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Authors: Hanssen L.J.J.; De Beer T., Pierre K., Pastoret S., Bonnegarde-Bernard A., Daoussi R., Vervaet C., Remon J.P.

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FTIR spectroscopy for the detection and evaluation of live attenuated viruses in freeze dried vaccine formulations

Laurent Hansen¹, Karin Pierre³, Soumya Pastoret³, Astrid Bonnegarde-Bernard³, Rim Daoussi³, Chris Vervae², Jean Paul Remon², Thomas De Beer¹.

¹Laboratory of Pharmaceutical Process Analytical Technology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

²Laboratory of Pharmaceutical Technology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium.

³Zoetis, Hoge Wei 10, 1930 Zaventem, Belgium.

Correspondence to: T. De Beer (Tel: +32-9-2648097; Fax: +32-9-2228236; E-mail: Thomas.DeBeer@UGent.be)

Abstract:

This article examines the applicability of Fourier Transform Infrared (FTIR) spectroscopy to detect the applied virus medium volume (i.e., during sample filling), to evaluate the virus state and to distinguish between different vaccine doses in a freeze dried live, attenuated vaccine formulation. Therefore, different formulations were freeze dried after preparing them with different virus medium volumes (i.e., 30µl, 100µl and 400µl) or after applying different pre-freeze-drying sample treatments (resulting in different virus states); i.e., (i) as done for the commercial formulation; (ii) samples without virus medium (placebo); (iii) samples with virus medium but free from antigen; (iv) concentrated samples obtained via a centrifugal filter device; and (v) samples stressed by 96h exposure to room temperature; or by using different doses (placebo, 25-dose vials, 50-dose-vials and 125-dose vials). Each freeze-dried product was measured directly after freeze-drying with FTIR spectroscopy. The collected spectra were analyzed using principal component analysis (PCA) and evaluated at three spectral regions which might provide information on the coated proteins of freeze dried live, attenuated viruses: (i) 1700-1600cm⁻¹ (amide I band), 1600-1500cm⁻¹ (amide II band) and 1200-1350cm⁻¹ (amide III band). The latter spectral band does not overlap with water signals and is hence not influenced by residual moisture in the samples. It was proven that FTIR could distinguish between the freeze-dried samples prepared using different virus medium volumes, containing different doses and using different pre-freeze-drying sample treatments in the amide III region.

Keywords: FTIR spectroscopy, principal component analysis (PCA), NIR spectroscopy, freeze drying, live, attenuated vaccines

Introduction:

Live, attenuated viruses are used in many vaccines because of the strong immune response they generate.¹ However, their potency is severely compromised by their low stability in aqueous solutions. To overcome this, most live, attenuated virus formulations are freeze-dried.²

After freeze-drying, the potency of live, attenuated viruses measured by titration is generally evaluated in a few randomly collected samples by assays measuring their biological response *in vitro* (i.e., cell-based assays). These tests are often considered imprecise, labor-intensive and time-consuming.³⁻⁵

Near infrared (NIR) and Raman spectroscopy are frequently used as non-invasive, non-destructive and fast analytical techniques for pharmaceutical products.⁶⁻⁸ One of the first freeze drying related applications of NIR spectroscopy was the determination of residual moisture content in the end products (i.e., through the glass vials).⁹⁻¹² Afterwards, NIR and Raman in-process monitoring of excipient¹³⁻¹⁵ and protein¹⁶ behavior during freeze drying was performed.

Recently, also the possibility of NIR spectroscopy to evaluate live, attenuated virus vaccine formulations has been examined.¹⁷ For a freeze-dried live, attenuated virus vaccine formulation, NIR spectroscopy was able to distinguish between samples prepared using different virus medium volumes or using different pre-freeze-drying treatments. This distinction could be made by evaluating two NIR spectral regions: (i) the 7300-4000cm⁻¹ region containing the amide A/II band which might reflect information on the coated proteins of the freeze-dried live, attenuated viruses; and (ii) the C-H vibration overtone regions (10,000-7500 and 6340-5500cm⁻¹) which might supply information on the lipid layer surrounding the freeze-dried live, attenuated viruses.

Fourier transform infrared (FTIR) spectroscopy is a reference technique frequently used to obtain information about the secondary structure of proteins.¹⁸ The amide I and amide II bands are two characteristic bands for proteins and polypeptides. Both arise from the amide bonds linking the different amino acids. The amide I (~ 1650cm⁻¹) is associated to stretching vibrations of the C=O bond of the amide with minor contributions from the out-of-phase CN stretching vibration, whereas the amide II (~ 1550cm⁻¹) is associated to bending vibrations of the N-H bond and stretching vibrations of the C-N bond.¹⁸ Since both, the C=O and N-H bonds, are involved in the hydrogen bonds responsible for the protein secondary structure, frequency shifts of these amide I and II bands are associated to protein secondary structure changes. The amide III band (1400 to 1200cm⁻¹) is the combination of the N-H bending and the C-N stretching vibration.^{18,19} The amide III is also influenced by side-chain vibrations that vary considerably making this band less suited for secondary structure analysis.¹⁸ However, in contrast to the amide I and II bands the amide III band offers the advantage not to show overlap with water signals, which is important when analyzing freeze-dried samples.²⁰⁻²²

Several studies have evaluated freeze dried proteins secondary structure^{16,23-25} and bacteria (lactic acid bacteria protein secondary structure and lipid CH₂ vibration)²⁶ using FTIR spectroscopy. However, to our best knowledge, there is no paper demonstrating the possibility of FTIR spectroscopy to evaluate live, attenuated viruses in freeze dried formulations.

The selection rule for molecules to be FTIR and NIR active is identical, being a change in dipole moment during their normal modes. However, FTIR spectroscopy mainly measures the fundamental vibrations of molecules, whereas NIR spectroscopy mainly measures overtones and combinations of fundamental vibrations. Overtone and combination bands have a lower intensity than fundamental bands, resulting in detection sensitivity differences at low levels of analyte (FTIR being more sensitive). Because of the anharmonicity of the atomic vibrations, the NIR bands related to particular functional groups of an analyte are often broad and overlapping²⁷ making their analysis complex and requiring chemometrics .

The aim of this study was to examine the suitability of FTIR spectroscopy to evaluate live, attenuated viruses in freeze dried vaccine formulations. Two different formulations provided by a pharmaceutical company were studied. Being available in large quantities, product 1 was freeze-dried after preparing it with different virus medium volumes (i.e., 30µl, 100µl and 400µl) or after applying different pre-freeze-drying sample treatments (resulting in different virus states); i.e., (i) as done for the commercial formulation; (ii) samples without virus medium (placebo); (iii) samples with virus medium but free from antigen; (iv) concentrated samples obtained via a centrifugal filter device; and (v) samples stressed by 96h exposure to room temperature. Product 2, containing another live attenuated virus, was provided by the company in its freeze dried form. These freeze-dried samples varied in the number of viral particles present in the virus medium, resulting in different multidose vials (i.e. placebo, 25-dose vials, 50-dose vials and 125-dose vials).

Material and Methods:

1. Materials

The live, attenuated viral vaccines were provided by Zoetis.

Trehalose, a well-known cryo-/lyoprotectant used in many freeze dried formulations, was used as stabilizer at a concentration of 9% (w/v) in product 1.

To study the capability of FTIR spectroscopy to evaluate the virus state, live, attenuated viruses were differently pre-treated (table 1b). Depending on the applied pretreatment, the virus quality, integrity or state might vary. The resulting formulations were named after their pretreatment: 'normal' (i.e., as used in the commercial formulations,), 'absent' (i.e., placebo formulations without virus medium), 'concentrated' via a centrifugal filter device (Millipore, Amicon® Ultra), 'medium' (i.e., placebo formulations where virus medium without viruses was used) and 'stressed' (by storage at room temperature for 96hours). For clarity, the 'concentrated' virus samples were prepared using a similar virus culture medium volume as the 'normal' virus samples. However due to the centrifugation pretreatment process, the 'concentrated' samples contain more viral particles in that volume. The other formulation components (stabilizers and buffer) were kept constant (table 1b).

Besides investigating whether FT-IR is able to evaluate the differently pretreated viruses, its ability to detect differences in added virus culture medium volume was studied as well. Three virus formulations varying in virus medium volume were prepared (table 1a). Stabilizer and buffer were kept constant (concentration and volume).

Table1: Product 1: Formulations used in the (a) virus volume study and (b) virus pre-treatment study.

	Virus pretreatment	Virus medium containing viruses (μl)	Medium without viruses (μl)	Buffer (μl)	Trehalose	<i>qsp</i> filling volume (μl)	Total volume (μl)
a)	Normal	30	0	y	9% w/v	i + 370	800
	Normal	100	0	y	9% w/v	i + 300	800
	Normal	400	0	y	9% w/v	i + 0	800
b)	Normal	x	0	y	9% w/v	j	800
	Stressed	x	0	y	9% w/v	j	800
	Concentrated	x	0	y	9% w/v	j	800
	Medium	0	x	y	9% w/v	j	800
	Absent	0	0	y	9% w/v	j + x	800

All symbols (x, y, i and j) represent absolute values in μl.

The stabilizer of product 2, containing another live, attenuated virus, is more complex as it contains several components (lactose, sorbitol, dextran, casein). Three formulations containing different doses (125, 50 and 25 doses) and one placebo formulation were prepared and freeze dried by Zoetis (table 2).

Table2: Product 2: Dose study

Virus pretreatment	Doses	Virus medium volume (ml)	Stabilizer solution	<i>qsp</i> filling volume, medium free from viruses (ml)	Total volume (ml)
Normal	125	e	33% (v/v)	h-e	5

Normal	50	f	33% (v/v)	h-f	5
Normal	25	g	33% (v/v)	h-g	5
Absent	0	0	33% (v/v)	h	5

All symbols (e, f, g and h) represent absolute values in ml.

2. Freeze-Drying

Product 1 was freeze dried using an Amsco FINN-AQUA GT4 freeze dryer (GEA, Köln, Germany). A conservative cycle resulting in elegant cakes, without any signs of collapse, was used (The process settings can't be disclosed for confidentiality reasons). All product 1 formulations (virus volume study and virus pretreatment study) were freeze dried together.

Product 2 was freeze dried using a Lyostar 3 (SP scientific, Stone Ridge, NY, USA) with a conservative cycle providing elegant cakes without signs of collapse (The process settings can't be disclosed for confidentiality reasons). The different dose formulations and the placebo formulation were freeze dried together.

3. FTIR spectroscopy

FTIR spectra were recorded using an ATR FT-IR spectrometer (Thermo Fisher Scientific, Nicolet iS5 ATR FT-IR spectrometer). The sample was grinded in the vial and then pressed against a diamond ATR crystal. One spectrum per freeze dried vial was collected in the 4000 - 550 cm^{-1} spectral range with a resolution of 8 cm^{-1} and averaged over 32 scans.

4. NIR spectroscopy

Besides the traditional applied reference method (titration, see further), NIR spectroscopy was also used as additional tool to evaluate the different freeze-dried formulations¹⁷ and to compare with the FT-IR observations. NIR spectra of all freeze-dried samples were collected off-line using a Fourier-Transform NIR spectrometer (Thermo Fisher Scientific, Nicolet Antaris II near-IR analyzer) equipped with an InGaAs detector and a quartz halogen lamp. All NIR spectra were recorded in the 10000-4000 cm^{-1} region with a resolution of 8 cm^{-1} and averaged over 16 scans. One NIR spectrum per freeze dried sample (in random order) was collected through the bottom of the glass vial using the integrating sphere device immediately after freeze-drying.

5. Data analysis

FTIR and NIR spectral data analysis was performed using SIMCA P+ v.13.0.3 (Umetrics, Umeå, Sweden).

Depending on the spectroscopic tool (FTIR or NIR spectroscopy) and analyzed spectral region, different pre-processing methods were applied (table 3). The evaluated spectral regions were selected in order to reflect information about the coated proteins (Haemagglutinin and Neuraminidase) from the freeze-dried live, attenuated viruses (see introduction). Therefore, the three amide bands (Amide I, II and III) in the FTIR spectra and the amide A/II band in the NIR spectra were analyzed.

Table3: Analyzed spectral ranges and corresponding applied pre-processing

	Preprocessing	Spectral region	Wavenumbers (cm ⁻¹)
FTIR spectroscopy	SNV	Amide I	1700-1600
	SNV	Amide II	1600-1500
	SNV	Amide III	1200-1350
	SNV and 2 nd derivative (17 Savitzky-Golay points)		
NIR spectroscopy	SNV and 2 nd derivative (37 Savitzky-Golay points)		7300-4000

FTIR spectra were analyzed using principal component analysis (PCA), allowing to examine the spectral differences between for instance the different sample types per batch (table 2). PCA produces an orthogonal bilinear data matrix (**D**) decomposition, where principal components (PCs) are obtained in a sequential way to explain maximum variance:

$$\mathbf{D} = \mathbf{TP}^T + \mathbf{E}$$

$$= \mathbf{t}_1\mathbf{p}'_1 + \mathbf{t}_2\mathbf{p}'_2 + \dots + \mathbf{t}_Q\mathbf{p}'_Q + \mathbf{E}$$

Where **T** is the $M \times Q$ score matrix, **P** is the $N \times Q$ loading matrix, **E** is the $M \times N$ model residual matrix, i.e., the residual variation of the data set that it is not captured by the model. Q is the selected number of PCs, each describing a non-correlated source of variation in the data set, and N is the number of collected spectra at M wavelengths.²⁸ Each principal component consists of two vectors, the score vector **t** and the loading vector **p**. The score vector contains a score value for each spectrum, and this score value informs how the spectrum is related to the other spectra in that particular component. The loading vector indicates which spectral features in the original spectra are captured by the component studied. These abstract, unique and orthogonal PCs are helpful in deducing the number of different sources of variation present in the data. However, these PCs do not necessarily correspond to the true underlying

factors causing the data variation, but are orthogonal linear combinations of them, since each PC is obtained by maximizing the amount of variance it can explain.²⁹

The number of PC included to describe the model was determined by cross validation. Each PC resulting on an increase of the predictive ability (Q^2) of the model was kept.

The collected NIR spectra were also evaluated using principal component analysis (PCA).

6. Titration

Titration of product 1 and 2 was done according to Zoetis internal SOPs. Each titer is expressed in $\log_{10}$ CCID₅₀ (Cell Culture Infection Dose 50 – Inverse of the highest dilution which produces a cytopathogenic effect in 50% of the cells). Titration provides information about the number of living viral particles contained in each vial. For each study (virus pre-treatment, virus volume and virus dose), statistical analysis was performed to evaluate if the titers of different vial populations (e.g. 125-dose, 50 and 25dose vials or ‘stressed virus’, ‘normal virus’ and ‘concentrated virus’) were significantly different. Minitab® 15.1.1.0 software was used to perform the statistical analysis.

When the titer distribution was normal (evaluated using a Kolmogorov-Smirnov test) and the variance equal (Levene’s test) an Anova I test was performed. When the distribution was determined as not normal or when the number of observation in each population was low, a non-parametric Kruskal-Wallis analysis of variance was used. In addition to the Kruskal-Wallis test, in order to determine which population is significantly different from the others, Dunn’s test was performed using a Matlab m-file³⁰ as follow-up test (Matlab 7.12, The Mathworks, Natick, MA).

7. Karl Fischer

The residual moisture content of freeze-dried samples was determined by Karl Fischer titration. For product 1, a Mettler Toledo V30 volumetric Karl Fischer titrator (Schwerzenbach, Switzerland) with Hydranal® titration solvent from Sigma Chemical Company was used. A known volume of dried methanol was added to the sampled vial, and left to equilibrate for a few minutes. From the solution, a known volume was then removed volumetrically using a syringe and injected into the titration cell. The water content of pure methanol was determined in duplicate prior to the measurement and subtracted from the result.

Product 2 was measured with a Metrohm 860 KF Thermoprep (oven) coupled to a Metrohm 852 Titrando (Herisau, Switzerland).

Results and discussion

The discussion of the results is divided into three parts: (i) virus volume study (table 1a); (ii) virus pre-treatment study (table 1b) and (iii) dose study performed using product 2 (table 2).

Virus volume study

To evaluate the ability of FTIR spectroscopy to distinguish between applied live, attenuated virus medium volume in the freeze-dried samples, three formulations were prepared containing 30 μ l, 100 μ l and 400 μ l of virus medium volume, respectively (table 1a). The 30 μ l virus medium volume vials had a titer of 6.93 ± 0.2 (n=3) which differed significantly from the 400 μ l virus medium volume vials having a titer of 8.67 ± 0.56 (n=3) (Kruskal-Wallis, $p < 0.05$, fig. 1). The 100 μ l virus volume vials had a titer of 7.82 ± 0.21 (n=3) which did not significantly differ from the 30 μ l and 400 μ l virus volume samples (Kruskal-Wallis, $p < 0.05$, fig. 1). Regarding the titration results fig. 1(left), a significant difference between all groups of sample was expected. This surprising result obtained via Kruskal-Wallis analysis (selected because of the small amount of titrated samples), might be due to the fact that this statistical analysis consider the population medians instead of the population means (Anova I),

The Karl fisher analysis revealed an increase in residual moisture with increasing virus medium volume. The 30, 100 and 400 μ l virus medium volume samples had a residual moisture of $1.28\% \pm 0.05$ (n=3), $1.83\% \pm 0.13$ (n=3) and $3.68\% \pm 0.89$ (n=3), respectively (fig. 1).

Fig.1: (left) Titration results of the different virus medium volume samples. Titers are expressed as log₁₀ CCID₅₀ (Cell Culture Infection Dose 50). Each titer is the average of three determinations (Fig.1, right). Residual moisture results of the different virus medium volume samples. Each result is the average of three determinations.

After freeze drying, FTIR spectra of 10 vials per virus medium volume class (i.e. 30 spectra in total) were collected.

As described in the materials and method section, the aim was to evaluate the three amide spectral regions (I, II and III) in the FTIR spectra. Figure 2 shows an FTIR spectrum of a freeze dried product from this virus volume study and the FTIR spectrum of water. The amide I (1700-1600 cm^{-1}) and II (1600-1500 cm^{-1}) bands clearly overlap with the strong water signal. As the residual moisture varies between the three different virus medium volume sample types (fig.1), the FTIR spectra of the virus volume study were not analyzed in the amide I and II regions. However, since water does not overlap

with the Amide III region (fig.2), the FTIR spectra of the virus volume study were analyzed using the amide III (1350-1200 cm^{-1}) spectral region using PCA.

Figure 2: Spectra of water (blue dashed line) and a freeze dried virus volume study sample (black line).

The resulting PCA model (composed of two components) described 99.6% of the spectral variability. The first PC explained almost all the spectral variability (99.3%) and distinguished the samples according to their virus medium content (fig. 3a). The distances in the scores plot between the virus medium volume groups are in agreement with the actual difference in virus medium content. Analysis of the loadings of the first principal component revealed one major peak at 1290 cm^{-1} (fig.3b). This peak is located in a spectral range (1300-1270 cm^{-1}) typical for α -helix.¹⁹ Analysis of the snv corrected spectra (fig. 3c) clearly showed a difference in peak intensity between the three virus medium volumes and confirmed the observation of the scores and loadings plots.

Fig. 3: Scores (a) and loadings (b) plots obtained after PCA on the FTIR spectra (1200-1350 cm^{-1} spectral region) from the virus volume samples 30, 100 and 400 μl . (c) SNV corrected FT-IR spectra of the 1200-1350 cm^{-1} spectral region (the arrow indicates the positive direction of PC1).

The scores plot of the FTIR PCA models allowed the distinction of the three different virus volumes (30, 100 and 400 μl).

To confirm this FTIR analysis, also NIR spectra of twenty vials per virus medium volume class were collected and analyzed using the 7300-4000 cm^{-1} spectral region (after snv and 2nd derivative preprocessing), as demonstrated in our previous study.¹⁷ The PCA model (composed of two components) developed from the 60 spectra described 84.3% of the spectral variability. The PC1 versus PC2 scores plot showed that the first PC (explaining 63.8% of the spectral variability) distinguished the samples according to their virus medium volume (fig.4a). Similarly as previously described in¹⁷, analysis

of the PC1 loadings plot revealed two major contributions: the water bands (~ 7000 and $\sim 5200\text{cm}^{-1}$) and the amide A/II band (4850cm^{-1}) (fig.4b).

Fig. 4: Scores (a) and loadings plots (b) obtained after PCA on the NIR spectra from the samples 30, 100 and 400 µl using the $7300\text{--}4000\text{cm}^{-1}$ spectral region.

To avoid the contribution of the water bands on the principal component analysis of the NIR spectra, a new PCA model was built for the $5029\text{--}4000\text{cm}^{-1}$ spectral range. The new two component PCA model explained 76.4% of the spectral variance and also distinguished between the different virus volumes (data not shown).

These results described above confirm that FTIR spectroscopy is able to distinguish between different virus medium volume in the freeze-dried formulations. Analysis of the loadings plot has shown that this distinction was caused by a difference in α -helix between the different virus medium volumes. The variation of this protein secondary structure between the different virus medium volumes was not possible with NIR spectroscopy. To avoid the impact of the residual moisture variation on the virus volume determination, the amide III spectral region should be used, since this spectral band does not overlap with water bands.

Virus pre-treatment study.

To evaluate the ability of FTIR spectroscopy to distinguish between differently pre-treated (prior to freeze-drying) live, attenuated virus samples, five formulations were prepared as overviewed in table 1b. The three formulations containing viruses were titrated. The “stressed virus” samples had a titer of 6.12 ± 0.1 ($n=3$) which significantly differed from the “concentrated virus” samples having a titer of 7.72 ± 0.06 ($n=3$) (Kruskal-Wallis, $p < 0.05$, fig. 5 left). The “normal virus” samples had a titer of 6.93 ± 0.2 ($n=3$) which did not significantly differ from the “stressed” and “concentrated” virus samples. A significant titer difference between all groups of sample was expected. The analyse of the population medians performed by the Kruskal-Wallis procedure might explain this surprising statistical results.

Karl fisher analysis revealed no significant difference in residual moisture between the different pre-treated samples. The normal, stressed, concentrated, absent and medium had a residual moisture of 1.28 ± 0.05 (n=3), 1.26 ± 0.18 (n=3), 1.42 ± 0.1 (n=3), 1.19 ± 0.11 (n=3) and 1.30 ± 0.2 (n=3), respectively (fig. 5 right).

Fig. 5: Titration results of the different pre-treated samples (left). Titters are expressed as log₁₀ CCID₅₀ (Cell Culture Infection Dose 50). Each titer is the average of three determinations. Residual moisture results of the different pre- treated virus samples (right). Each result is the average of three determinations.

After freeze drying, ten FTIR spectra per pre-treatment group (i.e. 50 spectra in total) were collected. Because the residual moisture was not significantly different between the different pre-treatment groups, the FTIR spectra were analyzed using PCA for different spectral ranges, i.e., the 1700-1600cm⁻¹ (amide I band), the 1600-1500cm⁻¹ (amide II band) and the 1200-1350cm⁻¹ (amide III band). According to the titration results, only two groups containing viruses have significantly different titers, being the 'stressed' and 'concentrated' samples. Therefore, PCA models were first built using the spectra of these both groups.

Spectral region 1700-1600cm⁻¹, Amide I band:

The PCA model (composed of three principal components) developed from the 20 FT-IR spectra of the 'stressed' and 'concentrated' samples described 95% of the overall spectral variability. It was not possible to distinguish between concentrated and stressed samples using the amide I band (data not shown).

Spectral region 1600-1500cm⁻¹, Amide II band:

The PCA model (composed of five principal components) developed using the 20 spectra described 99.2% of the spectral variability. Using the amide II band, it was also not possible to distinguish between concentrated and stressed samples (data not shown).

Spectral region 1200-1350cm⁻¹, Amide III band:

The PCA model (composed of four principal components) developed using the 20 spectra (from the ‘stressed’ and ‘concentrated’ samples) described 95.3% of the overall spectral variability. The first principal component (capturing 68.7% of the spectral variability) was able to distinguish between both groups (stressed and concentrated) (fig. 6a). Analysis of the loadings plot revealed a broad peak at 1290cm^{-1} (α -helical structure) responsible for the grouping according virus pre-treatment (fig.6b).

Fig. 6: Scores (a) and loadings plots (b) obtained after PCA of the FTIR spectra from the concentrated and stressed samples using the $1350\text{-}1200\text{cm}^{-1}$ spectral region.

Besides FTIR, NIR spectra of 40 vials (20 stressed and 20 concentrated pre-treated samples) were also collected and analyzed using the $7300\text{-}4000\text{cm}^{-1}$ spectral region¹⁷ to confirm the FTIR findings. The two component PCA model developed from these 40 spectra described 75.8% of the overall spectral variability. The PC1 versus PC2 scores plot showed that the first PC (explaining 45.9% of the spectral variability) and the second PC (explaining 29.9% of the spectral variability) allowed distinguishing the samples according their pre-treatment (fig.7).

Fig. 7: Scores plot obtained after PCA on the NIR spectra collected from the stressed and concentrated pre-treated samples using the 7300-4000cm⁻¹ spectral region.

In addition to the evaluation of 'stressed' and 'concentrated' pre-treated samples, characterized by a significantly different virus titer, the class 'medium' was also studied. This class was interesting to analyze since the only difference between these samples and the other pre-treated samples (i.e. 'normal', 'stressed' and 'concentrated') is the absence of virus particle in the vials (table 1b).

Using the amide III spectral range, different PCA models were built using: (i) the FTIR spectra from the 'normal' and 'medium' samples, (ii) the FTIR spectra from the 'concentrated' and 'medium' samples and (iii) the FTIR spectra from the 'stressed' and 'medium' samples (fig. 8). The first model ('normal' and 'medium' samples) was composed of five PCs describing 98.3% of the spectral variability. The PC1 versus PC2 scores plot distinguished between both groups of samples (fig. 8a). The loadings of the first and second principal component are presented in fig. 8d. The first principal component distinguished between the spectra by a large peak around 1290cm⁻¹. The second principal component distinguished between the spectra by a peak at 1315cm⁻¹. Both peaks are located in a spectral range typical for α -helical structure.^{21,22} A difference between the 'normal' and 'medium' samples in this spectral range was also visible in the snv and 2nd derived spectra (fig. 8e). The second PCA model was built using the 'medium' and 'concentrated' spectra and consisted of five PCs describing 97.2% of the spectral variability. The first principal component (73.3% of the spectral variability) clearly distinguished between the two groups (fig. 8b). The PC1 loadings plot revealed one peak at 1290cm⁻¹ (α -helical structure) (data not shown). The last PCA model ('stressed' and 'medium' samples) was composed of six PCs explaining 97.7% of the spectral variability. Loadings of the second principal component showed different peaks, one located at 1280cm⁻¹ typical for β -turn^{21,22} and another one located at 1233cm⁻¹ typical for β -sheet^{21,22} (data not shown).

Fig.8: Scores plots obtained after PCA using the 1350-1200cm⁻¹ (Amide III) spectral region of the FTIR spectra of; a) normal and medium samples, b) concentrated and medium samples, c) stressed and medium samples. The loadings plot of the PCA using normal and medium samples (d) (PC1 black and PC2 blue) and the spectra of the medium (yellow) and normal (red) samples (snv and 2nd derived) (e) are also presented.

These PCA models developed from ‘medium’ samples containing no viruses on the one hand and the differently pre-treated samples on the other hand, allowed distinguishing between the presence and absence of viruses. As can be derived from the loadings plots, one peak around 1290cm⁻¹ and typical for α -helical protein secondary structure was involved in the spectral distinction between normal and medium samples as well as concentrated and medium samples. Loadings of the principal component distinguishing between stressed and medium samples showed two different peaks, located at 1280cm⁻¹ (β -turn) and at 1233cm⁻¹ (β -sheet).

Virus dose study

In this part of the study, the ability of FTIR spectroscopy to detect another live, attenuated virus as well as to distinguish between vaccines containing different doses was evaluated.

The three formulations, different in virus dose (table 2), were titrated. The 125 doses samples had a titer of 5.09 ± 0.16 (n=8), the 50 doses samples had a titer of 4.73 ± 0.08 and the 25 doses had a titer of 4.41 ± 0.18 (n=8). These titers are significantly different (Anova I, $p < 0.05$, fig. 9 left).

Unlike the virus volume study, karl fisher analysis revealed no significant difference in residual moisture between the different dose samples. The 125, 50, 25 and placebo samples had a residual moisture of 1.44 ± 0.33 (n=3), 1.31 ± 0.16 (n=3), 1.44 ± 0.21 (n=3), and 1.36 ± 0.07 (n=3), respectively (fig. 9, right).

Fig. 9: Titration results of the different dose samples (left). Titers are expressed as log₁₀ CCID₅₀ (Cell Culture Infection Dose 50). Each titer is the average of eight determinations. Residual moisture results of the different pre-freeze-drying treated virus samples (right) (n = 3).

After freeze drying, ten FTIR spectra per dose group and five FTIR spectra of the placebo group (i.e. 35 spectra in total) were collected. The FTIR spectra, SNV preprocessed, were analyzed using PCA applying different spectral ranges, i.e., the 1700-1600cm⁻¹ (amide I band), the 1600-1500cm⁻¹ (amide II band) and the 1200-1350cm⁻¹ (amide III band).

Spectral region 1700-1600cm⁻¹, Amide I band:

The PCA model (composed of two principal components) developed from the 35 spectra described 97.7% of the overall spectral variability. The first PC capturing 78.2% of spectral variability distinguished between the samples according to their doses (fig. 10a). The distances in the scores plot between the dose groups is in agreement with their actual dose difference. Among the different peaks observed in the PC1 loadings plot (1632 cm⁻¹, 1645 cm⁻¹, 1660 cm⁻¹, 1680 cm⁻¹, 1695cm⁻¹) (fig.10b), the two peaks located at 1632 cm⁻¹ and 1645 cm⁻¹ also clearly varied in the snv preprocessed spectra (fig.10c). These two peaks might represent turn/ β sheet (1638cm⁻¹)²⁶ and α helix (1645cm⁻¹)²⁶, respectively.

Fig. 10: PC 1 versus PC 2 scores (a) and PC 1 loadings (b) plots obtained after PCA of the FTIR spectra from the samples 0 (placebo), 25, 50 and 125 doses using the 1600-1700cm⁻¹ spectral region. (c) SNV corrected spectra of the 1600-1700cm⁻¹ spectral region (the arrow indicates the positive direction of PC1).

Spectral region 1600-1500cm⁻¹, Amide II band:

The PCA model (composed of three principal components) developed using the 35 spectra described 99.3% of the spectral variability. No PCs combination was able to distinguish between the different doses using the amide II band (data not shown).

Spectral region 1200-1350cm⁻¹, Amide III band:

The PCA model (composed of two components) developed from the 35 spectra using the amide III spectral region described 91.4% of the spectral variability. The first PC explained 63.6% of the spectral variability and distinguished between the samples according to their dose (fig. 11a). In contrast to the amide I band, the clustering between the different doses was less clear. Nevertheless, the placebo was

better separated from the other samples. Analysis of the loadings of the first principal component revealed one major peak at 1290cm^{-1} (fig.11b). This peak is located in a spectral range ($1300\text{-}1270\text{cm}^{-1}$) typical for α -helix.¹⁹ Analysis of the snv corrected spectra (fig. 11c) clearly showed a difference in peak intensity between the different doses and confirmed the observation of the scores and loadings plots.

Fig. 11: PC 1 versus PC 2 scores (a) and PC 1 loadings (b) plots obtained after PCA of the FTIR spectra from the 0, 25, 50 and 125 dose samples using the $1350\text{-}1200\text{cm}^{-1}$ spectral region. (c) SNV corrected spectra of the $1350\text{-}1200\text{cm}^{-1}$ spectral region (the arrow indicates the positive direction of PC1).

NIR analysis was also performed using the virus dose samples. Spectra from 18 vials per virus dose were collected and analyzed using the $7300\text{-}4000\text{ cm}^{-1}$ spectral regions (after snv and 2nd derivative pre-processing), as demonstrated in our previous study.¹⁷ The PCA model (composed of four components) developed from the 72 spectra described 93.3% of the spectral variability. The PC2 (18.2% of the

spectral variability) versus PC4 (8.48% of spectral variability) scores plot allows distinguishing the samples according to their virus dose (fig.12).

Fig. 12: Scores plot obtained after PCA on the NIR spectra from the virus dose samples using the 7300-4000cm⁻¹ spectral region.

Conclusion:

This study is, to our best knowledge, the first one evaluating and demonstrating the ability of FTIR spectroscopy to detect live, attenuated viruses in a freeze dried formulation. FTIR was able to distinguish between freeze dried formulations varying in “virus volume”, “virus pre-treatment” and “virus dose”.

In the virus volume study, the amide III spectral region was used hence avoiding the influence of the residual moisture which was different for the different virus medium volume samples. Using this spectral region, it was possible to distinguish between the different virus medium volume samples.

No residual moisture difference was detected for the samples of the virus pre-treatment study. Among the three amide (I, II, III) spectral ranges studied, the amide III spectral range was the most appropriate one to distinguish the different pre-treated samples.

Finally, in the virus dose study, the three amide spectral ranges were also compared (as no difference in residual moisture was seen between the different doses). Both, the amide I and III spectral ranges distinguished the samples according to their doses. Using the amide I spectral range, the distance between the different groups in the scores plot was in agreement with the dose content. Using the amide III spectral range, the placebo samples were better separated from the samples containing doses but the distinction between the different dose samples was less clear.

The presented results demonstrate the ability of FTIR spectroscopy to detect and evaluate live attenuated viruses in freeze dried formulations.

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