Role of excipients in the quantification of water in lyophilised mixtures using NIR spectroscopy

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Abstract

The ratio between mannitol and sucrose in a freeze-dried formulation has a major impact on the processing and the stability of a lyophilised product. The ratio of these common excipients influences a critical quality attribute of the system, namely the overall amount of water, due to the different nature of their water-solid interactions. For this experiment samples containing various ratios of mannitol and sucrose and several other additives were freeze-dried, stored under different conditions and measured by NIR. Different spectral pre-processing methods and wavelength selections were tested. Multivariate analysis was applied to correlate the Karl Fischer titration to the NIR spectra. It was found that standard normal variate (SNV) transformation of the wavenumber range 4200 ï 7400 cm^{-1} yielded prediction errors close to the accepted measurement error of the Karl Fischer titration, when measuring samples of up to 5.5% (w/w) water. It was further found that there was a slight tendency for samples containing inorganic salts or histidine to be underestimated in the NIR measurements. However, no influence was found to be caused by the varying mannitol-sucrose ratio. By reducing the sample set to those samples containing up to around 2% of water, the error was found to be below the uncertainty originating from the reference method. Due to this it can no longer be determined whether the deviation originates from the NIR method or the reference method. It can therefore be concluded the NIR is a suitable tool for quantification of the water content in lyophilised samples with varying mannitol-sucrose ratios.

Keywords: NIR, freeze-drying, mannitol, sucrose, multivariate analysis, water quantification

1. Introduction

Freeze-drying is a complex unit operation which will become increasingly important in the future as it is the main approach for the processing of an increasing amount of pharmaceutically active biomacromolecules. Due to the potential instability of macromolecular systems and the fact that a high level of variation often takes place during processing, a robust process monitoring and control solution is needed to ensure a sufficiently good quality of the final product. The ratio between mannitol and sucrose in a freeze-dried formulation has a major impact on the production cycle and the stability of a lyophilised product. This is due to the fact that the two excipients show different modes of water-solid interaction [1]. Mannitol is known to result in crystalline cakes. However, it might crystallize in 3 polymorphic forms (α, β and δ) or as a hemi-hydrate upon freeze-drying [2, 3]. In a crystalline substance, water will not be absorbed into the core of the material but rather be adsorbed to the surface. In contrast, sucrose, which usually results in an amorphous product upon freeze-drying, will absorb water eventually leading to deliquescence of the preparation at the critical relative humidity. The ratio of the two compounds will thus influence a critical quality attribute of the system, namely the overall amount of water, due to the different nature their of water-solid interactions. The overall water can be seen as a combination of water that has not been removed during the drying process and water that has been taken up during storage. Thus, there is a direct connection between the composition of a product and its quality.

Currently, Karl Fischer titration is the standard method for the determination of water, though this is a time-consuming and destructive method that requires the handling of organic solvents and can only be applied for a limited number of samples. Furthermore,

vial-to-vial variability can lead to false positive or negative results, as the measured samples are removed from the production batch. NIR spectroscopy has gained more interest in the industry as it has the potential to be interfaced in several production processes and, due to the fast and non-invasive nature of the method, provides real-time data of a complete batch. Pharmaceutical applications of NIR were recently reviewed by Reich [4]. NIR has been shown to be useful for various applications in solid state analysis, e.g. the investigation of amorphous and crystalline state of both excipients and active compounds [5, 6]. Besides the investigation of the solid state properties of a compound, NIR has seen wide-spread use for quantification of water in pharmaceutical products. The applicability of NIR for the determination of water profits from the fact that water shows strong adsorption bands in NIR, most prominently the first overtone of OH stretching at around 6800 ï 7100 cm^{-1} and the combination band of OH stretching and bending at around 5100 ï 5300 cm^{-1}. The exact position of the bands is depending on the chemical and physical environment of the water molecule and may therefore vary between different systems. Therefore, it is not surprising that several models can be found in the literature describing the use of NIR for the determination of water in freeze-dried products. Though usually the final product is characterised, the use of both Raman and NIR spectroscopy for in-line spectroscopic measurements during freeze-drying has recently been described [7-9].

An early publication on the use of NIR for freeze-dried products describes the feasibility of measuring moisture in lyophilised sucrose through intact glass vials, thus enabling NIR to be used for non-destructive measuring [10]. Derksen et al. [11] determined the moisture content of a mannitol-based product of an undisclosed active compound and

several other excipients in a fixed ratio. They found a difference between moisture content based on KF and NIR between -0.6 and +0.4 mg/vial using 2nd derivative spectra in the range 4000 ï 9000 cm^{-1}. Another method for evaluating the content of moisture was used for moisture determination in PVP and cyclodextrin [12]. Here, a comparison of the standard normal variate corrected (SNV) water band at 5200 cm^{-1} with a reference peak was compared to results obtained by partial least square regression and found to be of similar quality but more robust. It has also been shown in an applied study that samples containing a non-specified active substance can be classified according to ñhighò or ñlowò water content by using multiplicative signal correction (MSC) of the NIR spectra between 4650 and 8330 cm^{-1} [13]. While most studies focus on a single active compound, Zheng et al. [14] investigated NIR for moisture determination of five vaccine formulations. Using KF titration as reference method, water contents between 0.17 and 1.5% were found. By applying second derivative transformation on the spectral range from 5100 ï 5260 cm^{-1}, a model based on 2 PLS components with a root mean square error of prediction (RMSEP) of 0.9% was found. The deviation between NIR and KF can mainly be seen at low water concentrations. It is furthermore stated that product specific models performed better than a multi-product model, and that using the whole range (4000 ï 14300 cm^{-1}) for a calibration will require more PLS factors due to more noise in the spectrum.

Besides the determination of the amount of water, also the state of water, i.e. surface or bound water, is of interest as this will have an influence on the design of the freeze-drying cycle. NIR was applied for differentiating between those two states of water using pure compounds. Zhou et al. [15] constructed a model with an RMSEP of 0.42 % for the

moisture determination of an unknown drug substance using 2nd derivative transformation of the spectral range of 5139 ï 5488 cm^{-1} for water contents between 1 and 10%. Furthermore, samples with surface and bound water were differentiated from samples with only bound water based on PCA score plots. The common excipient mannitol was investigated in detail by Cao et al. [16], who reported that the water band in the 2nd derivative spectra shifted from 5249 cm^{-1} for surface water to 5136 cm^{-1} for bound water. It can be concluded that several approaches are leading to acceptable calibration models using NIR, but it has to be mentioned that the composition of the formulation was usually kept constant and that change in composition and interference from unknown constituents will decrease the performance of the model [15-17]. This was investigated for freeze-dried monoclonal antibodies where surfactant and buffer did not influence the model, but the concentration of the disaccharide used as excipient had to be kept constant [17]. It is therefore still an open question whether more generally applicable models are feasible. It was thus the aim of this study to investigate whether it seems feasible to establish a method that can determine the amount of water in freeze-dried mannitol/sucrose mixtures regardless of their ratio and the presence of other commonly used excipients, in this case sodium chloride, calcium chloride and histidine.

2. Materials and methods

2.1. Materials

Mannitol (Unikem, Copenhagen, Denmark) and sucrose (Ferro Pfanstiehl, USA) were used as lyoprotectants and cryoprotectants. As relevant excipients, sodium chloride,

calcium chloride dihydrate (both Merck, Darmstadt, Germany) and histidine (Alsiano, Kawasaki, Japan) were used.

Samples consisted of 50 mg/ml solutions of various mannitol-sucrose mixtures. Weight ratios between mannitol and sucrose in the mix ratios of 1:9, 3:7, 5:5, 7:3 and 9:1 were investigated. All samples were prepared by pouring 2 ml of the solution into 2 ml borosilicate vials, followed by freeze-drying. In order to achieve samples with a wide span of moisture content, the dried samples were stored at relative humidities (RH) of 0, 11, 33 and 75% by using relevant saturated salt solutions. Besides those binary mixtures, tertiary mixtures based on a 50:50 mixture of mannitol and sucrose were composed containing either 3.2 mg/ml histidine, 2.92 mg/ml NaCl or 1.5 mg/ml $CaCl_2$. A quaternary mixture containing the above-mentioned quantities of both NaCl and histidine was also included.

2.2. Methods

2.2.1. Freeze-drying

A small pilot-scale Model FCM 10 freeze-dryer, made by Steris (Cologne, Germany) was used. The freeze-dryer was equipped with a stoppering system, had 3 shelves and a capacitance as well as a pirani vacuum gauge. The freeze-drying cycle had a duration of 91 h and is shown in Figure 1.

2.2.2. NIR analysis

For NIR analysis a Bomem FTLA 2000 series FT-NIR spectrometer (ABB Bomem, Quebec, Canada) was used. NIR sample measurement was performed through the glass

vial. Depending on the condition of the sample and the remaining filling height, samples were preferably measured through the side of the vial while placed on a customised spinning device, which rotated the vial perpendicular to the beam of the NIR spectrometer. In cases where measurement through the side was not possible, samples were measured through the bottom of the vial. Samples were measured in the range 3800 to 10000 cm^{-1} with a resolution of 8 cm^{-1}.

2.2.3. Reference methods

The residual moisture of samples was determined by coulometric Karl-Fischer titration using a Metrohm 831 titrator with an autosampler (Copenhagen, Denmark). Formamide was used as solvent. The percentage of water content (w/w %) was calculated based on the theoretical amount of solid matter. X-Ray powder diffraction data were collected at room temperature using a rotating anode X-Ray generator (Rigaku MicroMax-007FH, Cu K(α) radiation, λ = 1.5418 Å) with a MAR345 plate. Powders were transferred to a bottom-capped glass capillary (Hampton Research, USA) with an outer diameter of 0.7 mm and centrifuged at 2000g for 10 minutes to pack the crystals in the bottom. The capillaries were sealed and mounted on a goniometer head. The samples were exposed for 10 minutes under rotation.

2.2.4. Data analysis

Multivariate data analysis was performed using SIMCA-P 11.5 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used to gain an overview of the data, while partial least square (PLS) regression was used for correlating the NIR spectra to the

Karl Fischer titration. As the principles of PCA and PLS are becoming more and more familiar among scientists, the mathematical basis of PCA and PLS will not be explained in this article. It should be mentioned, though, that over-fitting should be avoided. In order to prevent over-fitting of the model, the number of components used for the PLS model was only increased until the goodness of prediction started to decrease. Regarding scaling methods, all NIR spectra were centred. As is usual for spectroscopic data, unit variance scaling was not performed. Centring was done regardless of which mathematical treatment, if any, was applied on the spectra later on. During model development, wavenumber selection was employed on several spectral ranges with clearly visible features:

- 4875 ï 5400 cm^{-1} (OH combination band, main water band)
- 4500 ï 5400 cm^{-1} (as above plus R-OH combination band)
- 4200 ï 7400 cm^{-1} (as above plus CH combination bands at lower wavenumbers and 1^{st} overtone of CH and OH at higher wavenumbers)
- 3950 ï 7400 cm^{-1} (complete spectrum without highly varying part below 3950 cm^{-1} and noisy baseline above 7400 cm^{-1})

3. Results and Discussion

It has been shown before that the molar ratio of mannitol-sucrose should be 3:1 or above to prevent collapse during primary drying [18]. It is therefore not surprising that samples with a high content of sucrose and stores at elevated humidity collapsed during storage. Due to the small remaining cake of the collapsed samples, extraction of water under Karl-Fischer titration was unreliable. The NIR spectra of these samples also deviated strongly

from the other samples. This could easily be seen in a principal component analysis (PCA) plot of the raw data (data not shown). Samples with a mannitol-sucrose ratio of 1:9 stored at 33% and 75% RH, as well as a mannitol-sucrose ratio of 3:7 stored at 75% RH, have therefore been excluded from all further models. Karl Fischer titration of the remaining samples yielded water contents of between 0.2 and 5.59%, with the majority (76%) of the samples containing less than 3% water. For a first visual inspection, all spectra were SNV-corrected in order to compensate for the baseline offset. Looking at Figure 2, which presents the standard deviation spectrum of the SNV-corrected spectra of the remaining 29 samples, it can clearly be seen that a major difference between the spectra can be found at 5160 cm^{-1}. This band corresponds to the combination band of OH-stretching and bending of water molecules and should be suitable for quantification. Another area with high variance can be found around 6900 cm^{-1}. In this area, the first overtone of the stretching of OH-bonds in water molecules can be found. It often overlaps with CH bands, however, and is thus less suitable for quantitative purposes. As a basis for further discussion, the spectra of mannitol and sucrose raw material are shown in Figure 3a. Investigating the spectra of the freeze-dried mixtures, a closer look on the spectra individually reveals that the mannitol-sucrose ratio (Fig. 3b) and the relative humidity under storage (Fig. 3c) show a clear influence on the obtained spectra. The excipients NaCl, $CaCl_2$ and histidine on the other hand do not show an obvious impact on the spectra, which is due to their low concentration and low NIR activity (Fig. 3d).

The variation can be explained as follows: In Figure 3b the mannitol-sucrose ratio is varied. It can thus be expected that the varying chemistry between the sugar alcohol (also

called polyol) mannitol and the disaccharide sucrose will be responsible for a part of the variation in the spectrum. This can be seen in changes in the CH bonds (combination bands at 4200 ï 4500 cm^{-1} and first overtone at 5600 ï 6200 cm^{-1}) as well as the R-OH bonds (4600 ï 4850 cm^{-1}). In Figure 3c the mannitol-sucrose ratio is kept constant so that no influence from this source can be expected. However, mannitol can crystallize in various forms, which will also be visible in the NIR spectra. These differences can also be seen in the above-mentioned areas. X-ray powder diffractometry (XRPD) of selected samples clearly showed that mannitol crystallized during freeze-drying (Figure 4). Both β-mannitol (at 10.5, 14.5 and around 19°2ɗ) and mannitol hemi hydrate (at 9.6, 16.5 and 18°2ɗ, according to [19]) were detected by XRPD, thus confirming the variations in the NIR spectra. For comparison, the diffractogram of sucrose raw material is included in Figure 4 and downscaled by factor 4 for reasons of clarity. No reflection confirming the crystallization of sucrose was observed. It can therefore be stated the sucrose was in an amorphous state after freeze-drying.

As several factors influence the spectra, a univariate calibration approach is not appropriate, and multivariate data analysis should be performed. Though it is not a common practice to look at the untreated data, as this will usually result in a less robust model, one can get an impression of physical and chemical information in this way as both scattered and absorbed light is taken into account. As mentioned in the introduction, common practice in the use of multivariate analysis for NIR data includes the transformation of the obtained data, with the use of 2^{nd} derivative seemingly being the most common. Transformation is performed in order to reduce the impact of the physical

state and focus on the chemical information. However, it should be considered to take a look at the untreated data as well in order to understand all the underlying spectral information. As the uncorrected spectra will deviate strongly from each other due to differences in baseline offset and noise, comparing the spectra by visual inspection will be of little use. For an investigation of the untreated data, PLS regression was performed using several wavenumber ranges (Table 1). The reasoning behind the choice of ranges is given in the methods section.

As can be seen in Table 1, even with the untreated data of the complete spectrum a root mean square error of estimation (RMSEE) of 0.52 % can be achieved. It has to be taken into consideration, however, that a rather weak correlation was obtained and that the first principal component, which contributes to more than 99.5% of the variation, merely describes the baseline offset, which is visible in the loadings of the first component (data not shown). Although the second component merely describes around 0.24% of the variation, it shows a clear domination by the water absorption band at 5160 cm^{-1} (Figure 5). Spectral focusing on the range 3950 ï 7400 cm^{-1} decreased the RMSEE to around 0.45% while further focussing merely had a marginal effect.

Due to the wide overlapping bands obtained by NIR, mathematical transformations are rather common. For all derivative transformations cubic smoothing with 15 points in each sub-model was applied. Regarding the quantification of water in the mannitol/sucrose mixtures both the 1^{st} derivative and the commonly used 2^{nd} derivative do not show any clear superiority compared to the untreated data (Table 1).

For further investigation of the available mathematical pre-treatments, standard normal variate transformation (SNV) was used. SNV correction removes the baseline offset of NIR spectra and adjusts all spectra based on an individual average spectrum. An overview over the performed models is given in Table 1. It can clearly be seen that models with lower error rates were achieved using fewer PLS components compared to both the untreated data and the 1st and 2nd derivative. SNV correction is therefore the most appropriate approach for quantification of sucrose/mannitol mixtures and will be investigated further.

Closer examination of the spectral range 4200 ï 7400 cm^{-1}, for instance, reveals that one PC is sufficient to achieve a good model with a low RMSEE of 0.42% (Figure 6).

Investigating the loading of the PLS component, which is the basis of this estimation, a clear influence of the water band at 5160 cm^{-1} can be seen (Figure 7). Besides the good correlation with the KF data, this also confirms that water is the main contributor to the model. However, some smaller features also seem to influence the model systematically, as the RMSEE in this model is lower than in a model in which only the main water band is taken into account. Two areas in the loading plot might contribute to this result. The first area contains the small peak at 4380 cm^{-1}, which can be attributed to the CH combination band of the two sugars. As the water uptake mechanism varies between the two sugars, this might also influence the result. The second area is around 6800 ï 7000 cm^{-1}, where the first overtone of the stretching vibration of water molecules can be found. As mentioned earlier, this band often overlaps with other signals, and the band in the

loading is rather flat and broad. Nevertheless, taking this band into account can be the reason for the improved model.

To summarise, it can be stated that lowest number of PCs needed to obtain to a suitable model was found with SNV correction. Regardless of the spectral range, the RMSEE was improved from above 0.45% (with 1^{st} or 2^{nd} derivative, or the untreated data) to around 0.42% with SNV correction. This corresponds to a relative improvement of around 5.5%. Based on practical experience, the accuracy of the Karl Fischer measurement is set to 0.4%. As the RMSEE is close to the accuracy of the reference method, the origin of the error cannot be determined. It can therefore be stated that moisture determination by SNV-corrected NIR spectra of freeze-dried mixtures is comparable to Karl Fischer titration. Although this model is applicable to all investigated mixtures, i.e. varying ratios of sucrose and mannitol as well as added excipients, it will be investigated, whether or not the composition shows a major influence on the model.
In connection with investigating whether or not the model predicts samples with excipients in different manner than samples without excipients, the differences between the predicted and the measured moisture content have been compiled and can be seen in Figure 8. For this evaluation the model obtained for SNV-corrected spectra in the range of 4200 ï 7400 cm^{-1} was used.

When first looking at the binary samples, no clear tendency in the moisture prediction according to the different ratio of mannitol and sucrose was observed. However, it can be seen that there is a tendency for samples containing excipients to result in higher moisture

contents when analysed by KF titration than by the NIR model, indicating that NIR might underestimate the moisture content in the presence of excipients. A possible explanation can be given as follows: It has been reported in the literature that both sodium chloride and the amino acid glycine can inhibit the crystallisation of mannitol [20, 21]. Based on these findings it can be assumed that sodium chloride and the amino acid histidine can lead to an increase in the amount of amorphous mannitol in the samples thus increasing the amount of absorbed water compared to surface water. It has been reported that this results in a shift of the water band [16]. This shift in the water band maximum will influence the shape of the peak and in turn the outcome of the quantification. This assumption can also explain why models that do not purely focus on the main water band can show a higher accuracy: changes in composition will also show other forms of systematic variation that will even out the change in water band shift caused by the change. Further studies with larger sample sets should be performed to investigate if this influence of inorganic salts and amino acids can be generalised.

In order to investigate the predictive properties of the model without excipients, a new model has been calculated based on SNV corrected NIR spectra in the range 4200 ï 7400 cm^{-1}. The correlation between the observed and the predicted moisture content showed an RMSEE of 0.387% and thus theoretically lies below the accuracy of the reference method.

Both models (with and without excipient) contain 10 ï 90% mannitol in the mannitol-sucrose mixture and result in errors close to the accuracy of the reference method. It is therefore a major finding that the ratio between mannitol and sucrose does not influence the usability of the models, which can be looked upon as general moisture quantification

models for mannitol-sucrose mixtures. As the accuracy of these models comes close to that of the reference method, further optimisation is not necessary. However, in order to show the potential of SNV corrected NIR data for the determination of moisture, a hypothetical model was established taking only samples with low moisture content into account. The rationale behind this idea is that the model will be more predictive due to a higher density of obtained moisture contents. Furthermore, such a model might be relevant for practical purposes also, as low moisture contents would be the normal working range. NIR spectra of all samples up to around 2% of moisture content (KF) were SNV-corrected in the range 4200 ï 7400 cm^{-1}. In this model a hypothetical RMSEE of 0.211% could be achieved. For verification of the precision of the NIR method, however, a more precise reference method would be needed.

4. Conclusion

It has been shown that multivariate analysis of NIR spectra can be used to predict the water content of freeze-dried mannitol-sucrose mixtures with moisture contents of up to around 5.5%. Several mathematical pre-treatments were applied in order to compare NIR with Karl Fischer titration. Untreated data were analysed, but as most of the variation is due to background variation, which leads to a less robust model, spectral pre-treatment is recommended. While 1^{st} and 2^{nd} derivative did not lead to improvement of the model, SNV correction simplified the model and resulted in a model with a lower error. SNV corrected NIR spectra correlate well with KF data in the magnitude of the insecurity given by the reference method. Although there seems to be a tendency for the moisture content of samples containing inorganic salts or the amino acid histidine to be

underestimated, it cannot be concluded that a model excluding those samples will resemble reality in a better way due to the insecurity of the reference method. No influence on the model performance due to changes in the ratio between mannitol and sucrose was detected. In summary, it can be stated that the establishment of multivariate NIR based models that predict the water content close to the error incorporated in the reference method is feasible - even if the mannitol-sucrose ratio is varied from 1:9 to 9:1.

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Literature

[1] G. Zografi, Drug Dev. Ind. Pharm. 14 (1988) 1905-1926.

[2] A.I. Kim, M.J. Akers and S.L. Nail, J. Pharm. Sci. 87 (1998) 931-935.

[3] L. Yu, N. Milton, E.G. Groleau, D.S. Mishra and R.E. Vansickle, J. Pharm. Sci. 88 (1999) 196-198.

[4] G. Reich, Adv. Drug Delivery Rev. 57 (2005) 1109-1143.

[5] L. Nørgaard, M.T. Hahn, L.B. Knudsen, I.A. Farhat and S.B. Engelsen, Int. Dairy J. 15 (2005) 1261-1270.

[6] M. Savolainen, A. Heinz, C. Strachan, K.C. Gordon, J. Yliruusi, T. Rades and N. Sandler, Eur. J. Pharm. Sci. 30 (2007) 113-123.

[7] M. Bruells, S. Folestad, A. Sparen and A. Rasmuson, Pharm. Res. 20 (2003) 494-499.

[8] T.R.M. De Beer, M. Alleso, F. Goethals, A. Coppens, Y.V. Heyden, H.L. De Diego, J. Rantanen, F. Verpoort, C. Vervaet, J.P. Remon and W.R.G. Baeyens, Anal Chem 79 (2007) 7992-8003.

[9] S. Romero-Torres, H. Wikstrom, E.R. Grant and L.S. Taylor, PDA J. Pharm. Sci. Technol. 61 (2007) 131-145.

[10] M.S. Kamat, R.A. Lodder and P.P. DeLuca, Pharm. Res. 6 (1989) 961-965.

[11] M.W.J. Derksen, P.J.M. Van De Oetelaar and F.A. Maris, J. Pharm. Biomed. Anal. 17 (1998) 473-480.

[12] M. Bruells, S. Folestad, A. Sparen, A. Rasmuson and J. Salomonsson, J. Pharm. Biomed. Anal. 44 (2007) 127-136.

[13] L. Sukowski and M. Ulmschneider, Pharm. Ind. 67 (2005) 830-835.

[14] Y. Zheng, X. Lai, S.W. Bruun, H. Ipsen, J.N. Larsen, H. Lowenstein, I. Sondergaard and S. Jacobsen, J. Pharm. Biomed. Anal. 46 (2008) 592-596.

[15] G.X. Zhou, Z. Ge, J. Dorwart, B. Izzo, J. Kukura, G. Bicker and J. Wyvratt, J. Pharm. Sci. 92 (2003) 1058-1065.

[16] W. Cao, C. Mao, W. Chen, H. Lin, S. Krishnan and N. Cauchon, J. Pharm. Sci. 95 (2006) 2077-2086.

[17] T.P. Lin and C.C. Hsu, PDA J Pharm Sci Technol 56 (2002) 196-205.

[18] R.E. Johnson, C.F. Kirchhoff and H.T. Gaud, J. Pharm. Sci. 91 (2002) 914-922.

[19] C. Nunes, R. Suryanarayanan, C.E. Botez and P.W. Stephens, J. Pharm. Sci. 93 (2004) 2800-2809.

[20] A. Pyne, K. Chatterjee and R. Suryanarayanan, J. Pharm. Sci. 92 (2003) 2272-2283.

[21] C. Telang, L. Yu and R. Suryanarayanan, Pharm. Res. 20 (2003) 660-667.