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Research Article

Near Infrared Spectroscopy - A Real-Time Method to Measure Aluminum Content and Protein Concentration in Adsorbed Vaccines

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Abstract

Development of analytical methods to streamline real-time measurements during process development is a priority for the evolution of bioprocess manufacturing. Current methods to measure downstream processes are limited, therefore, in this study, NIR and NMR spectroscopies have been successfully applied for the quantitation of aluminum content in the vaccine drug substance and drug product. In addition, NIR capabilities were demonstrated to measure concentration of adsorbed protein in-line. Statistical methods, such as Multivariate (PLS) Partial Least Squares and Principal Component Analyses (PCA) provided quantitative information about various components of vaccine drug substances that could be further developed to monitor downstream formulation processes. Samples of various protein antigens, such as Tetanus Toxoid, recombinant Neuraminidase, Pertactin and genetically detoxified Pertussis Toxin drug substance were shown to closely conform to modeled calibration data. ^{27}Al NMR spectroscopy was also applied as an orthogonal method to independently quantify aluminum content. This analytical technique tandem is shown to provide rapid determination of aluminum content in adsorbed drug substance.

Keywords: Adjuvants; Aluminum oxyhydroxide (AlOOH); Near-Infrared (NIR) spectroscopy; Nuclear Magnetic Resonance (NMR); Process Analytical Technology (PAT); Protein concentration, Bovine Serum (BSA)

Introduction

Vaccination is an essential tool for the control of diseases in humans and the development of vaccines has been one of the greatest public health achievements in modern science. Vaccines are complex biological products with various constituents; specifically, antigens and adjuvants are two common components that are necessary to produce an immune response for protection against infectious diseases [1-4]. Adjuvants are commonly used in human vaccines to enhance the immune response of a specific antigen and there are different formulations, including aluminum salts and oil-in-water emulsions [5]. It has been reported that aluminum salts can be employed to enhance the immunogenicity of antigens against bacterial and viral infections and specifically can be used to improve the immune response against diphtheria toxoid [6]. In general, aluminum salts are commonly used adjuvants in vaccines due to their long-term success [7].

The manufacturing of subunit vaccines commonly involves mixing of protein antigen with adjuvant to create an adsorbed drug substance complex. Therefore, a determination of protein and adjuvant concentration is essential for the formulation of vaccine drug substances to ensure consistency of the process and quality of the drug product. Protein concentration is commonly measured using spectroscopic techniques and colorimetric assays. However, UV/VIS spectroscopy is only possible if the sample is a transparent solution. Since many vaccine products are suspensions containing aluminum-based adjuvant, UV/VIS can not be applied as the samples contain non-transparent turbid particles. On the other hand, protein assays such as immuno-electrophoresis [8], Bicinchoninic Acid (BCA) protein assay [9], and UV absorbance assay [10] are used in vaccine quality control. However, these techniques can only determine the free protein concentration whereas the total protein concentration is required for calculating the fraction of protein adsorbed. Another disadvantage of these techniques is that they cannot directly measure the adsorbed protein concentration in a vaccine suspension. The inability to directly quantify the concentration of adsorbed protein in the amount of adjuvant in suspension by these techniques has prompted the investigation of alternative analytical methods such as light scattering, which is sensitive to the adsorptive capacity of the aluminum-containing adjuvant [11]. Moreover, since current methods for measuring protein concentration and aluminum content are done by various offline techniques, there is interest to develop a PAT method that can monitor both components in the formulation process in a real-time manner simultaneously. As reported recently, Near-Infrared (NIR) spectroscopy [12] have been used to quantify antigen and aluminum content individually by measuring protein concentration and in-direct measurement aluminum hydroxide respectively. Hence further development was pursued using FT-NIR process spectrometer equipped with an in-line probe to measure both components, aluminum content and adsorbed protein concentration in the drug substance. Furthermore, Nuclear Magnetic Resonance (NMR) spectroscopies have been studied for measuring protein and aluminum content in aluminum phosphate adjuvant and adsorbed drug product, therefore the exploration of using this technique as an orthogonal method to quantify aluminum content is also explored [13]. Development of methods to measure drug substance formulation during downstream processes using non-destructive analytical tools, could streamline current product testing in manufacturing suites, and be adapted for real-time product testing.

NIR is a versatile, non-destructive analytical technique that utilizes vibrational spectroscopy to analyze multiple properties of a vaccine suspension in a single spectrum. Moreover, it has been reported that NIR spectroscopy can be used for protein determination and to detect light-scattering material present in milk in the food industry [14]. NIR measures higher energy infrared wavelengths that create combination and overtone vibrations. This is possible since NIR measures higher energy infrared wavelengths which are better suited for monitoring light scattering effects due to the vaccine suspension components. These infrared wavelengths then create both overtone and combination vibrations. Baseline absorbance levels can be compared to absorbance levels when aluminum adjuvants are introduced to detect size change of the adjuvant-protein complex. Any changes in the absorbance will be detected by NIR and can be due to the difference between the free dissolved material and macromolecule suspensions in the vaccine since the macromolecules are subject to light scattering and will produce a different level of absorbance.

An advantage of NIR is that it displays weak absorbance bands that translate into strong signals reaching the detector and therefore, eliminating the need for any type of sample preparation or dilution. Although NIR is a very sensitive method of spectroscopy, the analysis of the spectra can be complex. This is due to most mixtures in fermentation containing a variety of different molecules which results in overlapping overtones. Therefore, differences in peak intensities resulting from the variables are not easily determined from the spectra and require multivariate statistical models to extrapolate information for different parameters. Thus, calibration models are built for each process parameter and are then validated against a reference set. Apart from metabolites and protein concentration, previous work has also demonstrated the feasibility of using NIR to successfully measure aluminum oxyhydroxide [15]. In this study, NIR was used with actual vaccine formulations to measure changes in various critical process parameters, including protein concentration, AlOOH concentration, and the data was used to build statistical models [16,17].

Furthermore, additional technologies to cross-validate NIR methods have been implemented Including Nuclear Magnetic Resonance (NMR). NMR has advantages including being easily reproducible over time, many different nuclei can be measured from a single sample, and the integral of the NMR signal is proportional to the concentration of the molecule being detected [18]. NMR has various applications including the measurement of media components and yield to provide information about titer, cell viability or even glycosylation patterns [19,20] Moreover, recent development for NMR in downstream applications have been further developed to measure aluminum content in aluminum and phosphate-containing and Tdap vaccines [21]. This study proposes a method for aluminum adjuvant measurement in downstream vaccine products. The development of complementary quantitative detection of aluminum using NMR as an orthogonal method could be implemented as a real-time technology to optimize the input and output variables to streamline both upstream fermentation and characterize downstream processes during vaccine manufacturing.

Material and Methods

Near-Infrared Spectroscopy (NIR)

The samples in the NIR study consisted of Drug Substance (DS), Bovine Serum Albumin (BSA) and aluminum hydroxide/ aluminum oxyhydroxide. The drug substances used were produced in-house (Sanofi Pasteur, Toronto, Canada). These samples were included: Recombinant Neuraminidase [rNA] at concentrations of 1.25, 0.625, and 0.31 mg/mL, Pertactin (PRN) at a concentration 0.19 mg/mL, Tetanus Toxoid (TT) at a concentration of 1.6 mg/ mL, and genetically detoxified Pertussis Toxin (gdPT) at a concentration of 0.4 mg/mL. Protein antigens were adsorbed with AlOOH to achieve a final concentration of 0.66 mg/mL. BSA (Albumin Standard 23209 lot#VH308072 Thermo Scientific, USA) had a stock concentration of 2 mg/mL in a 0.9% aqueous NaCl solution and was prepared at concentrations of zero, 0.1, 0.25, 0.5 ,0.75, 1.0 and 1.3 mg/mL. Each sample was adsorbed to the aluminum hydroxide at aluminum concentration of 0.66 mg/ mL. To achieve homogeneity, a vortex shaker was used to mix the samples prior to each experiment.

AlOOH adjuvant was prepared from the commercially purchased aluminum hydroxide suspension Alhydrogel® 2% (Cat #:250-845162EP Batch: 85643, Brenntag Biosector). To prepare aliquots with different AlOOH concentrations, Alhydrogel® was vortexed and then aliquoted into 50 mL conical tubes, Milli-Q® distilled H_2O (Milli-Q Water Purification System, from Millipore) was added and the samples were stored in a 4°C walk-in fridge. AlOOH diluted in Milli-Q® distilled H_2O at concentrations of 2.0, 1.61, 1.3, 1.05, 0.85, 0.686, 0.554, 0.447, 0.361, 0.235, 0.19 and 0.153 mg/ml were prepared. To get homogenization of the samples, the vortex machine was used to mix the samples prior of each experiment. The samples were left at room temperature for an hour prior to taking NIR measurements.

A FT-NIR spectrometer Matrix-F (Bruker, Billerica, MA, USA) equipped with an NIR source, a quartz beam-splitter using the TE-InGaAs detector and a low-pass filter of 10 kHz. The Matrix-F was coupled with a fiber optics Hellma Excalibur XP-25 Immersion probe with a diameter of 25 mm. NIR spectra are comprised of broad peaks resulting from molecular vibrations caused by interaction of molecules with light in the region from approximately 0-2500 mm. The resolution of the scans was set to 16 cm^{-1} and the number of sample scans was 256. A background measurement was taken before the sample measurements began in which no sample container was present. The background was subtracted automatically from each sample measurement by the instrument. To extract the necessary information from the broad peaks produced for the quantitative determination of protein concentration, the Partial Least Square (PLS) regression model was used to find the best correlation function between spectral and concentration data matrix. The OPUS/QUANT2 software (8.5.29) was used for the quantitative analysis of spectra. The software allows characterization of the concentration of the protein by using a Partial Least Squares (PLS) method. Several PLS fit regression models were built using different regions within the spectra, such as frequency regions, in combination with several data preprocessing methods on the OPUS/QUANT2 software. Each parameter was pre-processed separately to capture regions representing the largest amount of variation amongst the spectra in order to get the best model to use. The specific optimization for each parameter is generated by the software using various pre-processing methods, and the best models with the lowest RMSECV scores are used for the validation.

The PLS models were verified using a leave-one-out cross which is a statistical model validation technique in which the number of folds is equal to the number of data points in the set. The applicability of the model is validated by two factors - RMSECV and the determination coefficient (R^2). R^2 is the percentage of variance present in the true component values which is reproduced in the prediction. As R^2 approaches 100%, the predicted concentration values approach the true experimental values. Figure 1 shows the flow chart of experiment and experiment set-up consisting of the FT-NIR Spectrometer in a lab setting, to perform individual offline analysis to create multivariate statistical models.

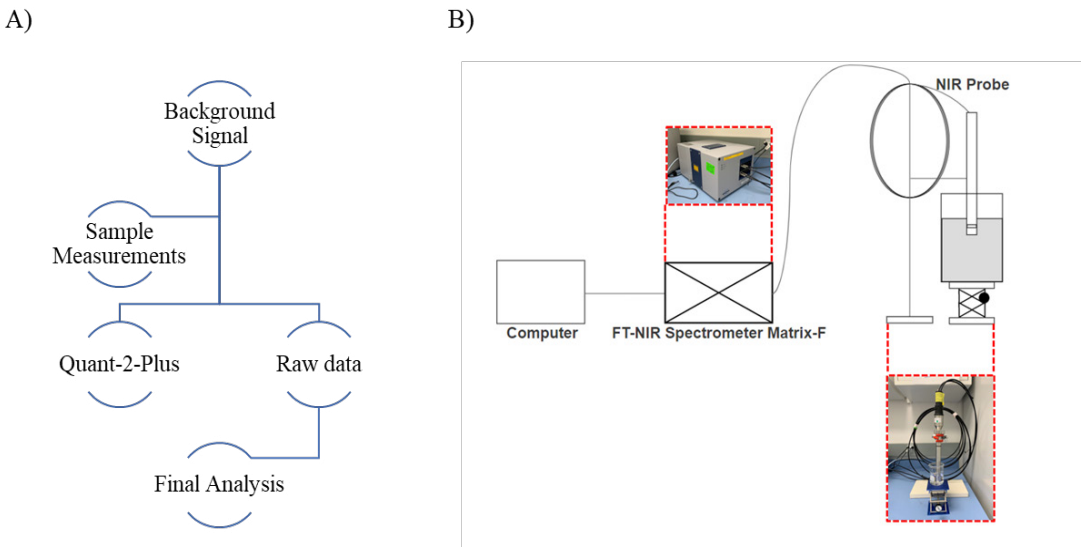


Figure 1: A) Flow chart for the design and execution to preform NIR experiments. Quant2 program in OPUS (Version 7.8.44) is used to create and analyze raw spectral data to create multivariate statistical models **B)** Model of experimental set-up consisting of the FT-NIR Spectrometer, fiber optics probe, computer, and samples.

Nuclear Magnetic Resonance (NMR)

$Al(NO_3)_3$ samples with concentrations of 0.1, 0.4, 0.5, 0.6, 0.66, 0.7, 0.8, 0.9, 1.0 and 1.4 mg/ml were prepared and acidified to pH 0.8 for the NMR experiments. 60 µl of D_2O (10% deuterium oxide) was added to 540 µl of sample and the total 600 µl volume was then transferred to 5mm NMR tubes. Similar sample preparation was also done for AlOOH adjuvanted drug substances (Tdap- AlOOH Control, Tdap-TLR9 agonist-AlOOH, Tdap-TLR4 agonist-AlOOH), the samples were all spiked with 12% D_2O and acidified to pH 0.8, then 600 µl volumes were transferred to 5 mm NMR tubes. 1D ^{27}Al NMR data for all samples were collected using a Bruker AV 400 NMR spectrometer equipped with a 5 mm z-gradient automatic tune and match broad-banded probe. The data was processed using TopSpin version 2.1 software. The acquisition parameters for 1D ^{27}Al NMR used in this study included a 2048 scans, a 90 degree pulse of 9.70 µsec, a spectral width of 52083.3 Hz, an acquisition time of 0.629 seconds acquired using 65536 data points, a relaxation delay of 1.00 seconds and D_2O was used as the sample solvent.

Results and Discussion

Measurement of Drug Substance using NIR

The determination of protein concentration in the presence of salt adjuvants such as aluminum hydroxide is an important yet challenging task due to the turbidity of the particles and the strong antigen adsorption to adjuvant particles [22]. Near-Infrared spectroscopy was used to characterize both AlOOH and protein concentrations. Raw NIR spectra of AlOOH, BSA, and protein samples with added aluminum hydroxide are shown in Figure 2 in the wavelength range of 11500-4000 cm^{-1}, which has three absorption peaks at approximately 6700 cm^{-1}, 5300 cm^{-1} and 4200 cm^{-1} of 11500-4000 cm^{-1}which has three absorption peaks at approximately 6700 cm^{-1}, 5300 cm^{-1} and 4200 cm^{-1}.

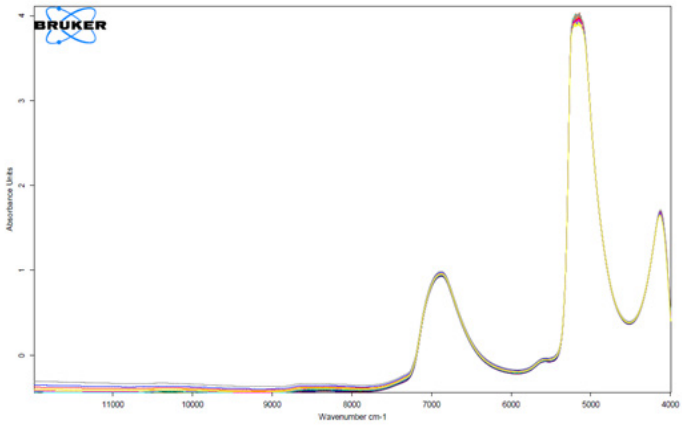


Figure 2: Raw NIR spectra represented by different colors for the measurement of Protein Drug Substance, Protein BSA, and AlOOH.

Two regions associated with protein spectra include 4605-4242 cm^{-1} and 9403-7498 cm^{-1}. These peaks can be seen at approximately 6700 cm^{-1} because of the second overtone of OH stretching as well as at 5300 cm^{-1} and 4200 cm^{-1} because of the

combination band of the first overtone OH stretching and OH bending. Moreover, there is a significant light scattering intensity that appeared in the spectra of AlOOH. However, differences in NIR peak intensities resulted from different concentrations are not easily determined from the spectra and requires multivariate statistical models to extrapolate information about different parameters. Therefore, calibration models were applied for each process parameter and are then validated against a reference set [19]. Using vector normalization and first derivative pre-processing the optimized regions were 9403.3-6094 cm^{-1} to 4605.2-4242.7 cm^{-1}, yielding a RMSECV value of 0.11 and R^2 value of 95.3% as shown in Figure 3A. The linearity of the correlation indicates that the technique is applicable for the determination of protein concentration in vaccine samples. The addition of the data for TT, rNA, gdPT, and PRN agrees with the calibration data. The PLS model can also be used as a suitable technique to monitor a variety of antigens as well as the AlOOH adjuvant content in the region of interest as shown in Figure 4. Using vector normalization and first derivative and vector normalization (SNV) pre-processing the optimized regions were found to be 4605-4497 cm^{-1} and 9403-7498 cm^{-1} which yielded a RMSECV value of 0.0478 and R^2 value of 99.3. The obtained linearity indicates the technique is well suited for the aluminum content. The aluminum content measurement for adsorbed TT, rNA, gdPT, and PRN agrees with the calibration data.

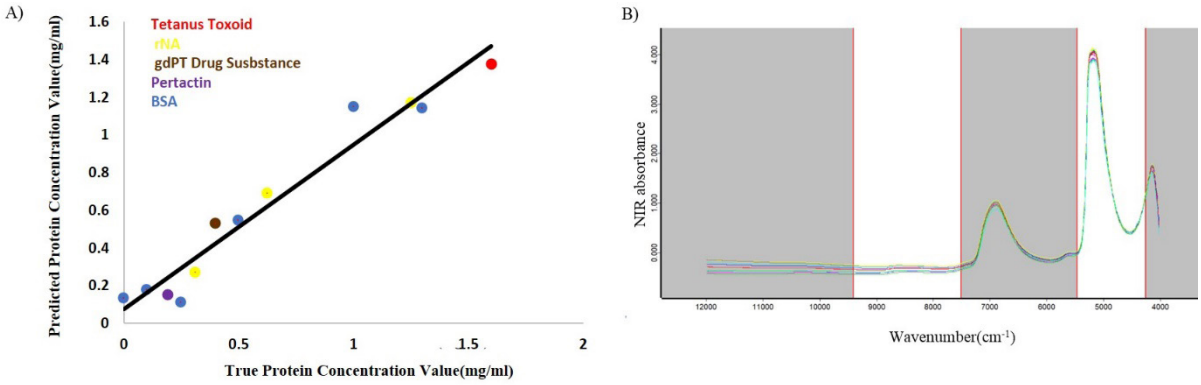


Figure 3: A) Leave-one-out cross-validation for protein concentrations (mg/ml). Diagrammatic representation of the validation results, showing the predicted versus the experimental total protein values following cross-validation of the calibration for BSA and Drug Substance with a RMSECV of 0.11 and a R^2 of 95.31. All data was visualized and processed using OPUS 7.8**. B)** Raw NIR spectra with highlighted optimized spectral regions of interest.

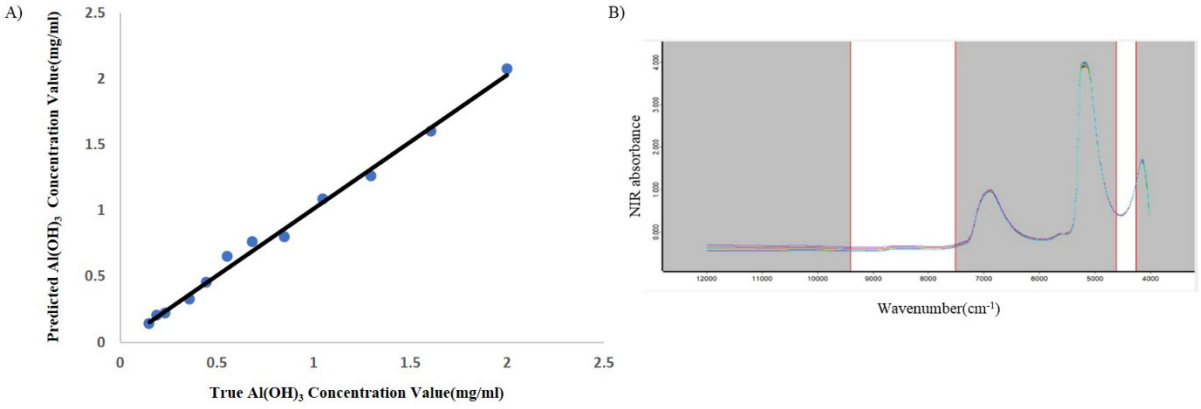


Figure 4: A) Leave-one-out cross-validation for AlOOH concentrations (mg/ml). Diagrammatic representation of the validation results, showing the predicted versus the true total concentration AlOOH values following cross-validation of the calibration for AlOOH with a RMSECV of 0.0478and a R^2 of 99.3 All data was visualized and processed using OPUS 7.8. **B)** Raw NIR spectra with highlighted optimized spectral regions of interest.

PCA models are a statistical method used to interpret data sets that displays a collection of points on a coordinate graph by applying a mathematical eigenvalue process such that the points on the graph are a linear function of the original dataset but are uncorrelated with each other while maximizing variance [23]. The purpose of PCA is to select optimal spectra for the test data sets, to select spectra for calibration set, to provide an overview of the acquired spectra and to recognize outliers. Figure 5 shows that the scores plot of the PCA models. The spectral data of each group that is identified using red represents AlOOH of different concentrations whereas blue represents BSA adsorbed to AlOOH at a final concentration of 0.66 mg/ml. The PCA plot of AlOOH and BSA concentrations, respectively, have been shown in Figure 5 in the range of 4242.7-9403.3 cm-1 with first derivative and vector normalization data pre-processing treatments. NIR spectral data were used alternatively to determine if there were detectable differences in adsorbed concentrations (mg/mL) between various AlOOH sets and BSA. Principal component analysis allowed for a simple regression of components in the matrix without the need of model calibration to detect variable differences, thus producing simpler correlative analysis. Therefore, using PCA analysis, in response to various samples, two defined clusters were observed based on the samples. The PCA model in Figure 5, demonstrated that the datasets of concentrations AlOOH and BSA concentrations do not overlap and therefore, the sets of samples can be distinguished between each other by the NIR absorption. Furthermore, PLS models demonstrated that NIR can be used as a suitable technique to monitor a variety of antigens as well as the adjuvant content. Thus, the development of an NIR method that can measure both adjuvant and antigen concentration in real-time would help streamline characterization of drug substance products in downstream processes and replace the necessity of various offline tests. One of the main advantages of NIR as a protein determination method in comparison to traditional methods is the ability to measure a variety of parameters simultaneously. Therefore, by using one probe to measure multiple sample characteristics simultaneously, this provides the opportunity for the single-use application of inserting the NIR probe into a downstream process to monitor the protein concentration and particle size during manufacturing [24]. NIR probe can be incorporated in manufacturing process as PAT solution to replace off-line assays with Real-Time Release Testing (RTRT) during manufacturing of vaccine product. Therefore, the implementation of real-time non-destructive PAT methods such as NIR, can help increase testing efficiency and consistency which in turn will reduce over-processing and process defects [22].

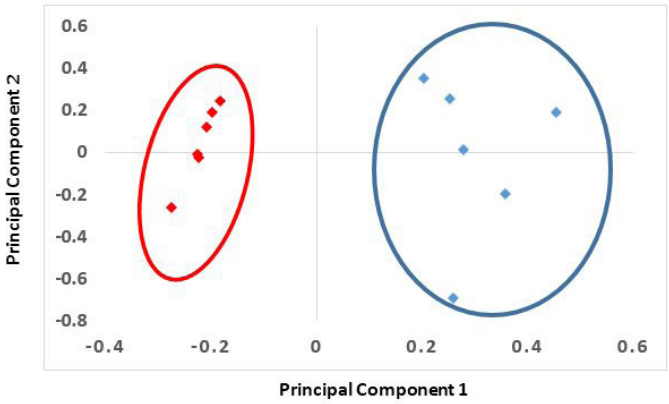
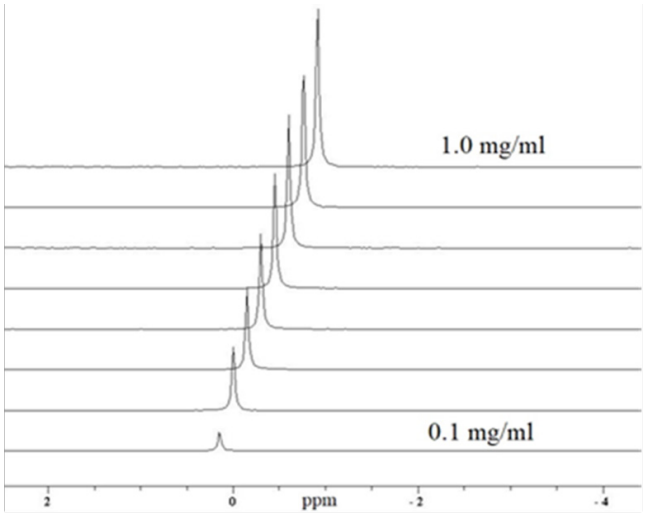


Figure 5: The scores plot of the PCA models. Red points are AlOOH with different concentrations values versus blue points are BSA that was mixed with AlOOH to reach the final concentration of 0.66 mg/ml.

Measurement of Aluminum Adjuvant using NMR

Since, NMR is a direct non-destructive analytical method that could complement NIR primary measurements, we explored the capability of using NMR to measure aluminum content in drug substances. To prepare vaccine samples for ^{27}Al NMR analysis, the pH was lowered to release bound aluminum from the adsorbed media. Once the aluminum ions reach low pH, they become completely solvated by water and assume a symmetrical environment. As ^{27}Al is a quadrupolar nucleus with small quadrupole moment, symmetry surrounding the aluminum nucleus ensures that the electric field gradient will be minimized leading to sharper NMR resonances. Figure 6 shows the ^{27}Al NMR spectra from aluminium samples ranging from 0.1 mg/ml-1.4 mg/ml of $Al(NO_3)_3 \cdot 9H_2O$ dissolved in H_2O/D_2O with a pH 0.80. Figure 6A, shows aluminium peak intensity correlated with increasing concentrations in solution. Peak area integral values were quantified and a correlation of peak area and aluminum concentration results in a calibration line with an R^2 value of 0.9947, showing aluminum could be accurately quantified in a concentration dependant manner (Figure 6B).

A)



B)

Al Concentrations (mg/ml)	Integration Value
0.1	8.3852
0.4	29.4113
0.5	37.0262
0.6	44.6912
0.66	49.8456
0.7	52.3091
0.8	61.8157
0.9	62.6969
1	75.4666
1.4	100

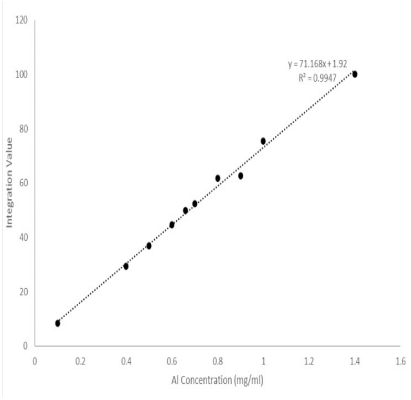


Figure 6: ^{27}Al Standard Curve of Al(NO3)3 .9H_2O dissolved in H_2O/D_2O at pH 0.80. A) Raw spectra of peak intensity changes. Bottom to top 0.1, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/ml $Al(NO_3)_3$ B) Calculated integration values of Aluminum peak intensities.

The NMR technique was applied to actual vaccine samples and Figure 7 shows ^{27}Al NMR spectra of three different drug substance sample lots in D_2O that have been acidified to pH 0.80. Aluminum can be detected in all Tdap samples and calculated relative integration values for the Al^{3+} content in each of the final bulk products yielded very similar aluminum content in the samples. Thus, there is no significant difference in peak intensities between the Tdap-TLR4 agonist-AlOOH, Tdap-AlOOH, and Tdap-TLR9 agonist-AlOOH based on the associated integration values, showing NMR can reliably predict aluminum content in drug substances regardless of formulation variations. Variations between triplicate sample re-measurements for each drug substance also remain relatively consistent over time, and small variations may have occurred due to pH changes over time during measurement (Figure 8). Although based upon a totally different technique, NMR provides an excellent complement to NIR spectroscopy for the determination of total aluminum content in vaccine samples. Therefore, since NMR data could be used to detect aluminum content directly in drug substances, it could be adapted as an orthogonal method to complement NIR data measurements. Moreover, since there has been recent development of low-field NMR spectrometers, these methods could potentially be adapted as a primary measurement for NIR data or used as a potential at-line tool to measure downstream process samples. The synergy between these two techniques provides an opportunity for the development of real-time acquisition of analytical data that could be adapted and stream-lined for process development during manufacturing.

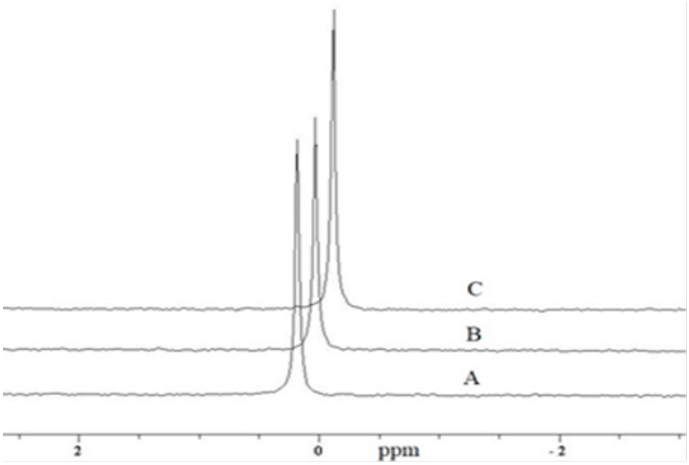


Figure 7: ^{27}Al NMR spectra Peak intensities of Tdap Drug Product lots with 12% D_2O at pH 0.80. Bottom to top **A)** Tdap-TLR4 agonist-AlOOH, **B)** Tdap -TLR9 agonist-ALOOH, **C)** Tdap-AlOOH control.

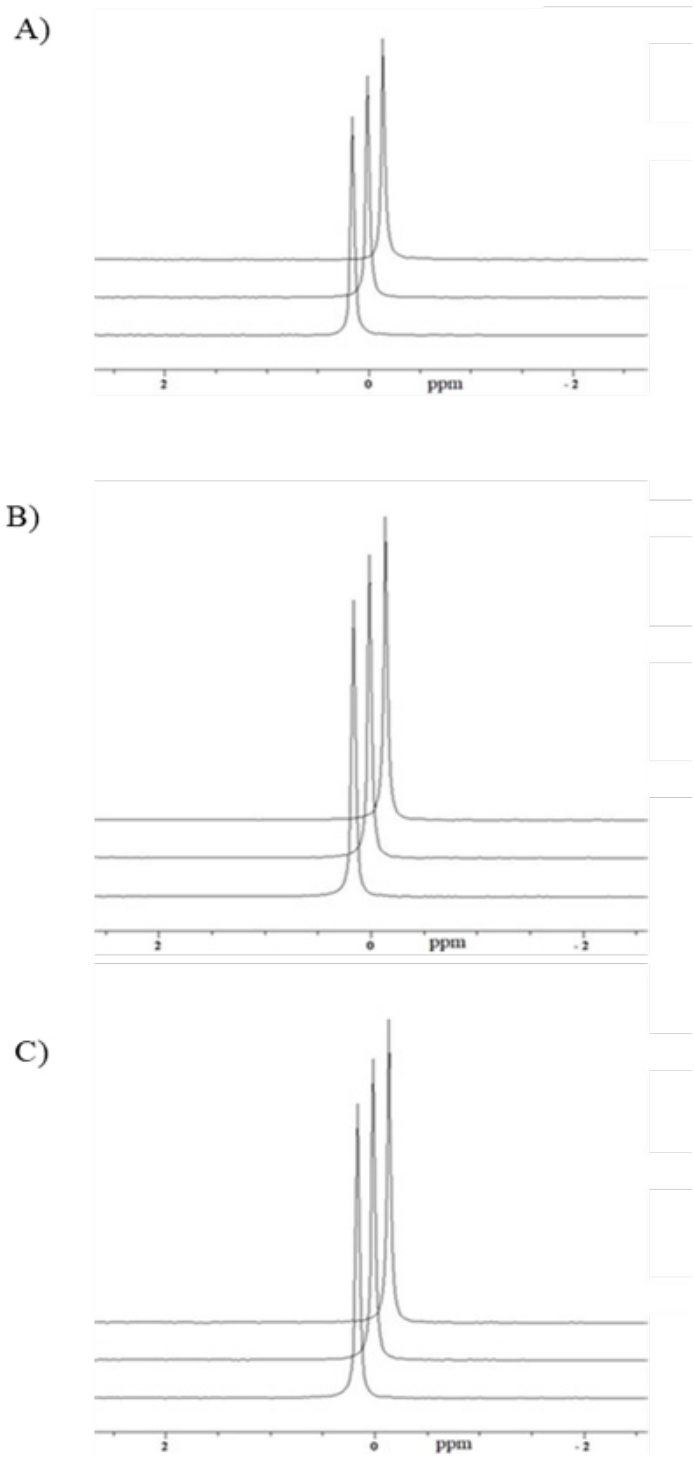


Figure 8: Peak intensities of three consecutive ^{27}Al spectra of **A)** Tdap-TLR9 agonist-AlOOH, **B)** Tdap-TLR4 agonist-AlOOH, **C)** Tdap-AlOOH in D_2O pH 0.80.

Conclusion

In this study, the measurement of adsorbed protein concentration and protein drug substance in aluminum hydroxide suspensions has been determined using near-infrared transmittance spectroscopy and nuclear magnetic resonance. This study shows the ability of NIR to determine different adsorbed protein drug substances, and BSA protein concentrations in aluminum hydroxide suspensions using NIR spectroscopy. Moreover, this study also determined an orthogonal method to measure Al^{3+} content using NMR spectroscopy. Compared to the UV absorbance method and other methods, the advantages of both NIR and NMR methods, are that they are both quantitative, non-destructive, and fast, and it can be developed for real-time monitoring for downstream vaccine manufacturing processes. Considering the rapid development and need for various PAT methods and improved technologies for digital real-time monitoring during biopharmaceutical product manufacturing, the impact of NIR and NMR as non-destructive analytical methods could be implemented for various stages of the manufacturing process. Therefore, both technologies have promising potential to be used as analytical tools that can be applied in quality control and quality assurance of vaccine production for faster real-time product release.

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