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Reliable and fast quantitative analysis of active ingredient in pharmaceutical suspension using Raman spectroscopy

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Abstract

The concentration of acetaminophen in a turbid pharmaceutical suspension has been measured successfully using Raman spectroscopy. The spectrometer was equipped with a large spot probe which enabled the coverage of a representative area during sampling. This wide area illumination (WAI) scheme (coverage area 28.3 mm^2) for Raman data collection proved to be more reliable for the compositional determination of these pharmaceutical suspensions, especially when the samples were turbid. The reproducibility of measurement using the WAI scheme was compared to that of using a conventional small-spot scheme which employed a much smaller illumination area (about 100 μm spot size). A layer of isobutyric anhydride was placed in front of the sample vials to correct the variation in the Raman intensity due to the fluctuation of laser power. Corrections were accomplished using the isolated carbonyl band of isobutyric anhydride. The acetaminophen concentrations of prediction samples were accurately estimated using a partial least squares (PLS) calibration model. The prediction accuracy was maintained even with changes in laser power. It was noted that the prediction performance was somewhat degraded for turbid suspensions with high acetaminophen contents. When comparing the results of reproducibility obtained with the WAI scheme and those obtained using the conventional scheme, it was concluded that the quantitative determination of the active pharmaceutical ingredient (API) in turbid suspensions is much improved when employing a larger laser coverage area. This is presumably due to the improvement in representative sampling.

Keywords: Acetoaminophen; Pharmaceutical suspension; Raman spectroscopy; Wide area illumination; Process Analytical Technology

1. Introduction

Raman spectroscopy has found increasing use for the analyses of diverse pharmaceutical products [1–3]. Raman offers the advantages of fast and non-destructive analyses with minimal or no sample pretreatment or preparation. Additionally, it is an analytical method that can be widely adopted for Process Analytical Technology (PAT) as the guideline proposed by the Food & Drug Administration (FDA) in 2004 [4,5]. Among those pharmaceutical applications for which Raman can be used, the quantitative analysis of pharmaceutical suspensions is one of the most difficult and challenging because they contain suspended particles that can adversely affect the measurement reproducibility. Several factors such as re-absorption (self absorption), elastic (non-Raman) scattering and beam attenuation are potential sources of variability.

Baeyens' group has recently evaluated Raman spectroscopy for the analysis of medroxyprogesterone acetate in a pharmaceutical suspension and in-line monitoring of the homogenization process of a pharmaceutical suspension using a fiber optic probe [6,7]. A similar approach has been used to monitor the formulations of topical gels and emulsions by Ackermann and coworkers [8]. Although these studies were successful, the possibility of non-representative sampling and its affect on prediction accuracy was not addressed. A pharmaceutical suspension is heterogeneous and is typically a mixture of solid active pharmaceutical ingredient (API) and a supporting syrup matrix. Due to the heterogeneity, the resulting Raman spectrum might not be representative of the correct API composition. The potential for non-representative sampling with solid matrices

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was shown in a previous study by our group using tablets [9].

Due to the potential for particle settling inherent with suspensions, Raman measurements of such samples are generally performed employing stirring in order to correctly represent the sample composition [6]. However, the reproducibility of Raman data collection during sample stirring could be significantly affected by several factors such as the mixing speed and the sample turbidity. Moreover, if Raman measurements are used for the analysis of suspensions contained in their final packaging, it is not possible to stir the sample without contamination or damage.

Quantitative analyses of APIs in turbid suspensions could be improved by the collection of Raman data with a larger illumination spot in order to cover a larger sampling area. In this manner, the Raman spectra should correctly represent the average sample composition of suspension samples. Also, the spectral reproducibility, which is otherwise flawed by troublesome effects such as elastic scattering and beam attenuation, could be improved by such an approach.

Using this reasoning, we have developed a novel Raman collection system using a wide area illumination (WAI) scheme that employs a 6-mm diameter circular laser spot (area 28.3 mm^2) for sample analysis. To evaluate the performance of this WAI scheme for the analysis of pharmaceutical suspensions, the quantification of acetaminophen suspended in a syrup formulation has been attempted. Simultaneously, we have installed a layer of isobutyric anhydride as an external standard in front of the suspension samples to correct for fluctuations in laser intensity. The Raman spectra collected with the WAI scheme were much more reproducible compared to those using the conventional Raman collection scheme. When acetaminophen concentrations were in the higher range (visually very turbid), the corresponding Raman intensity dropped unexpectedly. Because of this, the data were segregated and two different partial least squares (PLS) [10–12] models for the low and high concentration ranges were developed. By acquiring Raman spectra using appropriate representative sampling and effectively correcting the Raman intensity using an external standard, the concentrations of acetaminophen in suspension were successfully determined.

2. Experimental

2.1. Suspension preparation

Appropriate amounts of acetaminophen were suspended in a 70% (w/w) aqueous sucrose medium. The relative concentrations of acetaminophen varied from 79 to 130% (2.24–4.16 g/100 mL) based on the target concentration. The acetaminophen concentration for each sample was expressed as the percentage relative to the amount of acetaminophen in a suspension. A suspension containing 3.2 g/100 mL was designated as 100% acetaminophen. Propylene glycol (1 g/100 mL) and β-cyclodextrin (1 g/100 mL) were added as solubilizers. Methyl *p*-benzoic acid (0.09 g/100 mL) and propyl *p*-benzoic acid (0.01 g/100 mL) were added as preservatives. A total of 18 suspensions were prepared with different concentrations of acetaminophen (increments of 3%).

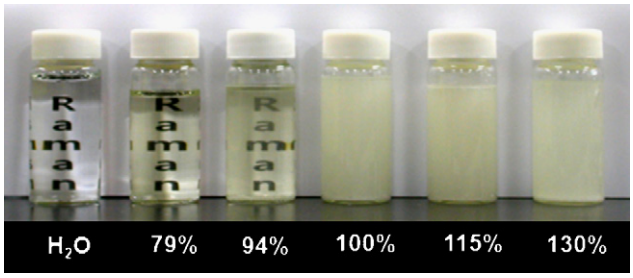

Fig. 1. Aqueous pharmaceutical suspensions with five different concentrations (79, 94, 100, 115 and 130%) of API in glass vials.

Fig. 1 displays photographs of five different suspensions with relative concentrations of 79, 94, 100, 115 and 130% (based on the nominal level) in glass vials. The letters "Raman" printed on white paper were placed behind these samples to compare the relative transparencies. With relative acetaminophen concentrations less than 94%, the samples are fairly transparent. The sample containing a nominal concentration of 94% was slightly hazy in appearance. The sample with a nominal concentration of 100% appeared markedly turbid. The degree of turbidity increased with the concentration of acetaminophen.

2.2. Raman spectral collection and data processing

The detailed description and schematic diagram of the wide area illumination scheme used in this study can be found in the previous publication [9]. The wide area illumination scheme involves the application of a circular laser image to the sample for excitation. The laser spot applied is 6 mm in diameter (area 28.3 mm^2). The WAI scheme was shown previously to improve the reliability of Raman measurements by significantly increasing surface coverage area [9]. This advantage can be extended to other heterogeneous samples such as pharmaceutical suspensions. As in the previous studies, isobutyric anhydride sealed in a 2-mm thick rectangular quartz cell was installed in front of the sample of suspension to correct the Raman intensity variation resulting from the uncontrollable fluctuation of laser power.

Raman spectra were collected using 785 nm laser (HoloLab, Kaiser Optical Systems, Ann Arbor, MI, USA), with an exposure time of 3 s with 32 scans (96 s total time). The suspension sample was vigorously shaken before data collection and positioned so that the beam illuminated the center of the sample vial. Since the suspension samples were viscous, there was insignificant settling of the solids during spectral collection. Triplicate spectra were collected for each suspension. After each measurement, the sample was shaken vigorously and re-positioned. Fifty-four spectra collected from 18 suspensions were used as the calibration set to construct PLS models. For the validation set, the same samples were collected again with two different exposure times of 2.5 and 2 s. This was done to simulate the circumstances likely to be encountered long term in the lab as the power of the laser source deteriorates. A total of 36 spectra (2 spectra per sample) were collected. All of the calculations including baseline correction, intensity normalization and par-

tial least-squares regression, were accomplished using Matlab version 7.0 (The Math-Works Inc., MA, USA).

3. Results and discussion

3.1. Raman spectral features

Since the suspension was mainly composed of acetaminophen and sucrose syrup, the individual spectral features of these two components were initially examined. Fig. 2 shows the Raman spectra for acetaminophen (pure powder), sucrose syrup and 100% acetaminophen suspension (3.2 g/100 mL). The spectrum of 100% acetaminophen suspension was collected by positioning isobutyric anhydride in front of the sample as a synchronous external standard. The pure isobutyric anhydride spectrum is also shown in Fig. 2 along with the Raman spectrum of the glass vial (sample container). A broad spectral feature occurs for the glass in the 1800–1200 cm^{-1} range.

Many grades of glass fluoresce in a broad region as shown in Fig. 2 (1800–1200 cm^{-1} range). This fluorescence could be avoided by choosing a different excitation wavelength and thus excluding the background resulting from the fluorescence of the containers. However, choosing a shorter excitation wavelength increases the chance of intractable sample fluorescence and using a longer excitation wavelength could result in decreased sensitivity since Raman efficiency is decreased by the power of four with increasing wavelength. Hence, 785-nm excitation is usually a good universal choice if the glass fluorescence does not obfuscate the underlying features of interest from the sample.

The Raman spectrum of 100% acetaminophen suspension is the composite of the spectral features from acetaminophen, sucrose syrup, isobutyric anhydride and the glass vial. Since the concentration of sucrose is very high, the overall spectral features of 100% acetaminophen are similar to those of sucrose syrup. Several peaks corresponding to acetaminophen appear in the 1700–300 cm^{-1} range. The four major bands of the active are marked as asterisks on the spectrum of the 100% acetaminophen suspension.

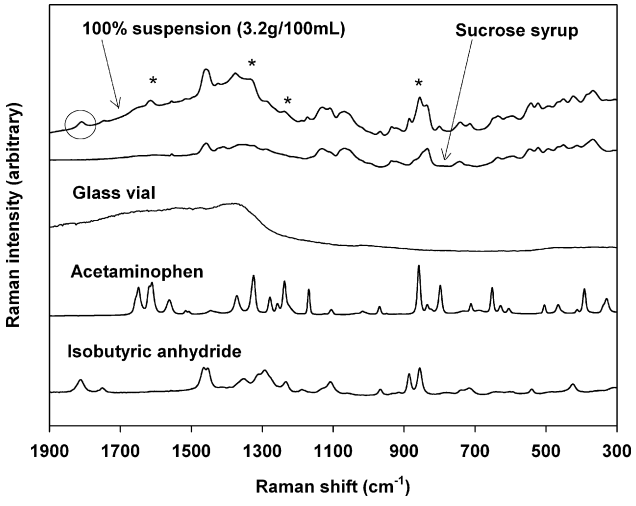

Fig. 2. Raman spectra of acetaminophen (pure powder), sucrose syrup, 100% acetaminophen suspension (3.2 g/100 mL), isobutyric anhydride and glass vial only.

To utilize an external standard to correct Raman intensity, it is required to have at least one band of the standard that does not overlap with the sample bands. The intensity of such an isolated band from a standard can be used to compensate for laser power variation. The non-overlapping 1810 cm^{-1} band of isobutyric anhydride (marked as a circle) was selected for this purpose.

3.2. Comparison of spectral reproducibility for the conventional and WAI schemes

Since the pharmaceutical suspensions used in this study were viscous and turbid (over 97% acetaminophen), the resulting Raman spectrum could fail to represent correct compositional information if the laser illumination spot were small. This observation was made during the previous study of naproxen tablets [9]. In such situations, the acquisition of Raman spectra by interrogation of a larger sample area could be useful to achieve accurate quantitative analysis.

To investigate whether the representative sampling of these pharmaceutical suspensions could improve quantification compared to the conventional scheme, Raman spectra of a 100% acetaminophen suspension collected by both the WAI scheme and a conventional small-spot scheme (non-contact Raman probe with a 10-mm focal length) were compared. Fifty spectra of a 100% acetaminophen suspension were collected using both schemes. For this experiment, a rectangular, non-fluorescent quartz cell (1 cm × 1 cm) was used as the sample holder to examine spectral variation without influence from a glass vial. The spectra were collected without the use of external standard (isobutyric anhydride). With each measurement, the sample was shaken vigorously and re-positioned.

Fig. 3 shows the resulting 50 spectra (1700–700 cm^{-1} range) of the 100% suspension using the conventional (top) and WAI (bottom) schemes. The broad glass background in the 1800–1200 cm^{-1} range, shown in Fig. 2, was no longer apparent in either cases. Even when the same sample is measured repeatedly, the resulting Raman spectra vary significantly when the conventional scheme is employed. The spectra are much more reproducible when using the WAI scheme. Since the laser spot of the conventional scheme is about 100 μm, the local chemical composition where the laser is focused could potentially vary when different sample aliquots are measured. The Raman spectra acquired using the conventional scheme could be representative only of a localized area and, if so, may not be satisfactory in representing the average composition of the acetaminophen suspension. By contrast, the Raman spectra collected using the WAI scheme are more reproducible due to the wide area coverage of the laser.

To quantitatively compare the spectral reproducibility, the spectra were baseline corrected and zeroed at 1700, 960 and 700 cm^{-1}, and the relative standard deviation (RSD) at each wavenumber was calculated for both cases. The RSD at each wavenumber is indicative of the degree of spectral variation. The RSD for both schemes at each wavenumber is plotted in Fig. 4 (top plot). The sudden increases of RSD at 970 and

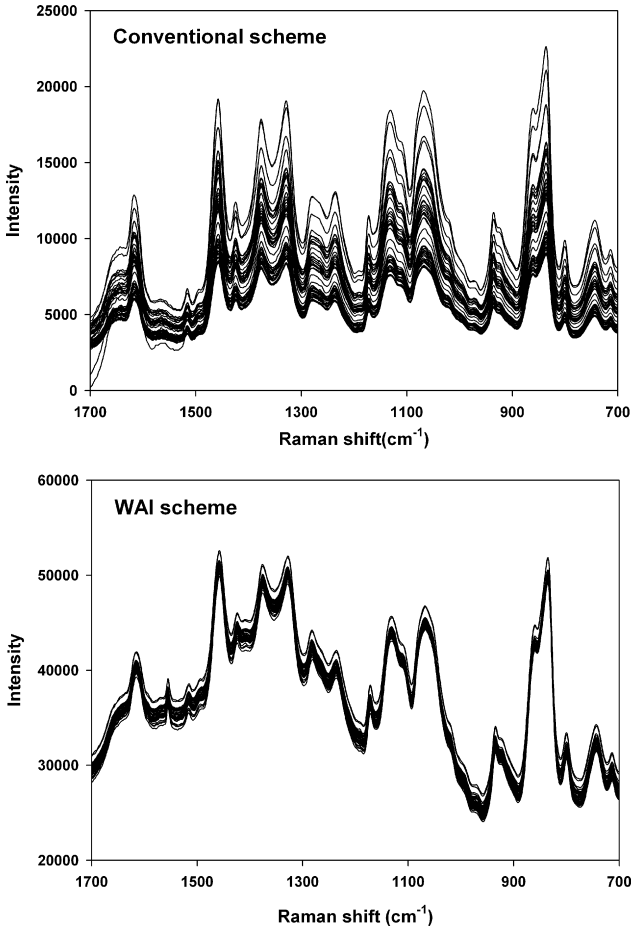

Fig. 3. Fifty raw spectra (1700–700 cm^{-1} range) of a 100% suspension sample collected using the conventional (top) and WAI (bottom) schemes. With each measurement, the sample was shaken vigorously and re-positioned.

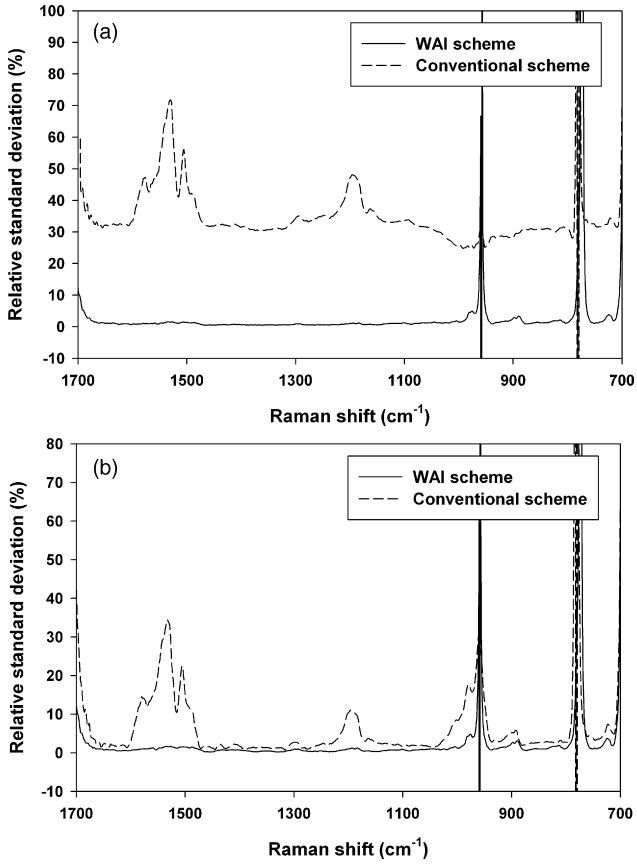

Fig. 4. Relative Standard Deviation (RSD) variation of 50 spectra calculated using baseline corrected spectra (a) and normalized spectra (b). RSD variations are compared for both the conventional and the WAI schemes.

780 cm^{-1} are from baseline correction and zeroing. By zeroing, the corresponding average will be zero or nearly zero around these wavenumbers. Therefore, the calculated RSDs are artificially high. Therefore, the 1650–1000 and 950–815 cm^{-1} ranges which exclude these anomalous regions, were evaluated to compare the RSDs for both schemes. As expected, the relative variation is much larger for the spectra collected by the conventional scheme. Average RSDs for the conventional and WAI schemes were 32.9 and 1.6%, respectively.

If the spectral variation from the use of the conventional scheme originates only from the intensity change with correct compositional information, the resulting RSD should be very small after spectral normalization. If the RSD is still high even after normalization, it is an indication of the lack of successful representation of the sampling. To investigate this, the same calculation outlined in the previous paragraph was performed using the normalized spectra. The data were normalized by dividing the original spectra by the corresponding total spectral peak area. The result is shown in Fig. 4 (bottom). When normalizing the Raman intensity, the RSD for the conventional scheme is significantly decreased (from 32.9 to 4.3%); however, it is still comparatively higher (approximately four times) than that for the WAI scheme (1.1%). This clearly suggests that the Raman spectra acquired with the conventional scheme might not be as representative of the overall API concentration of the suspensions. The spectra acquired using the WAI scheme appeared to be more representative based on the lower RSD of the repetitive measurements.

3.3. Quantitative calibration using PLS

Fig. 5(a) shows all of the uncorrected Raman spectra collected from 18 suspension samples (calibration set). The spectra can be divided into two groups that can clearly be distinguished visually by their spectral characteristics. The spectra that are offset more from the baseline correspond to suspensions containing 79–97% acetaminophen and the spectra that are less offset represent the suspensions containing 100–130% acetaminophen. The turbidity of the suspensions with API contents greater than or equal to the nominal 100% level result in an overall decrease in the corresponding Raman intensity. Large suspended solids attenuate the Raman scattering in these samples. Beyond that point of notable turbidity, the Raman intensity is not dramatically changed with the higher suspension concentrations.

Due to the non-linear baseline effects from changing solids contents and from the scattering emanating from the glass vial, a baseline correction scheme was employed using a six-point (1845, 1775, 1183, 955, 690 and 283 cm^{-1}) non-linear cor-

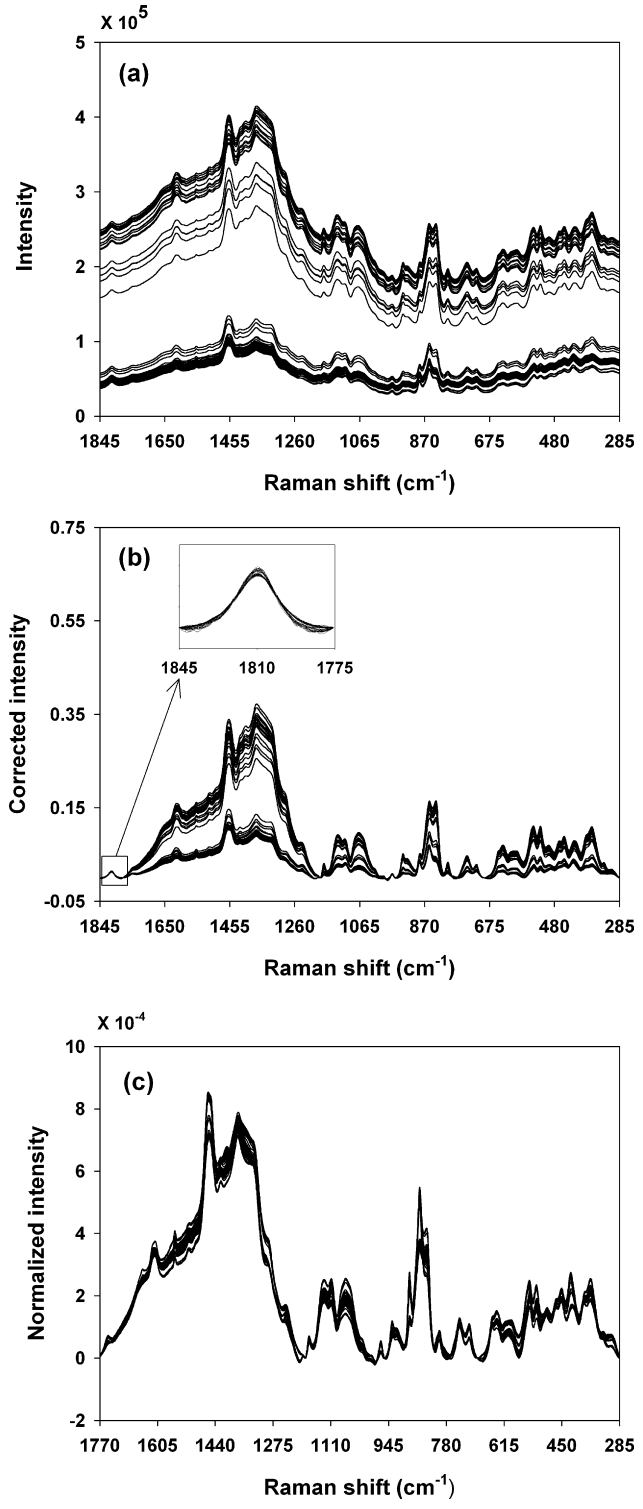

Fig. 5. Raman spectra collected from 18 suspension samples (a), the corresponding intensity-corrected spectra (b) and then normalized spectra (c).

rection method. The band area of isobutyric anhydride in the 1845–1775 cm^{-1} range was calculated and each sample spectrum was divided by that area to compensate for any Raman intensity variations from occasional laser power fluctuations. The results are shown in Fig. 5(b). The enlarged band featured in the inset is from the isobutyric anhydride band at 1810 cm^{-1}.

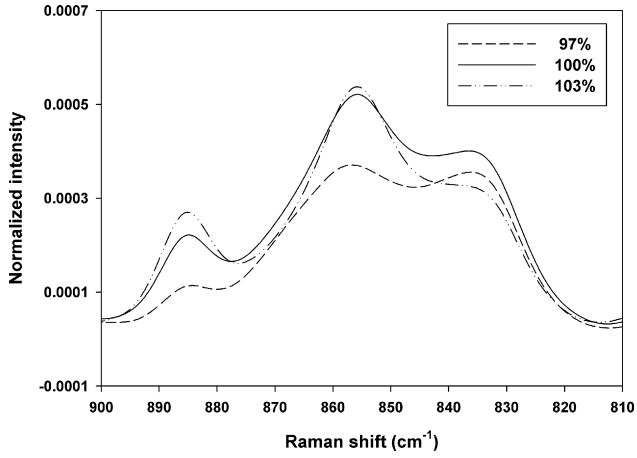

Fig. 6. The normalized spectral variation of the acetaminophen peaks in the 900–810 cm^{-1} range (94, 97, 100 and 103% acetaminophen).

Since the Raman intensity is significantly decreased with the increased turbidity of the suspensions, this effect should be compensated or minimized for reliable quantitative performance. For this purpose, the intensity-corrected spectra (Fig. 5b) were normalized. The area under the 1775–283 cm^{-1} range was calculated and each sample spectrum was divided by the calculated area to minimize the Raman intensity variation from the difference in the turbidity of the samples. The resulting spectra are shown in Fig. 5(c). To develop partial least squares [10–12] calibration models, the intensity-corrected and normalized spectra as represented in Fig. 5(c) were used.

Fig. 6 shows the normalized spectral variation of acetaminophen peaks in the 900–810 cm^{-1} range (97, 100, and 103% acetaminophen). The bands at 885 and 858 cm^{-1} correspond to acetaminophen, and the band at 835 cm^{-1} is from sucrose syrup. The acetaminophen peaks clearly increase with the API concentration while the band for the sucrose simultaneously decreases. However, the most striking observation is the sudden increase of Raman intensity for the spectrum representing the 100% suspension compared to the sample containing the API at a nominal level of 97%. This leads to the conclusion that there is a significant deviation from linearity in the spectra due to the solids contents. Such non-linearity can often be accounted for in PLS calculations.

Three different spectral ranges of 1845–283, 1845–1183 and 1183–283 cm^{-1} were used to evaluate the potential for quantitative modeling using PLS. The 1845–283 cm^{-1} range incorporates all of the information from acetaminophen. The 1183–283 cm^{-1} range utilizes the spectral region that avoids the broad underlying background from the glass vial in the 1800–1200 cm^{-1} range. The 1845–1183 cm^{-1} range represents the region where Raman scattering from the glass vials occurs.

The resulting standard errors of calibration (SECs) from the PLS calibrations using the three different spectral ranges are summarized in Table 1. The numbers in parentheses correspond to the number of factors used. To validate the calibration models, the suspension samples were independently collected with two different exposure times of 2.5 and 2 s to simulate the gradual degradation of laser power. Fig. 7(a) shows the baseline-

Table 1
Overall PLS calibration results for whole, low and high concentrations using three different spectral ranges

Spectral range (cm^{-1})	Whole concentration (79–130%)		Low concentration (79–97%)		High concentration (100–130%)	
	SEC	SEP	SEC	SEP	SEC	SEP
1845–283	2.1 (6)	2.2	0.8 (5)	0.9	3.3 (5)	3.0
1183–283	2.5 (4)	2.4	0.6 (5)	0.8	2.1 (6)	2.2
1845–1183	2.5 (6)	2.2	0.8 (5)	1.0	3.3 (5)	3.4

Numbers in parentheses correspond to the number of factors used.

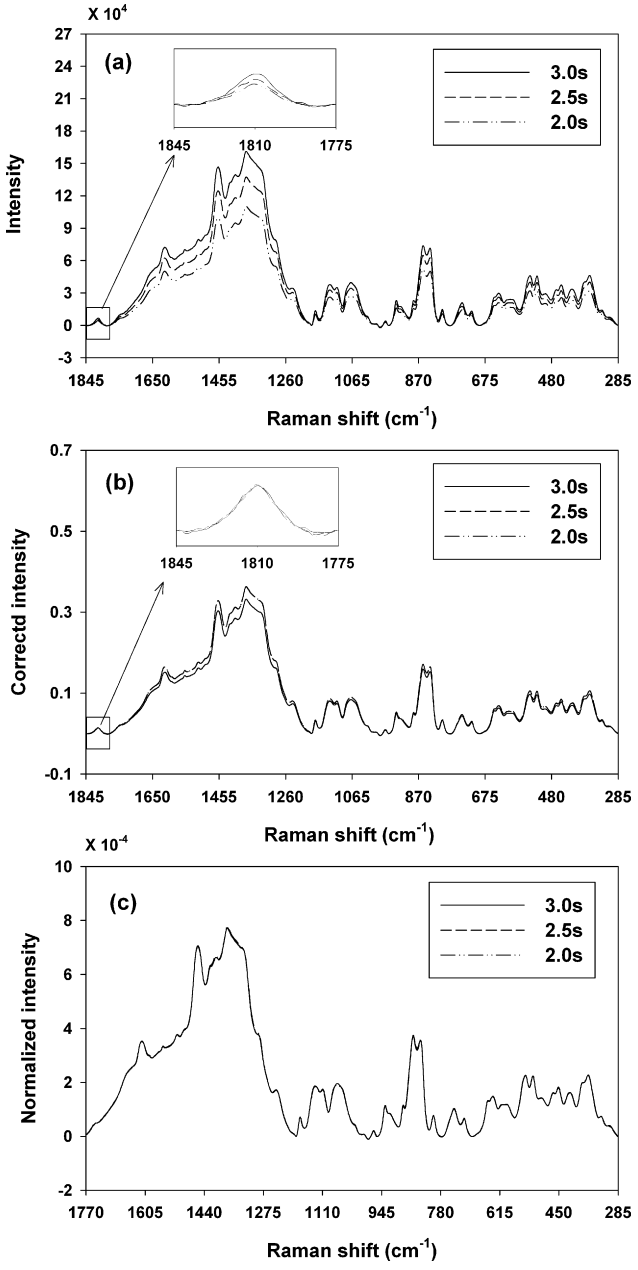

Fig. 7. Baseline-corrected Raman spectra of the 97% acetaminophen suspension at three different exposure times (a), the corresponding spectra that have been intensity-corrected based on the area of the external standard peak (b) and then followed by the normalization (c).

corrected Raman spectra of the 97% acetaminophen suspension at three different exposure times (3.0, 2.5 and 2.0 s). As shown, the intensity of the isobutyric anhydride band at 1810 cm^{-1} (enlarged in the plot) and the overall Raman intensity decrease with shorter exposure time. Fig. 7(b) shows the corresponding spectra that have been intensity-corrected based on the area of the external standard peak as described previously. Fig. 7(c) shows the normalized spectra by the total spectral area. The intensity variation from different exposure times is substantially reduced using the isolated isobutyric anhydride band and area normalization.

A total of 36 spectra (2 spectra per sample) were predicted and the resulting standard errors of prediction (SEPs) were calculated. As shown in Table 1, the prediction results are similar for the three cases although the calibration from the 1183–283 cm^{-1} region required only four factors to adequately represent the spectral variation whereas six factors were required in the other two cases. This is likely due to the need to factor out more background when the higher shift range was employed. Fig. 8 shows the correlation plot resulting from the PLS modeling procedure with the use of 1845–283 cm^{-1} range. The filled and open circles correspond to calibration and prediction data, respectively. Up to the concentration of 106% acetaminophen, the correlation between the reference and calculated (predicted) values is quite good. However, the data points begin to exhibit greater error cor-

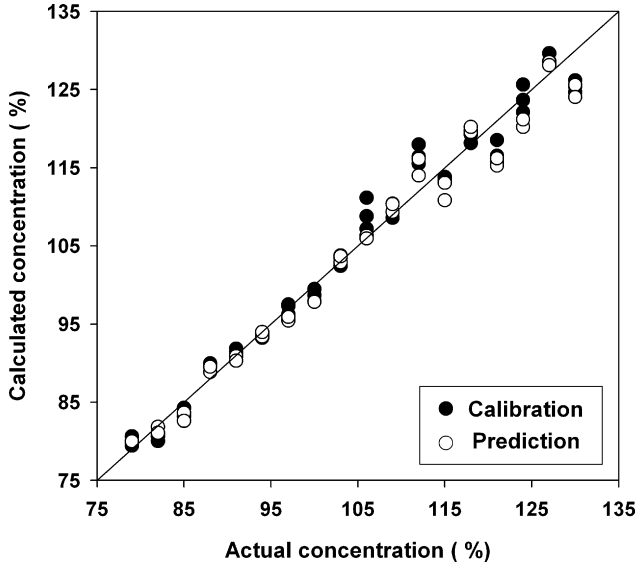

Fig. 8. Concentration correlation plot resulting from PLS analysis using the 1845–283 cm^{-1} range. Filled and open circles correspond to calibration and prediction data, respectively.

responding to the observed spectral deviations that occur with API concentrations greater than 106%. This indicates that the reproducibility of the Raman spectra worsens for turbid suspensions. The corresponding intensity is also decreased when the concentration of acetaminophen is higher. The resulting signal-to-noise decrease may contribute to the increased variability.

The acetaminophen concentrations were accurately predicted even with the variation in the Raman intensity that occurred with the turbidity increase at higher API concentrations. However, the prediction performance, as expected, was not as good for the more turbid suspensions. The SEP for the whole concentration range was 2.2%. The SEPs for the low (79–97%) and high (100–130%) concentration ranges were recalculated independently and were found to be 1.1 and 2.7%, respectively. Hence, as a possible alternative to rectify the anomalies caused by the turbidity, the construction of separate calibrations was investigated.

3.4. Dual Models for low and high concentration ranges

In an effort to improve the PLS calibration performance, separate calibration models for the low (79–97%) and high (100–130%) concentration ranges were used. Because the spectral intensity and baseline behavior in the low and high concentration ranges are clearly different, it should be possible to pre-assign an unknown spectrum *a priori* to either the low or high concentration range by the use of a simple principal component analysis (PCA) [13]. Fig. 9 shows the score plots (using the first and second scores) indicating the clear separation of the two groups (79–97% and 100–130%). The normalized spectra (Fig. 3(c)) were used for this analysis. Circles and triangles correspond to the samples in the low and high concentration ranges, respectively. Filled and open symbols correspond to calibration and prediction data, respectively. The calibration and prediction samples in the two different groups are consistent with each other.

Two different calibration models were developed using the samples in the low and the high concentration ranges separately. The three spectral ranges identified previously in Table 1 were used to develop the calibration models. For predictions, PCA screening was initially performed to determine to which group (low or high concentration) the particular sample belonged. The PCA model was developed using the first and second scores for the calibration samples. The Mahalanobis distance was used to set up the discrimination limit of the score cluster for the low and high concentration samples.

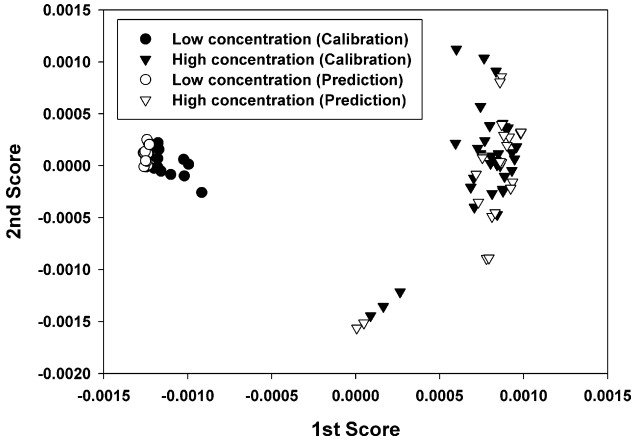

Fig. 9. Score plots (using first and second score) showing the unambiguous separation of the low (not turbid 79–97%) and the high (turbid 100–130%) concentration samples.

To determine the Mahalanobis distance in any given case, an ellipsoidal boundary defined by the variation in the sample data is calculated. This boundary circumscribes the data cluster in question and is related to the standard deviation (σ) from the mean (centroid). The proximity of an unknown sample to the data cluster in question can be measured as a function of the distance from the centroid. The Mahalanobis distance is such a measure and the distance from the centroid is defined as a function of the deviation of the original data set from which the elliptical boundary was calculated.

Using the PCA analysis just described, each prediction spectrum was first assigned to its proper group (high or low concentration of active) by using the Mahalanobis distance with 3σ level (99.7% confidence level). Once the group was assigned, the relevant PLS model could be used to predict the concentration of acetaminophen. The results are summarized in Table 1. When two separate PLS models were developed for the two concentration ranges, the 1183–283 cm^{-1} range provided the best results and the corresponding accuracy was improved compared to the use of one PLS model for the whole concentration range. An additional benefit from this approach was that the spectral range of the glass interference was avoided. This likely aided in the construction of more accurate PLS calibration models.

4. Conclusion

The data in this study support the conclusion that the concentration of the active pharmaceutical ingredient in both clear and turbid suspensions can be accurately measured with the use of the WAI scheme. By using a laser that illuminates a relatively large sample area, spectra could be obtained that were more representative and more reproducible compared to the conventional small-spot scheme. Without the WAI scheme, the quantitative analysis of a turbid pharmaceutical suspension using Raman spectroscopy would be less accurate because of the inadequate representation by the small laser spot in a traditional measurement scheme. By the same token, the reproducibility of the Raman spectra collected by the conventional small-spot scheme would be significantly degraded when measuring highly turbid samples.

Because of the changing turbidity of the samples used in this study, it was found that two separate calibrations yielded better results than one overall calibration. Fortunately, the appropriate calibration needed for a given sample can be identified using a PCA model without the need for subjective definition by the user. This mode of operation should still work well in a Quality Control application.

The use of the WAI scheme allows Raman to be used in a manner that provides acceptably accurate quantitative analyses because of the improved reproducibility and representative sam-

pling compared to traditional small-spot schemes. Raman also offers the advantage of coupling to fiber optics which engenders the potential for the application of this technique for in-line analyses. The same advantages exist for other pharmaceutical applications such as in-line monitoring of mixing homogeneity in pharmaceutical blending as well as other common pharmaceutical applications.

Acknowledgment

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