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Research Paper

Characterization of Mannitol Polymorphic Forms in Lyophilized Protein Formulations Using a Multivariate Curve Resolution (MCR)-Based Raman Spectroscopic Method

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Purpose. To develop a novel multivariate curve resolution (MCR)-based Raman spectroscopic method to characterize and quantify five known mannitol solid-state forms in lyophilized protein formulations.
Materials and Methods. The multivariate quantitation method was developed based on second derivative Raman spectra of three anhydrous crystalline forms (α-, β-, and δ-mannitol), a hemihydrate and an amorphous mannitol form. The method showed a 5% quantitation limit of mannitol forms in lyophilized model protein formulations. Binary mixtures of β- and δ-mannitol combined with evaluation of the prediction residue were used for the method validation. X-ray powder diffractometry data was used to confirm the existence of mannitol forms in the sample.
Results. The various polymorphic forms of mannitol were characterized and quantified based on the Raman spectra of the existing pure forms, and the results are consistent with the X-ray powder diffraction data. This Raman method has been demonstrated for the application of monitoring and controlling of mannitol polymorphic forms in the lyophilized drug products during formulation and process development. It has implications in monitoring and improving the quality of the drug product.
Conclusions. An MCR-Raman method has been developed for the quantitative determination of five different mannitol polymorphic forms in the presence of sucrose and protein.

KEY WORDS: lyophilized protein products; mannitol; polymorphism; quantitation; Raman spectroscopy.

INTRODUCTION

Mannitol is a commonly used bulking agent in drug products, especially in lyophilized protein formulations. Different polymorphic forms of mannitol have various effects on the stability and other quality attributes of protein formulations on long-term storage. According to the definition used in ICH guidance Q6A, polymorphic forms here refer to crystalline and amorphous forms, as well as solvate forms (1,2).

There are five known polymorphic forms of mannitol reported in the literature, which include three anhydrous crystal forms [α-, β-, and δ-mannitol forms (3)], one hemihydrate form (4), and the amorphous form (5). At ambient conditions, β-mannitol was identified as the thermodynamically most stable form, while α- and δ-mannitol show significant kinetic stability (3). Anhydrous mannitol crystalline forms provide a stable matrix structure for the lyophilized protein product. The hemihydrate form and the amorphous form of mannitol tend to dehydrate or crystallize under ambient conditions (6–8), and they each can be formed during lyophilization of protein formulations (4,7). As these mannitol phase transitions can harm the stability and the appearance of the protein product during long-term storage (7,9), a systematic understanding of mannitol polymorphism is desired in order to improve the quality of the lyophilized protein product. Reliable analytical methods are therefore needed to identify and quantify mannitol polymorphic forms in the lyophilized drug product.

A variety of different technologies can be used for the quantitative analysis of solid-state forms (10). In principle, specific polymorphic forms can be identified and quantified by using X-ray powder diffractometry (XRPD) (7,11), vibrational spectroscopic techniques including IR (12), NIR (13), Raman (14–16), solid-state NMR (17), and thermoanalytical methods like differential scanning calorimetry (DSC) (18). The use of XRPD may be limited by uncertainty in peak intensity caused by preferred orientation and limited sensitivity, even though it is still the most popular technique used for polymorphic

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ABBREVIATIONS: ALS, alternative least squares; CCD, charge-coupled device; DSC, differential scanning calorimetry; ICH, The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; IR, infrared; MCR, multivariate curve resolution; NIR, near infrared; NMR, nuclear magnetic resonance; RMSEP, root mean square error of prediction; XRPD, X-ray powder diffractometry.

identification. Vibrational spectroscopic techniques usually can give each polymorphic form a unique spectrum, and consequently are well suited for the quantitative analysis of polymorphism, especially with the increasing popularity of multivariate analysis. Analytical methods that are developed based on a spectroscopic technique usually need a calibration curve using gravimetric or volumetric techniques for preparing calibration standards (16). The use of physical mixtures for solid samples dictates that the method should be insensitive to changes in moisture and particle size. Otherwise, the application would be limited to a narrow range of unknown samples with well-controlled particle size and moisture content. Raman spectroscopy is a non-destructive laser technique which is based on the inelastic scattering of monochromatic light from a substance (19). In the context of the above issues, the characteristics of Raman spectroscopy make it a competitive tool for the quantitation of polymorphic forms. Some special advantages of Raman spectroscopy lie in the facts that water is a poor Raman scatterer, the particle size effect can be avoided by using a Raman probe with relative large sampling volumes (20), and the intensity of the Raman scattering signal is also proportional to the number of analyte molecules in the sample volume being analyzed (21).

Several quantitative studies using Raman spectroscopy for the quantitation of mannitol polymorphism have been reported in the literature. However, due to various limitations, none of them could be directly applied for the analysis of a lyophilized protein cake. Quantitative studies based on binary mixtures of β- and δ-mannitol forms were reported for mannitol in tablet formulations (15,22). However, the preparation of mixtures of all five mannitol forms is far more challenging due to the potential for phase transitions during mixing. Some efforts to establish analytical methods not based on reference mixture standards for the determination of mannitol crystallinity have been reported (5,23). Vehring reported the quantitation of amorphous mannitol and the three anhydrous crystal forms of α-, β-, and δ-mannitol from spray-dried mixtures of salmon calcitonin and mannitol. Although this work shed light on the mannitol crystallinity in a protein-mannitol mixture, it can not be extended for the quantitation of mannitol hemihydrate unless a reliable binary mixture of protein and hemihydrate mannitol can be prepared. By using a linear combination of elements analysis, Bettie *et al.* reported a method for the semi-quantitative determination of all five different mannitol forms and the study of crystallization behavior of mannitol within a frozen system (23). However, there are still some challenges remaining for the quantitation of mannitol forms in a lyophilized protein formulation, due to the complexity caused by the coexistence of protein and the other excipients.

The work described in this paper introduces an MCR-Raman spectroscopic method for reliably quantifying mannitol polymorphic forms in lyophilized model protein formulations, which are prepared from solutions containing (% *w*/*v*, g/ml): 4% mannitol, 1% sucrose, 0.1% Tris buffer and 2.5% or 5% model protein. The multivariate calibration model used in the MCR-Raman method was based on the estimated second derivative Raman spectra of the five mannitol forms. The performance of this method was evaluated using the binary mixtures of stable β- and δ-mannitol forms, and the prediction residue. This MCR-Raman method offers a promising approach for the determination of mannitol polymorphism in lyophilized protein formulations. Detailed discussions of how to remove Raman interference from protein and other components (such as sucrose) are also described in this paper. We propose that this technique can be applied to control mannitol polymorphic forms in the lyophilized drug product during manufacturing as well as storage, thereby improving the quality of the drug product during formulation and process development.

MATERIALS AND METHODS

Chemicals. Besides the spray dried mannitol (Mannogem™ EZ, SPI Pharma. Inc, Wilmington, DE, USA), mannitol (Catalog No. 152540, Lot No. 8935F) used was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). The model protein formulations used in this study were provided by the protein drug product group at Amgen Inc. (Thousand Oaks, CA, USA). The lyophilized protein formulations contained 10 mM Tris buffer, 4% mannitol and 1% sucrose at pH 7.4. All other chemicals and solvents used were reagent grade or better.

Mannitol Polymorphic Forms. α-Mannitol was crystallized by a slow solvent evaporation process with a saturated mannitol solution in a mixed solvent system of acetone, ethanol and water with 5:5:2 ratio by volume (24). β-Mannitol was obtained directly as a commercial powder form. δ-mannitol was prepared by first dissolving 2 mg mannitol in 50 ml methanol/water (7:3 *v*/*v*) at 50°C, and then cooling the resulting solution at room temperature to crystallize out the mannitol. Mannitol hemihydrate was obtained by following a very slow lyophilization process (25). A commercial lyophilizer (VirTis 1.2 EL, FTS System, NY) was used for preparing the mannitol hemihydrate. Amorphous mannitol was produced by using a three-step process. First, 5 μl of a 4% mannitol aqueous solution was quickly cooled down to −50°C at the rate of 20°C/min to prevent crystallization during the freezing step. Then the sample was warmed up to −35°C at the rate of 1°C/min under 50 mTorr vacuum. The temperature at −35°C is a critical setting to prevent the crystallization of amorphous mannitol. Finally, after the ice was completely sublimed, the amorphous mannitol was slowly warmed to room temperature under 50 mTorr vacuum to minimize moisture absorption from air which can also lead to the crystallization of amorphous mannitol. XRPD was used to check the purity of the resulting mannitol forms by comparison with the literature data (3,4,25). Due to the instability of amorphous mannitol under ambient conditions, amorphous mannitol was confirmed by comparing its Raman spectrum with the Raman spectrum of a concentrated mannitol solution (5).

Raman spectroscopy. The Raman spectra were obtained using a Falcon II™ Molecular Chemical Imaging (MCI) system from ChemImage Corporation (Pittsburgh, PA, USA). The excitation source was the Millennia IIi Laser (Spectra-Physics, Mountain View, CA, USA) at 532 nm with a power of ca. 150 mW at the sample, coupled with a Model BX51 microscope platform (Olympus, Center Valley, PA, USA). The laser beam size was enlarged with optics so that it filled the entire field of view through the objective, and Raman signals were collected with a 5× or 10× Olympus objective

from a sample area of ca. 450 or 250 μm in diameter, respectively. Multiple measurements (N=20) were performed with different sampling positions for each sample. A back-illuminated CCD (1340×100B XP, Princeton Instruments, Trenton, NJ, USA) cooled at −70°C was used to record the Raman signal after a 1,200 grooves/mm grating in the Raman spectrograph. A cryostage (FDCS 196 from Linkam Scientific Instruments Ltd., Tadworth, Surrey, UK) used for preparing amorphous mannitol was customized for *in situ* Raman measurement of samples through a transparent quartz window with a precise temperature control from −196°C to 125°C and vacuum control from 50 mTorr to 750 Torr.

Unless otherwise specified, all Raman measurements were performed under ambient conditions. Due to the instability of amorphous mannitol, its Raman spectrum was taken at room temperature but under 50 mTorr vacuum control. The 10× objective was used for all Raman measurements, except that the 5× objective was used in measuring Raman spectra of binary mixtures of β- and δ-forms in order to obtain a larger sampling area. The Raman spectra from five mannitol polymorphic forms were obtained with similar spectral intensities. The typical exposure times were 20 s for the crystalline forms, 600 s for the amorphous form, and 200 s for the lyophilized protein samples. The laser power effect was excluded with no apparent spectral change after extended laser exposure of samples.

X-Ray Powder Diffraction. X-Ray powder diffraction (XRPD) analysis was carried out on a θ/θ diffractometer with sample spinning capability (X'pert MPD, Philips Analytical, Natick, MA, USA) using Cu Kα radiation. All samples were analyzed with Bragg–Brentano geometry from 3° to 40° 2θ at a step size of 0.01° 2θ on zero-background silicon wafers.

Preparation of β- and δ-Mannitol Mixtures. Crystals of δ-mannitol were first ground using mortar and pestle, and then the powder form with particle size of approximately 120~180 μm was selected using suitable sieves. The powder form of β-mannitol with a similar particle size was also collected. Ratios between β- and δ-mannitol forms were calculated by their weights. Uniform mixtures were achieved using suspension mixing which was done by first adding hexane (in which mannitol has very limited solubility) to the mixtures, followed by filtering the powder sample with a membrane filter and drying the samples.

Multivariate Data Analysis. All multivariate data analysis was done using Matlab 7.01 (MathWorks Inc., Natick, MA, USA) and PLS toolbox 3.5 (Eigenvector Research, Inc., Wenatchee, WA, USA). The multivariate curve resolution (MCR) analysis is based on the alternative least squares (ALS) algorithm (26), and used for the second derivative Raman spectra. The normalized second derivative steps can be found elsewhere (27–29). The root mean square error of prediction (RMSEP) can be calculated based on the measured (y_i) and the predicted ($\hat{y}_i$) reference values of the test samples (N=N_p), and is defined by (30):

$$RMSEP = \sqrt{\frac{1}{N_p}\sum_{i=1}^{N_p}(\hat{y}_i - y_i)^2}$$

RESULT AND DISCUSSION

MCR-Raman Method Development

As the preparation of reliable reference calibration mixture standards for all five mannitol forms is not practical, an attempt was made in this work to apply an MCR algorithm for the Raman spectra of a mixture sample and quantitatively determine its polymorphic composition by comparison to the Raman spectra of the five individual pure forms. Mathematically, the Raman spectrum s of a polymorphic mixture is described as the sum of the linear combination of Raman spectra s_i (s_1, s_2, s_3, s_4, s_5: Raman spectra of pure α-, β-, δ-, hemihydrate, and amorphous mannitol forms), in addition to a fluorescent background b and a noise spectrum e, which can be expressed as:

$$s = \sum_{i=1}^{5} a_i s_i n_i + b + e \quad (1)$$

where a_i and n_i stand for the normalization factor of spectral intensity and the molar fraction of each polymorphic form in a given mixture, respectively.

To obtain representative Raman spectra s_i of five individual pure forms and use them to obtain the quantitative information n_i in Eq. 1, sources of error in Raman spectroscopy need to be fully evaluated; these can be separated into sample, spectrophotometer, and operational errors (16). Detailed evaluations of these various error sources were previously reported in the literature (15, 21, 31). The instrumental effects (laser power and instrument variability) were found to be minor with proper calibration. Therefore, the representative Raman spectra s_i (i=1 to 5) ideally should be the same as the Raman measurement of the pure polymorphic forms. In addition, this approach also relies on the hypothesis that there should be no spectroscopically detectable interaction among the mixed components in the Raman spectral region used for quantitation.

However, it is known that fluorescence interference in Raman spectroscopy can be a major issue, especially for biological samples. The fluorescent background b measured from most biological samples is unpredictable and different for every Raman measurement, and its spectral interference needs to be removed before applying the MCR data analysis. In the literature, curve fitting is a commonly used method to reduce the Raman background (23), although it is not always reliable, especially for highly overlapped spectral regions, such as the 1,000–1,500 cm^{-1} region in the mannitol spectrum. As a more robust approach, second derivatives have often been used to minimize the low-frequency baseline contribution such as the fluorescent background in Raman spectroscopy (27), and Eq. 1 can be converted into a new format as:

$$s'' = \sum_{i=1}^{5} a_i s_i'' n_i + e'' \quad (2)$$

Since a_i and n_i are not frequency dependent factors, the negligible background b'' is not included in Eq. 2, and the same linear correlation as in Eq. 1 is maintained. The normalization factor a_i is typically obtained by measurements of uniform physical mixtures with known compositions, which is again not practical for samples that cannot maintain their polymorphic

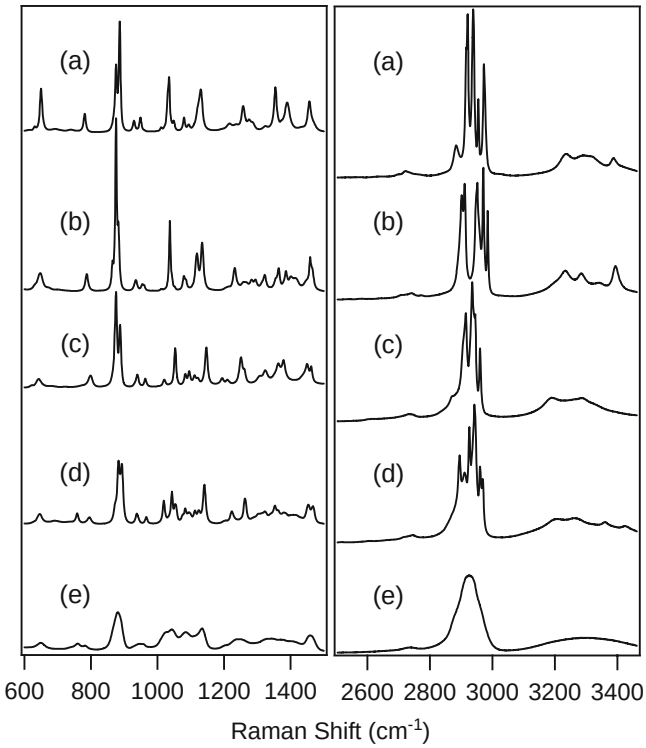

Fig. 1. Raman spectra of pure forms of (*a*) α-mannitol, (*b*) β-mannitol, (*c*) δ-mannitol, (*d*) mannitol hemihydrate, and **e** amorphous mannitol.

forms during mixing. Therefore, this study measured the Raman peak area assigned to the same molecular moiety (C–H stretching modes, $\nu_{CH,}$ 2,600~3,100 cm^{-1} in Fig. 1) of different polymorphic forms, and normalized Raman spectra of the five individual mannitol solid forms based on the peak area of the ν_{CH} Raman bands. Although the Raman peaks and their relative intensity of the C–H modes are different due to the different crystalline structures of the solid-state forms (23), the overall integrated intensity of all the C–H bands should be the same. This normalization approach has also been applied and validated with another pharmaceutical compound [unpublished work in (32)].

An MCR algorithm was tested for the determination of quantifying the mannitol forms, and the second derivatives of the average Raman spectra (N=20) of the five pure mannitol forms were used as the initial guess. The MCR-estimated second derivative spectra of the five mannitol forms in the calibration model obtained based on the MCR-analysis of the Raman spectra taken from pure mannitol forms and the binary mixtures of β- and δ-mannitol were found to be almost the same as the second derivatives of the average spectra of the pure forms (N=20). This supports the hypothesis that there is no Raman detectable interaction between mannitol forms in the spectral region used for quantitation. This MCR calibration model was later used for the analysis of the lyophilized protein samples. The quantitation limits of the MCR-Raman method for the analysis of mannitol polymorphism in different types of mannitol samples are discussed in the following sections.

MCR-Raman Analysis for Mannitol Samples

The Raman spectra of mannitol in both the finger-print region (left) and the spectral region covering ν_{CH} modes (right) are shown in Fig. 1. All Raman spectra were normalized to the same peak area of the corresponding Raman spectral region at 2,600~3,100 cm^{-1}. The Raman spectra of these two spectral regions were taken from the identical sampling area with the same signal integration time. The Raman spectra (a)–(c) were obtained from the three anhydrous crystal forms (α-, β-, and δ-forms). They were confirmed by comparison to the work done by Burger *et al.* (3). Fig. 1 (d) is the spectrum of mannitol hemihydrate. The peaks at 759 and 1,018 cm^{-1} can be used as an indication for the existence of mannitol hemihydrate. The purity of the three anhydrous crystal forms and mannitol hemihydrate were confirmed by comparison to XRPD data in the literature (3,4). Finally, Fig. 1 (e) shows the spectrum of amorphous mannitol at 25°C under 50mTorr vacuum. The spectral similarity of spectrum (e) to the aqueous mannitol solution spectrum (5) and a broad and smooth C-H stretch band at 2,800~3,100 cm^{-1} both confirmed the amorphous state of mannitol. The data in the spectral region of 600–1,500 cm^{-1} were used for the MCR-analysis. The RMSEP values calculated based on the replicate Raman measurements of the pure mannitol forms are less than 2% using the proposed MCR-Raman method.

For the performance evaluation of the MCR-Raman method, binary physical mixtures of the two stable mannitol forms (β-, δ-mannitol) and the prediction spectral residue were used to make sure that the results obtained are meaningful, accurate, sensitive, and robust. Physical mixtures of β- and δ-mannitol were prepared gravimetrically. In order to reduce variances related to the sample heterogeneity, Raman measurements of each mixture were repeated 20 times with different sampling areas. The predicted results based on the MCR calibration model gave RMSEP values of 3.2% for β-mannitol and 3.1% for δ-mannitol, as shown in Fig. 2. For the

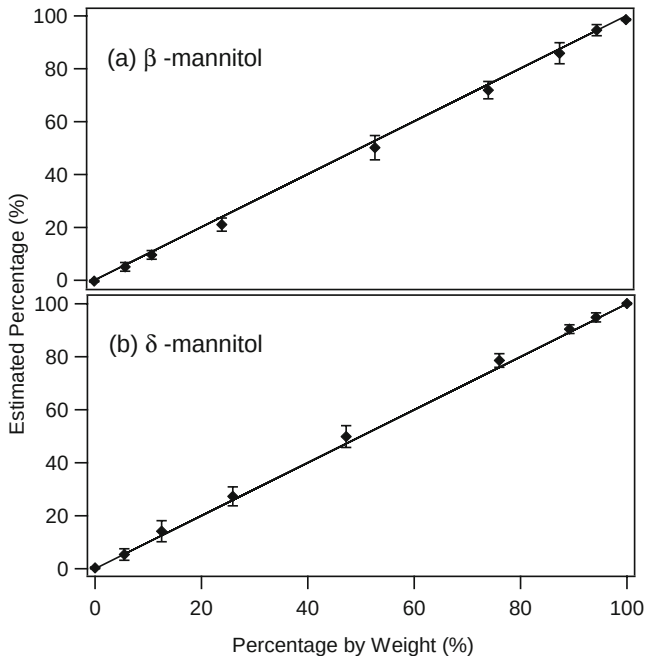

Fig. 2. **a** β-mannitol and **b** δ-mannitol estimated relative percentages in mixtures predicted by the MCR-Raman method *versus* their content determined by weight.

other three forms that physically did not exist in the mixture, the RMSEP values were 0.6%, 1.0%, and 1.3% for α-mannitol, mannitol hemihydrate and amorphous form, respectively. Based on the 2% RMSEP values for the pure mannitol forms, the calculated amounts of the other three mannitol forms are considered to be insignificant, which is also consistent with the nature of the binary mixture samples. These accurate prediction results for the known synthetic mixtures also confirm that the intensity normalization based on the peak areas of the C–H stretch Raman bands of mannitol is appropriate for the β- and δ-mannitol forms. The linear correlation between the predicted percentages and their actual values validates that the fact that the proposed MCR-Raman method can be used for the quantitative determination of β- and δ-mannitol, while the determination of a mannitol sample contains other mannitol forms are still considered to be semi-quantitative in this study.

In order to evaluate the performance of the proposed MCR-Raman method for real mixtures that contain mannitol forms other than β- and δ-mannitol, a spectral residue analysis was utilized to evaluate the unexplained Raman prediction residue signal. For this purpose, some mannitol samples were prepared to contain multiple mannitol forms during their crystallization procedures, and a commercial available spray dried mannitol (Mannogem™ EZ) was also tested. Raman spectra 3(a) from a representative lab-prepared mannitol mixture and 3(d) from spray dried mannitol are shown in Fig. 3. The quantitative data obtained by the MCR-Raman method described above showed mainly 5% β-mannitol, 61% δ-mannitol and 33% mannitol hemihydrate for sample 3(a), and 72% α-mannitol and 29% β-mannitol for sample 3(d), respectively. In order to demonstrate the spectral residue after prediction, spectra 3(b) and 3(e) were built based on the linear combination of the average spectrum of each individual mannitol form according to the predicted results. The difference spectrum 3(c) between the actual Raman spectrum 3(a) and the simulated Raman spectrum 3(b) indicates a close-to-ideal fit with about 3% residual intensity of the original Raman signal based on the mannitol bands around 850 cm^{-1}, which is also defined as the quantitation limit for the MCR-Raman method. A similar result is also shown with the difference spectrum 3(f).

XRPD was also used to characterize the mannitol samples as a reference method, shown in Fig. 4. As is evident from the figure, the lab-prepared mannitol mixture with its Raman spectrum listed in Fig. 3 (a) contains β-, δ-and hemihydrate mannitol forms with δ-mannitol as the major polymorphic form, and the spray dried mannitol sample has both α- and β-mannitol polymorphs. The absence of other mannitol crystalline forms quantified by the Raman method is also confirmed by the XRPD pattern of those two samples. Since different sampling sizes were used for the XRPD (ca. 10mm) and the Raman measurements (ca. 250 μm), the Raman quantitation result may not completely match what was observed in the XRPD pattern. The XRPD result here only provides the information on the identity of the mannitol forms in the samples. The quantitative XRPD analysis of mannitol samples is not explored further in this study, although semi-quantitative analysis can be done based on the relative integrated XRPD peak intensities for the different crystalline forms (12), but not for the amorphous form.

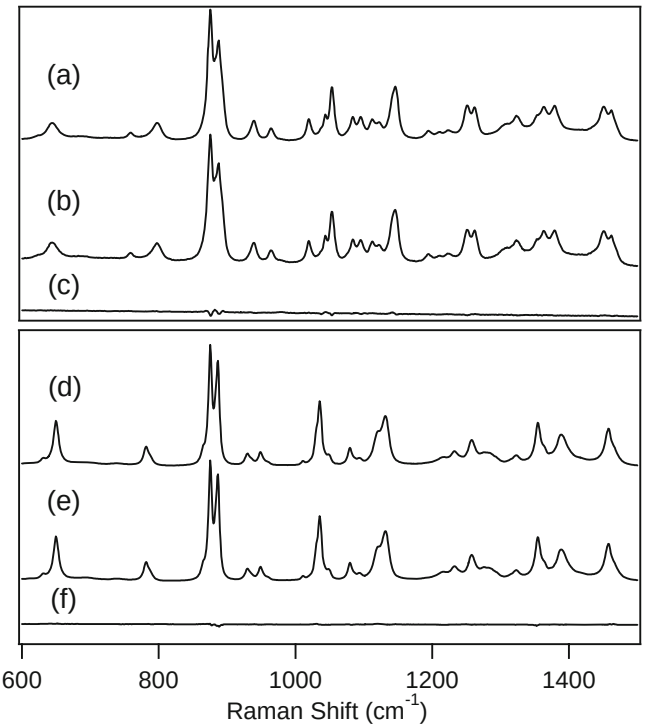

Fig. 3. Raman spectra of mannitol samples containing multiple polymorphs: (*a*) unknown mixture showing 5% β-mannitol, 61% δ-mannitol and 33% mannitol hemihydrate, and (*d*) Mannogem™ EZ having 72% α-mannitol and 29% β-mannitol. Simulated Raman spectra (*b*) and (*e*) were generated by the MCR predicted percentages of each polymorph shown above for (*a*) and (*d*), respectively. Difference spectra between the actual spectra and the simulated spectra are also shown in (*c*) *top*, and (*f*) *bottom*. Their prediction residue spectra have about 3% of the original signals.

MCR-Raman Analysis for Lyophilized Protein Formulations

The successful application of the MCR-Raman method to the analysis of mannitol alone encouraged us to extend its application to more complicated samples, such as the lyophilized protein product. For example, one tested 50 mg/ml model protein formulation in 10 mM pH 7.4 Tris–HCl buffer contains (g/ml as % *w*/*v*): 5% protein, 4% mannitol, 1% sucrose, and 0.1% Tris in aqueous solution. As mannitol, sucrose, and Tris all have non-aromatic molecular structures, their Raman signal intensity can be roughly estimated based on their relative weight percent, assuming similar Raman cross-section per weight. In the lyophilized solid, the weight of Tris is about 3% (% *w*/*w*) of the weight of mannitol, which indicates the signal intensity of Tris is only about 3% signal of mannitol, which is also the quantitation limit of mannitol forms described in the previous section. Therefore, the signal interference from Tris is estimated to be negligible, and this estimation is consistent with the observed spectra that showed no spectral features of Tris. On the other hand, the signals from sucrose and protein both show significant Raman spectral features together with the mannitol Raman contribution in the Raman spectrum of the lyophilized protein sample. Fig. 5 shows the Raman spectra of δ-mannitol, amorphous sucrose, and protein dialyzed from the protein formulation. The amorphous sucrose was prepared from a

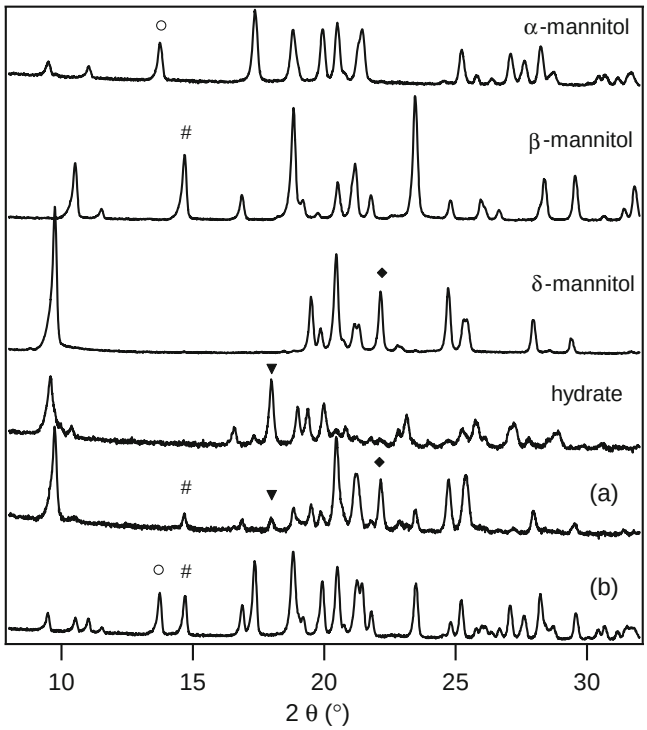

Fig. 4. XRPD pattern of mannitol samples. Characteristic peaks of α-mannitol (*circle*), β-mannitol (*number sign*), δ-mannitol (*diamond*) and mannitol hemihydrate (*inverted triangle*) have been indicated for (*a*) same unknown mixture listed in Fig. 3 (*a*), and (*b*) Mannogem™ EZ.

1% sucrose solution following a fast freeze-drying step on the cryo-stage, and the protein reference sample used was first dialyzed against 2% sucrose solution and then freeze-dried. The reason for choosing 2% sucrose solution instead of water was to attempt to retain native protein structure during lyophilization using sucrose as a stabilizer (33). δ-mannitol is usually the main mannitol polymorphic form in the lyophilized protein product.

There are two important facts about a lyophilized protein product that each relates to the understanding of its Raman spectral features. One is that sucrose and protein may not have a uniform distribution in a lyophilized cake as compared to the original solution phase. Therefore, their relative Raman intensities can vary compared to that of mannitol. Secondly, sucrose and protein both remain in the amorphous state after freeze-drying (34). This fact actually simplifies the Raman analysis for the signal taken from a lyophilized protein cake, as only the polymorphism of mannitol needs to be considered. As a consequence, the Raman spectrum taken from a lyophilized protein cake can be considered to be the sum of the Raman spectra of the various mannitol polymorphic forms, amorphous sucrose, and amorphous protein.

The pretreatment steps for the lyophilized protein Raman spectra began with the removal of the protein Raman spectral features, followed by the subtraction of the sucrose Raman features. By looking at the protein spectrum shown in Fig. 5 (c), the isolated amide I band located at 1,668 cm^{-1} is the unique Raman spectral feature coming from the protein. It then becomes straightforward to use the reference protein Raman spectrum to do a direct subtraction which removes the protein Raman features based on its second derivative spectral features (35). Although the reference protein spectrum contains both Raman spectral features of the protein and sucrose, there might be some residual sucrose Raman signal in the subtracted spectrum. A further data processing step was therefore needed to remove or to compensate the sucrose Raman spectral features not completely removed or possibly over-subtracted.

Due to the similar molecular structures between sucrose and mannitol, most of their Raman spectral features are overlapped. The only distinctive Raman spectral peak of amorphous sucrose is located at 832 cm^{-1}, but it is difficult to perform a reliable direct subtraction based on this weak, broad and partially overlapped peak. Fortunately, it was found that the second derivative spectra can actually illustrate a more differentiable sucrose spectral feature. For example, Fig. 6A shows the second derivatives of the Raman spectrum of pure δ-mannitol and the simulated Raman spectra of its mixtures with various amounts of sucrose. Five mixture spectra of mannitol and sucrose were simulated by the linear combination of the Raman spectrum of δ-mannitol and the reference sucrose Raman spectrum. The 1–5% sucrose label refers to the starting solution conditions containing 1–5% sucrose and 4% mannitol (% *w*/*v*, g/ml), while relative intensity ratios between amorphous sucrose and mannitol were estimated based on the solution Raman spectra of mannitol and sucrose. The absolute peak intensity at 832 cm^{-1} consistently grows with increasing amounts of sucrose. The broken curve that represents pure δ-mannitol gives zero intensity at 832 cm^{-1} in Fig. 6B. By taking advantage of this second derivative property of the mannitol

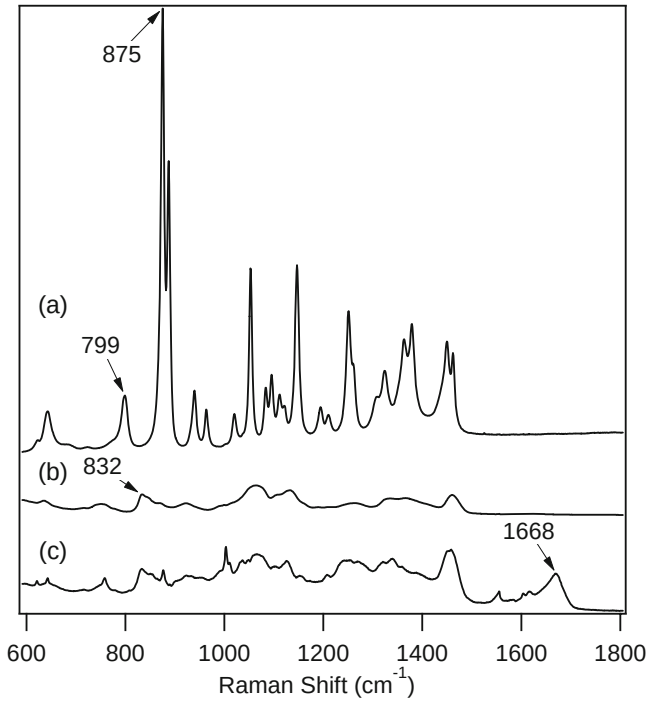

Fig. 5. Raman spectra of (*a*) δ-mannitol, (*b*) sucrose, and (*c*) the model protein from the protein formulation dialyzed against a 2% sucrose solution.

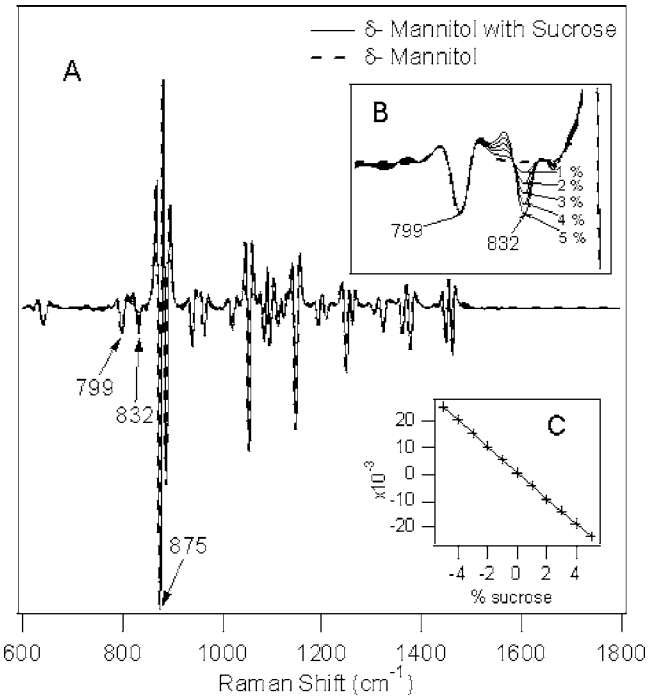

Fig. 6. **A** Second derivative spectrum of δ-mannitol and simulated spectra of δ-mannitol mixed with 1–5% sucrose, **B** their zoom-in spectra, and **C** the linear correlation between the second derivative peak intensities at 832cm^{-1} and the sucrose composition.

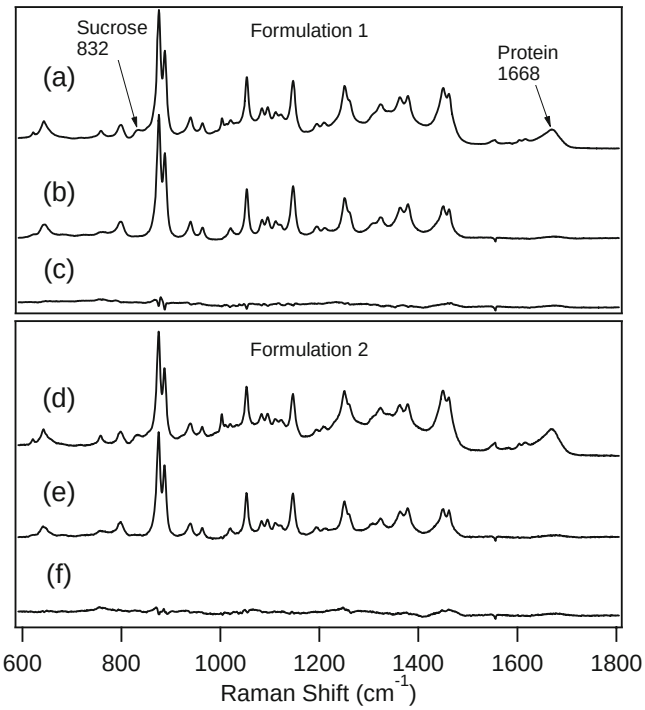

Fig. 7. Raman spectra of lyophilized model protein formulation with (*a*) 2.5% (*w*/*v*) protein and (*d*) 5% (*w*/*v*) protein. Their Raman spectra after protein and sucrose Raman feature subtraction are shown in (*b*) and (*e*), and the prediction residue spectra are shown in (*c*) and (*f*), respectively. Their prediction residue spectra have about 5% of the original signals.

and sucrose mixture, a linear univariate calibration curve based on the second derivative peak intensity at 832cm^{-1} was generated, as shown in Fig. 6C. It was used to determine the sucrose Raman contribution, which was subsequently removed or compensated.

Fig. 7 demonstrates the applications of removing the protein and sucrose Raman features, followed by mannitol polymorphic quantitation using the MCR-Raman method in the model protein formulations with different protein concentrations. Formulation 1 contains mannitol (4%, % *w*/*v*), sucrose (1%, % *w*/*v*), Tris buffer (0.1%, % *w*/*v*) and the model protein (2.5%, % *w*/*v*), and formulation 2 has the same excipient concentrations but with 5% (% w/v) of the model protein. The raw Raman spectra 7(a) and 7(d) of the lyophilized formulations 1 and 2 were first treated to remove the protein Raman feature, followed by the removal of the sucrose contribution. The negative residue peak at 1,556cm^{-1} is caused by the difference in the acquisition time of the spectra used for subtraction and is assigned to the background peak from the laser source. The resulting Raman spectra 7(b) and 7(e) were then analyzed using the MCR-Raman method to extract quantitative information on mannitol crystallinity inside the lyophilized protein cake. The final calculated results for the relative amounts of mannitol polymorphic forms in formulation 1 show 95% δ-mannitol and 5% mannitol hemihydrate. Similar results are obtained for formulation 2, with about 89% δ-mannitol and 10% mannitol hemihydrate. The predicted results for the other unlisted mannitol forms are all less than 5%. The vial-to-vial prediction variance was also evaluated by taking four replicate measurements from three different vials of formulation 1. Both δ-mannitol and mannitol hemihydrate forms show a standard deviation of 1% from a total of 12 measurements.

The prediction residue spectra for the two formulations are also listed as spectrum Fig. 7 (c) and (f). The residue intensity was estimated to be about 5% of the original Raman signal, based on the intensities of the main mannitol Raman peaks around 850cm^{-1}. Table I lists the quantitation limits of the MCR-Raman method for the samples from pure mannitol forms to the lyophilized protein samples. The larger quantitation limit follows the increased complexity of the tested sample types. The 5% quantitation limit that comes from the data processing of the model protein formulations may also be related to incomplete subtraction of the protein Raman spectral features. The reference protein Raman spectrum in Fig. 5 (c) was measured from the lyophilized protein sample after being dialyzed in a sucrose solution without mannitol, which is one of the best conditions that we can achieve for the model protein. However, the change in the dialyzed protein sample from its original formulation

Table I. The List of Quantitation Limits of the MCR-Raman Method

Sample types	Quantitation limits
Pure mannitol forms	±2%[a]
Binary mixtures of β-and δ-mannitol	±3%[a]
Mannitol samples	±3%[b]
Lyophilized protein samples	±5%[b]

[a] RMSEP value
[b] The result is estimated based on the prediction residual signal.

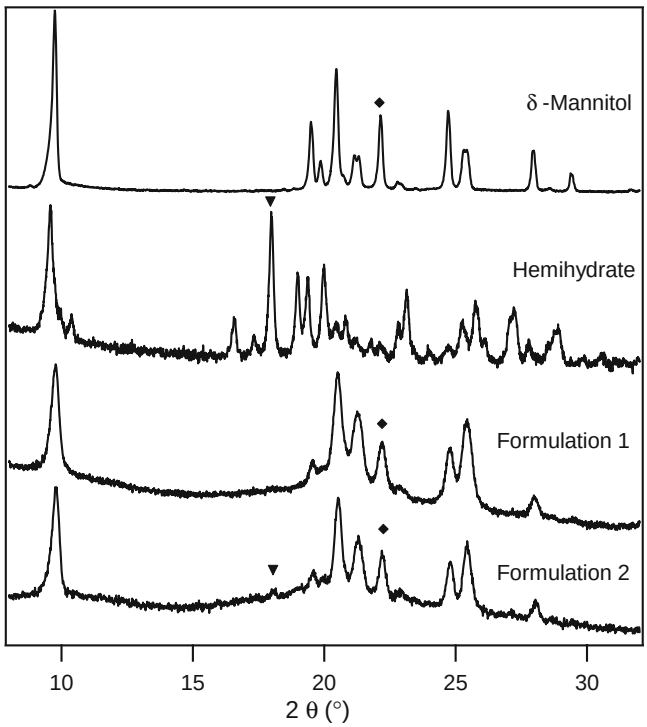

Fig. 8. XRPD pattern of δ-mannitol, mannitol hemihydrate and the protein samples with formulation 1 (2.5% *w/v* protein) and formulation 2 (5% *w/v* protein). Characteristic peaks of δ-mannitol (*diamond*) and mannitol hemihydrate (*inverted triangle*) have been indicated.

might still result in some differences in the protein Raman peak shapes since the protein conformation may be slightly different. The interaction between mannitol forms, sucrose and the model protein can also potentially cause some spectral changes, which may also contribute to the prediction residual signal. In addition, the multiple subtraction steps involved could also be a cause of the decreased signal-to-noise ratio of the resulting Raman spectra, and thus cause a larger prediction variance.

The XRPD measurements of the protein samples are listed in Fig. 8. The existence of the δ-mannitol and mannitol hemihydrate are each indicated by their XRPD peaks. The noisy baseline and notable amorphous hump was caused by the dilution of mannitol in the amorphous sucrose and protein in the formulation, which was also confirmed by the absence of their crystalline peaks. Interestingly, the existence of mannitol hemihydrate was only detected in formulation 2. Considering the 5% prediction residue signal, the 5% predicted mannitol hemihydrate falls close to the quantitation limit of the MCR-Raman method for the mannitol forms. In addition, detection of the low percentage of mannitol hemihydrate may not be possible due to the low signal-to-noise ratio of the XRPD signal. Again, the quantitative result based on the Raman method may not exactly match the results from the XRPD pattern due to the differences between their sampling sizes. However, the MCR-Raman method itself can be easily applied to cover much larger sampling sizes when appropriate Raman probes are available.

Differences in the mannitol polymorphism were observed with alternating temperature control parameters used for the lyophilization. Two lyophilized formulation 2 samples were each obtained with a low temperature control cycle and a high temperature control cycle by varying the temperature with ±5°C for all temperature control parameters used during the lyophilization. The cooling/heating rates were unchanged. The raw Raman spectra of the two formulation 2 samples are shown in Fig. 9. The predicted polymorphic forms of mannitol are 39% δ-mannitol, 48% hemihydrate and 14% amorphous for the low temperature control cycle, and 8% α-mannitol, 75% δ-mannitol, 11% hemihydrate and 6% amorphous for the high temperature control cycle. The low temperature control cycle clearly results in a higher yield of mannitol hemihydrate, which may be associated with the low annealing temperature, insufficient annealing time, and the low secondary drying temperature (7). The existence of mannitol hemihydrate can also be identified by its two characteristic Raman peaks at 759 and 1,018 cm^{-1}, as shown in Fig. 9 (b) after the removal of the Raman spectral features from both the protein and sucrose. As mannitol hemihydrate is not the preferred mannitol form in a lyophilized protein formulation, its dehydration requires higher annealing temperatures which may affect the protein stability (7). The changes in the temperature control cycles also result in a change in the protein secondary structure, which can be observed as the amide I peak (at 1,680 cm^{-1}) and amide III peak (at ~1,220 cm^{-1}) in the residue spectra. The observed Raman spectral change in the protein secondary structure may also potentially affect the stability of the protein product.

When applying the MCR-Raman method for protein samples with undesired conditions, there are potentially two aspects that can lead to limitations of this method. First, the

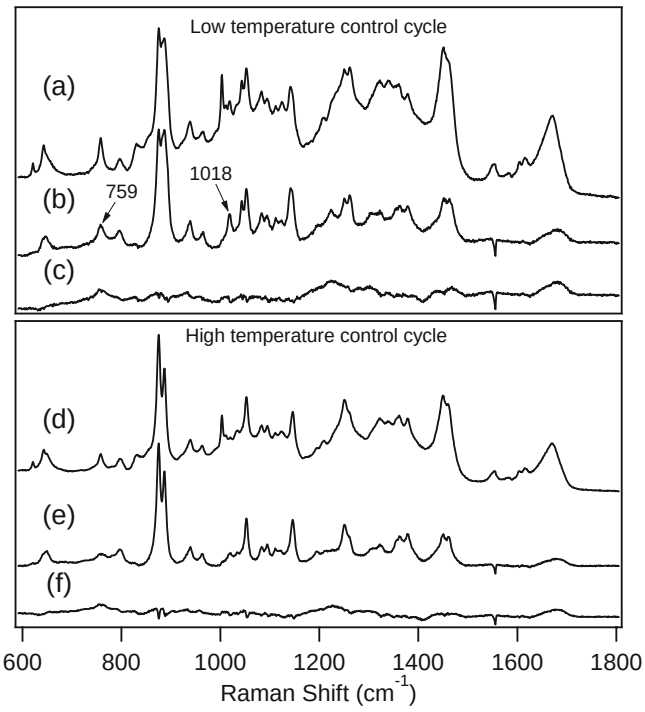

Fig. 9. Raman spectra of lyophilized model protein formulation with 5% (*w/v*) protein after (*a*) low temperature control cycle and (*d*) high temperature control cycle. Their Raman spectra after protein and sucrose Raman feature subtraction are shown in (*b*) and (*e*), and the prediction residue spectra are shown in (*c*) and (*f*), respectively.

reference protein spectrum may not match the protein Raman signal taken from the test protein sample, as shown in Fig. 9. The residue protein Amide I signal is close to 10% of the original protein signal. Therefore, the quantitation limit may become worse due to the interference of remaining protein spectral features. As there is not enough data for the determination of the actual impact of the residual protein signal to the quantitation limit, the careful selection of a reference protein sample for the minimization of the residue protein signal (<5%) is desired for the success of this MCR-Raman method. Secondly, ingredients other than sucrose may also lead to a larger quantitation limit for the conditions that they may have their own polymorphism in the lyophilized protein product or have completely overlapped Raman spectral features with the mannitol forms. Theoretically they can be resolved as additional components in the MCR calibration model without affecting the quantitation limit if spectral noise is negligible. However, noise from the Raman measurement is a limit and causes the larger prediction variances when the sample compositions become more complex.

Although the application of the MCR-Raman method to the lyophilized protein product may still have a few limitations, it appears to be a very useful tool which can provide quantitative information on mannitol polymorphic forms. This information can help us develop a systematic understanding of how changes in mannitol crystallinity can affect the final lyophilized drug product quality. This is especially applicable when screening different formulations and lyophilization parameters to achieve the optimal formulation and lyophilization process resulting in a high quality drug product.

CONCLUSIONS

An MCR-Raman prediction method for the quantitative determination and characterization of mannitol polymorphic forms in a model protein lyophilized product is described in this paper. With the available Raman spectra of pure mannitol forms, this MCR-Raman method has provided an analytical tool that avoids the difficulties of preparing synthetic calibration standards with unstable or metastable polymorphic forms.

The MCR-Raman method was tested with the reference binary mixtures of β- and δ-mannitol forms. The prediction residue was also used for determining the quantitation limits. The RMSEP values of 3% for the β- and δ-mannitol mixtures, and the quantitation limits of the mannitol forms of 3% for the mannitol samples and 5% for the lyophilized model protein formulations demonstrate that the MCR-Raman method is an accurate, sensitive and robust method for the semi-quantitative determination of the mannitol forms in lyophilized model protein products. The results from the XRPD measurements are consistent with the Raman data for the four crystalline forms in the actual samples.

The strategy of combining the MCR-Raman method with the removal of the protein and sucrose Raman spectral features has been demonstrated to be a useful and novel approach to directly determine the mannitol crystallinity in a lyophilized protein product. More work is needed in the future to demonstrate that this general Raman spectroscopic quantitation method can be efficiently developed and validated in order to study polymorphism in drug substances and products at various stages during the drug development process.

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