering [44] can for instance be used to compensate for these fluctuations. Other pretreatments can be applied to Raman spectra like baseline correction (for instance detrending), normalizations techniques, like the Standard Normal Variate (SNV), or even first or second derivatives [45,46].

2.2. Samples

2.2.1. Genuine samples

Twelve different protein-based medicines have been analyzed by Raman spectroscopy, each of them possessing at least one formulation. 33 different formulations have then been measured on the whole. The list of the products, anonymized for confidentiality reasons, is presented in Table 1. The studied medicines were available under three forms: liquid in glass vials, liquid in glass syringes, and lyophilizate in glass vials. The three lyophilized samples were only measured by Raman spectroscopy through the packaging and not by Raman microscopy since they are high potent products. All the other medicines present in liquid form have been analyzed by Raman microscopy, with in total 29 formulations. The products were stored at 5 °C until the analysis.

2.2.2. Confirmed counterfeits

Seven counterfeits were measured by the Raman spectrometer. Five are counterfeits of the product B (under the form of liquid in vials), one is a counterfeit of the product K (under the form of a liquid in a vial) and the last one, a liquid in a syringe, is a counterfeit of the product L.

One counterfeit was tested on the Raman microscope, Counterfeit 1 of the product B, under the form of liquid in vial.

3. Results and interpretation

3.1. Results of the parameter optimization

3.1.1. Parameters used for the spectrometer

Several tests were performed on the liquids in vials, liquids in syringes and lyophilizates in vials in order to define the best parameters for the acquisition of the spectra through the