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Research Article

Characterisation of Crystalline-Amorphous Blends of Sucrose with Terahertz-Pulsed Spectroscopy: the Development of a Prediction Technique for Estimating the Degree of Crystallinity with Partial Least Squares Regression

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Abstract. The control of the amorphous and crystalline states of drugs and excipients is important in many instances of product formulation, manufacture, and packaging, such as the formulation of certain (freeze-dried) fast melt tablets. This study examines the use of terahertz-pulsed spectroscopy (TPS) coupled with two different data analytical methods as an off-line tool (in the first instance) for assessing the degree of crystallinity in a binary mixture of amorphous and polycrystalline sucrose. The terahertz spectrum of sucrose was recorded in the wave number range between 3 and 100 cm^{-1} for both the pure crystalline form and for a mixture of the crystalline and amorphous (freeze-dried) form. The THz spectra of crystalline sucrose showed distinct absorption bands at ~48, ~55, and ~60 cm^{-1} while all these features were absent in the amorphous sucrose. Calibration models were constructed based on (1) peak area analysis and (2) partial least square regression analysis, with the latter giving the best LOD and LOQ of 0.76% and 2.3%, respectively. The potential for using THz spectroscopy, as a quantitative in-line tool for percent crystallinity in a range of complex systems such as conventional tablets and freeze-dried formulations, is suggested in this study.

KEY WORDS: crystallinity; partial least squares; sugars; terahertz-pulsed spectroscopy.

INTRODUCTION

The quality and stability of the pharmaceutical product is frequently dictated by the physical state of the ingredients, such as the polymorphic form of the drug, the hydration state of an excipient, and the proportion of crystalline and amorphous phases of each component. The control of the amorphous and crystalline states of drugs and excipients is important in many instances of product formulation, manufacture, and packaging, such as the formulation of certain (freeze-dried) oral disintegrating tablets (ODTs), the size reduction of powders by micronisation process, and the ingress of moisture through the packaging material during storage.

The amorphous state of any material is always thermodynamically less stable than crystalline state and hence the material may devitrify (i.e. crystallise from the amorphous state) under poor storage conditions, inappropriate packaging, or further processing. An increase in the crystalline component of a drug substance will slow the dissolution rate which could then impact bioavailability. For instance, when certain amorphous sugars, such as sucrose, are humidified following freeze drying, the amorphous sugar is fully or partially converted to the crystalline form (1). However, the propensity for re-crystallisation may be exploited to an advantage in the formulation of freeze-dried rapidly disintegrating tablets (RDTs) to enhance the mechanical properties without impacting the disintegration profile (2,3).

Different techniques such as thermal analysis, X-ray diffraction (XRD), powder X-ray diffraction (PXRD), and a number of optical spectroscopies have been used to study crystallinity in pharmaceutical formulations. Although XRD is the benchmark method for studying crystallinity (3,4), both the single crystal and PXRD techniques are technologies which are best suited to off-line analysis. The emerging reliance on process analytical technologies has led to an increase use of the optical spectroscopies such as near infrared (NIR), Raman spectroscopy, and THz spectroscopy which offer a potential solution for on-line freeze-drying process control (2,5).

Terahertz-pulsed spectroscopy (TPS) exploits the frequency band which lies between the microwave and infrared ranges (1×10^{-12}–3×10^{-12} Hz) of the electromagnetic spectrum and has been used in the characterisation of crystalline materials in particular. The coupled interactions that co-exist between the intermolecular modes of vibration, within an ordered crystalline lattice, leads to the formation of a single collective mode which propagates through the entire crystal (6–8) and results in the absorption bands which are detected by terahertz spectroscopy.

The applications for terahertz spectroscopy in characterising pharmaceutical materials have been reviewed in a number of articles (7–9). Studies have shown that

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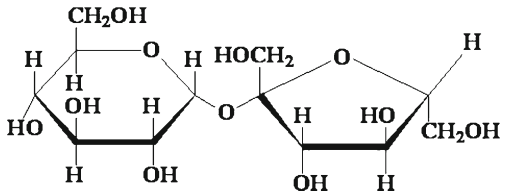

Fig. 1. Chemical structure of polycrystalline sucrose in the conformation relevant to the present investigation

different types of crystalline and amorphous materials can be easily differentiated through their terahertz absorption spectrum (6,10,11). In a study on saccharides (12), it was shown that a series of distinct absorption bands were detected for the polycrystalline form whiles the amorphous sugars showed a broad, featureless absorption spectrum. These contrasting features of the spectrum are distinct from the mid- and near-infrared spectra in which intermolecular vibration modes are evident irrespective of the physical state of the material.

In traditional quantitative analysis of spectral data, certain absorption bands are usually selected for peak area analysis. In this approach, the area under an absorption peak or the peak height is estimated relative to the apparent location of the baseline. This method of analysis is straightforward but may sometimes carry the risk of over- or underestimating the intensity of the peak because of the subjective choice of the base line. Other methods based on robust statistical analysis may avoid this problem and give more reliable results.

When combined with chemometrics, the terahertz absorption spectra of a material can be used to create calibration models for future crystallinity predictions. Previously, univariate and multivariate data analysis has been applied to analyse spectroscopic datasets from spectroscopic techniques (10,13–15). In this article, TPS and chemometrics are engaged to determine the degree of crystallinity in complex systems comprising freeze-died amorphous sucrose and polycrystalline sucrose. The purpose of this study is to develop a calibration model that can be applied for evaluating the crystallinity of pharmaceutical materials which are susceptible to physical transformations (amorphous to crystalline states) during manufacturing processes or storage. A model substrate (sucrose) has been selected for this work, as it is used frequently in the formulation of freeze-dried products but has a propensity to devitrify when exposed to atmospheric moisture.

EXPERIMENTAL

Materials

Polycrystalline sucrose (Fig. 1) was obtained from Fluka and manually sieved to produce powders with particle size of ≤63 μm. Polyethylene (PE) powder (Mw, ~4,000) with particle size of <80 μm was supplied by TeraView Ltd (Cambridge, UK) and used as received. Particle sizes of >90 μm have been associated with scattering as described elsewhere (16). PE was chosen as the diluent to prepare pellets because it exhibits negligible absorption in the terahertz region, as described in the previous literature (12) thereby allowing for well-defined spectra features, associated with only the polycrystalline sucrose to be observed within the terahertz region of 0.3 to 1.2 THz. Single distilled water was used to prepare all the sucrose solution used in this study.

Sample Preparation

Amorphous sucrose was prepared by a freeze-drying process following the protocol applied in previous studies (17) (Table I). For this purpose, a 10% (*w*/*w*) solution of sucrose was prepared in triplicate and then freeze-dried in a Heto model FD8 freeze dryer (Denmark): 10 mL clear glass vials (Schott) were filled with 3 g (±0.008 g) corresponding to 1 cm (fill depth) of the sucrose solution. The shelf temperature of the freeze dryer was set at −35°C (i.e. below the glass transition temperature, T_g', of sucrose, −34°C to −32°C) (17,18). The chamber pressure was set at 0.1 mbar (for both primary and secondary drying stages) corresponding to approximately half of the water vapour pressure at the ice surface (0.22 mbar) (19).

The freeze-dried sucrose was analysed by a Perkin Elmer Jade DSC (data not presented in this article) at a heating rate of 5°C min^{-1}. The thermogram showed phase transitions corresponding to glass transition (T_g), devitrification, and melting. The enthalpies of devitrification (crystallisation of an amorphous state on re-heating below the melting point) and melting were similar (±1%) confirming that the freeze-dried samples were in the amorphous state, prior to TPS measurement.

Disc-shaped pellets of PE powder (400 mg total mass, each incorporating up to 14% (*w*/*w*) of the polycrystalline sucrose were prepared with a hydraulic Carver press using 2.0 ton compaction force at RH ~4% (in glove box) and 25°C.

Table I. Freeze-Drying Protocol Adapted from Smith *et al.* (15)

Step	Temperature (°C)	Duration (h)	Time (h)	Vacuum (mbar)	Ramp/hold	Stage
I	−35	1	1	1,000	Ramp	Ramp to freezing temperature
II	−35	2	3	1,000	Hold	Hold at freezing temperature
III	−15	1	4	1,000	Ramp	Ramp to annealing temperature
IV	−15	3.5	7.5	1,000	Hold	Hold at annealing temperature
V	−35	1	8.5	1,000	Ramp	Ramp to freezing temperature
VI	−35	1	9.5	0.1	Vacuum	Vacuum application
VII	−35	25	34.5	0.1	Hold	Hold at primary drying temperature
VIII	25	6	40.5	0.1	Ramp	Ramp to secondary drying temperature
IX	25	2	42.5	0.1	Hold	Hold at secondary drying temperature

Table II. The Different Proportions of Amorphous Sucrose Homogeneously Blended with Polycrystalline Sucrose

Sample ID	Amorphous concentration (%, *w*/*w*)	Polycrystalline concentration (%, *w*/*w*)	Percentage crystallinity (%, *w*/*w*[a])
1	20.0	0.0	0.0
2	16.8	3.2	16.0
3	12.7	7.4	36.8
4	10.0	10.0	50.0
5	9.5	10.5	52.5
6	7.5	12.5	62.5
7	7.0	13.0	65.0

The percentage (*w*/*w*) is the amorphous and polycrystalline concentrations which represents the percentage of sugar in the total weight of the pellet

[a] The percentage crystallinity was estimated relative to the total weight of the disaccharide ($100\times W_a/(W_a+W_c)$, where W_a and W_c are weights of amorphous and crystalline components of sugar

These samples were used as the references to develop a calibration model. Although 2 ton compaction force was used to prepare the sample discs, transient heat was insufficient to cause changes in the properties of the freeze-dried sample. This is evident from the featureless THz spectra of polyethylene and freeze-dried sucrose measured (data not presented) with both the transmission and attenuated total reflectance (ATR) modules. The ATR module attached to TPS does not require any sample preparation (compaction). Thus, comparing the spectral measurements acquired with ATR and the transmission module for both freeze-dried sucrose and polyethylene, we were able to prove the preparation method had no impact on the amorphous samples.

A similar procedure was applied for the preparation of pellets with partially crystalline or pure amorphous sucrose. For test samples, a combination of freeze-dried sucrose and polycrystalline sucrose at different ratios (Table II) making up 20% (*w*/*w*) were incorporated into a 400 mg disc shaped PE pellets. The three components (polycrystalline sucrose, amorphous sucrose, and PE) were mixed manually within 3 min.

The thickness and diameter of all the pellets was measured to be ~3.6 (±0.06 mm) and 12.8 mm (±0.02 mm), respectively. All samples were prepared in triplicate to confirm that the minutes of PE and sucrose were homogeneous. The THz spectrum of each pellet was measured at three points across its diameter and a mean taken of the resultant nine measurements (n=3 samples×3 points).

Terahertz Spectroscopy

The experimental setup and principles of TPS have been documented elsewhere (6,11,20–22). A TPS spectra 3000 (TeraView Limited, UK) in transmission mode was used to acquire THz absorption spectra. A measurement rate of 30 scans/s provided the spectral resolution of 1.2 cm^{-1} and used to measure all samples and the reference. For polycrystalline sucrose, the reference sample was a 400-mg disc made from PE while for the test samples, the reference included 360 mg PE and 40 mg of freeze-dried sucrose. Each terahertz waveform was obtained by averaging 1,800 scans. All samples were individually placed in a 13 mm (diameter) sample holder, which helps to focus the terahertz beam onto the centre of the pellet (21). The sample holder is placed into a compartment which is dried by purging with nitrogen gas for 10 min prior to and during each measurement. The reproducibility of the measured spectra was determined by repeating the analysis on each sample at three discrete points across the entire pellet: one point was measured at the central position of the pellet and the other two measurements were done at ~1 mm from the edge of the pellet. This was because, preliminary studies showed that increasing the number of measurements (scans) for one sample above three repeats did not yield any significant improvement in accuracy and standard deviation, but rather increased the resident time of the sample in the sample compartment which could potentially lead to devitrification. Each spectrum is the average of three spectra recorded from three separate samples between 5 and 95 cm^{-1}. Given that the beam diameter is 2 mm and the diameter of the compact is 12.8 mm, then the ratio of the beam area to tablet area is 2.4%. With three measurements at different positions on each

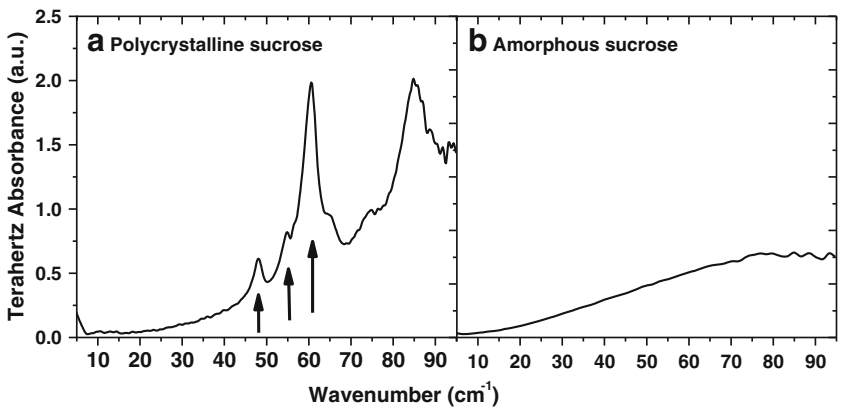

Fig. 2. The THz absorption spectra of a binary mixture of a 400 mg polyethylene disc incorporated with 10% (*w*/*w*) polycrystalline **a** and 10% (*w*/*w*) amorphous sucrose **b**. The *arrows* indicate an absorption band

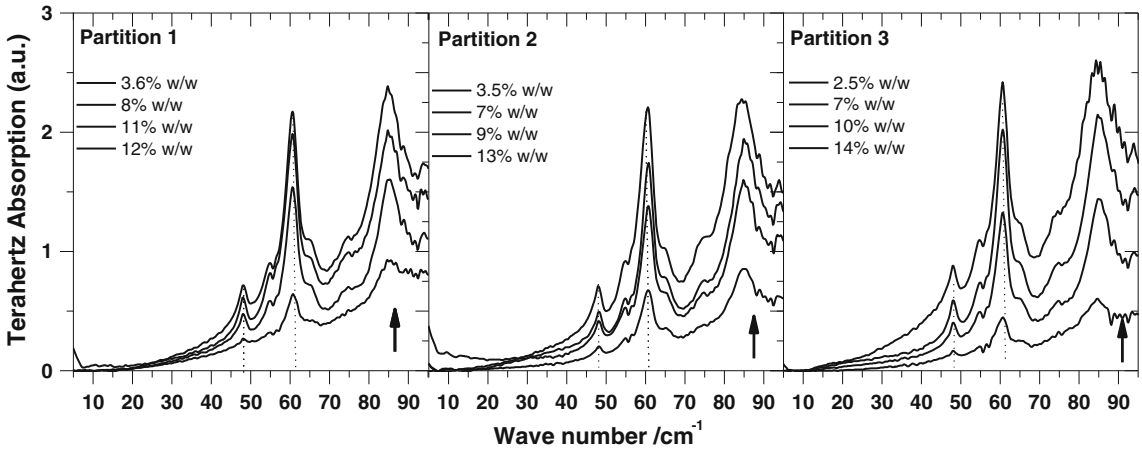

Fig. 3. The THz absorption spectra of a binary mixture of PE and polycrystalline sucrose (400 mg pellet). The *arrows* indicate an increasing concentration of the polycrystalline sucrose component in the disc. The recordings are divided into three partitions for CV purpose

tablet, this equates to 7.5% of the sample area (and hence volume) that is under scrutiny.

RESULTS AND DISCUSSION

Comparing the Spectra of Polycrystalline Sucrose and Amorphous Sucrose

The THz absorption spectrum of sucrose shows absorption peaks at ~48 cm^{-1}, ~55 cm^{-1}, and a more distinct peak centred on ~60 cm^{-1} with a visible shoulder at ~65 cm^{-1} (Fig. 2). The observed shoulder becomes more pronounced in samples containing high concentrations of the polycrystalline sucrose.

These well-resolved features have been reported in the literature previously (12). A generalised explanation is that these absorption bands originate from intermolecular vibrational modes of long range order, maintained by the hydrogen bond network of the crystalline structure. In contrast, the spectra recorded for the amorphous sucrose showed no discernible features owing to the fact that an amorphous material lacks the coordinated hydrogen bond vibrations of the crystalline state (Fig. 2a, b) (10,23).

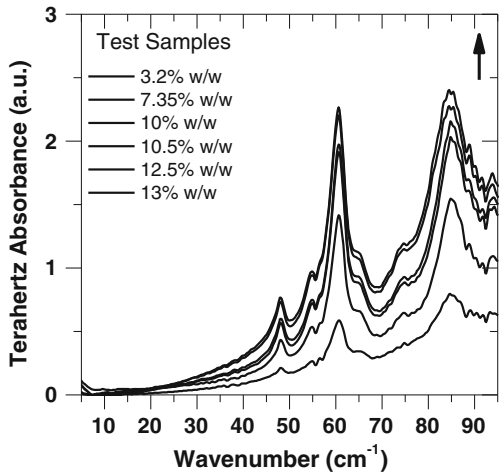

Fig. 4. The THz absorption spectra of a complex mixture of varying proportions of polycrystalline and amorphous sucrose (both making up 20% (*w/w*) in 400 mg pellet). The *arrow* indicates an increasing concentration of the polycrystalline sucrose component in the disc

THz Spectra for Partially Crystalline Sucrose

Quantitative analysis that relies on data from spectroscopic techniques such as TPS requires the differentiation between absorption bands and absorption features which are characteristic of the test sample when compared with the spectrum of a reference sample. Figures 3 and 4 indicate the effects of increasing the concentration of the polycrystalline component in the sample.

The spectra recorded for all samples share the distinctive absorption band attributed to the polycrystalline form of sucrose (Fig. 2). Furthermore, the spectra also revealed what may be speculated as an absorption band at the high frequency region ~84 cm^{-1} and becomes more pronounced at higher concentrations of the polycrystalline sucrose.

Quantitative Analysis

The conventional method for the analysis of spectra is usually based on either an estimation of the height or area under one or more of the principal absorption peaks. The former method is used if the shape of spectra does not change and peaks are simply scaled as a result of some stress or concentration. The latter method is more universal as it doesn't take in account the shape of the peaks and could be more suitable for analysis of THz spectra. Analysis based on

Table III. An Example of the Concept of Cross Validation

Test set	Training set
Partition 1	Partitions 2 and 3
Partition 2	Partitions 1 and 3
Partition 3	Partitions 1 and 2

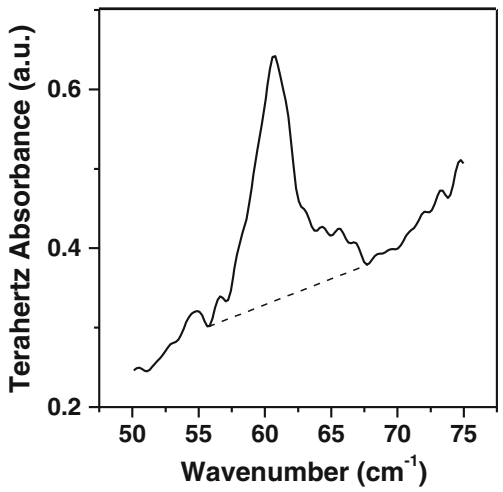

Fig. 5. Estimating peak area using linear function baseline fits. All estimates are repeated three times and the average used to calculate the prediction model

the peak height is more sensitive to noise while the area based analysis is related to the integration of the absorption band and therefore less affected by noise. Moreover, the area of the absorption band is directly proportional to the concentration of the absorbing component, given that it accounts for any broadening or tailing of the peak. The accuracy of both methods is dependent on how the baseline of each absorption band is estimated.

In comparison to the linear regression based on the 'peak area' analysis, the partial least square (PLS) regression method has also been identified as a powerful tool calculating prediction models. Unlike the peak area analysis (which does not account for the equipment noise and other external variables), the PLS method identifies a number of prediction variables and scores each as a different principal component, with the highest score being the factor that contributes the most to the observations.

Terahertz data from the pure polycrystalline sucrose was used to create calibration models using the PLS analysis package built into the open source software of the *R Project for Statistical Analysis* (24). Similarly, the selected spectral region for this analysis was based on the largest spectral difference between the spectrum of pure PE and the spectra of PE incorporated with sucrose. The calibration models were calculated using PLS algorithms with the concept of k-fold cross validation (CV). The concept of k-fold CV has been explained in detailed elsewhere (14,25,26). This approach partitions the original sample into k subsamples (For example, in Fig. 3, the three partitions will be k-1, k-2, and k-3). One of these partitions (partition 1) is used as a test set, and the remaining sub-samples (partition 2 and 3) are used as the training sets. This CV is repeated k times (the folds) in the way that each of the subsamples is used once for the test set. To reduce variations, the validation results are then averaged over all the folds. For instance, in a CV involving three sets of data collected from three independent partitions, k-fold CV will be applied as shown in Table III.

Table IV. Percent Contribution by the Four Principal Components

	Contribution (%)		
PCA	Partition 1	Partition 2	Partition 3
PC 1	99.73	99.67	99.87
PC 2	0.19	0.30	0.10
PC 3	0.078	0.02	0.01
PC 4	Negligible	Negligible	Negligible

The root-mean-squared errors (RMSE) of the cross-validated dataset were then determined for each sample. The model was repeatedly calculated until none was identified as an outlier within the 95% prediction interval relative to the CV. Limits of quantitation (LOQ) and detection (LOD) were further calculated using Eq. 1 as the signal response is linear to the degree of crystallinity.

$$\mathrm{LOD} = {}^{3.3\sigma}/_{S} \text{ and } \mathrm{LOQ} = {}^{10\sigma}/_{s} \quad (1)$$

where σ is the standard deviation (In our case σ was taken from the root mean squared error between the regression line

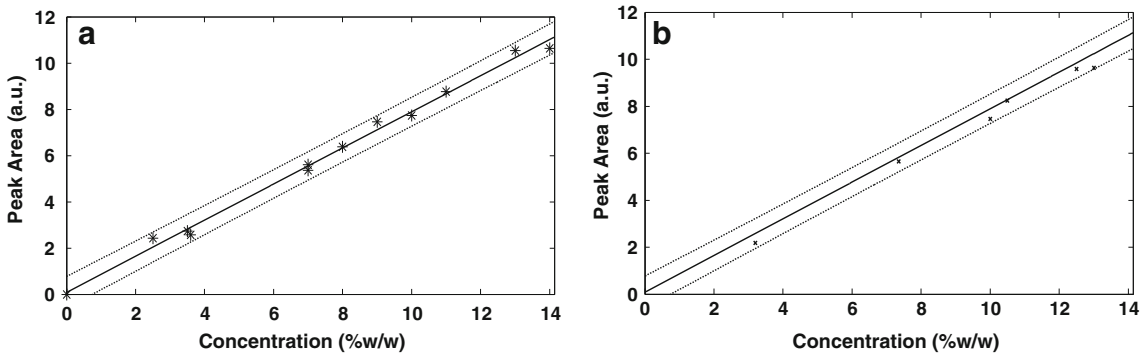

Fig. 6. Linear calibration model based on the concept of "area under the peak" using linear function to manually fit the baseline **a**. The linear calibration model **a** was used to predict the crystallinity in a complex mixture of PE, amorphous, and polycrystalline sucrose **b**. The model and predictions are fitted within with 95% confidence level and prediction bands (*dashed lines*)

Table V. Parameters of Function $f(x)$ Estimated for the Three Different Partitions

	a	b	R^2
Partition 1	0.077	−0.606	0.998
Partition 2	0.071	−0.585	0.998
Partition 3	0.069	−0.564	0.997

and the experimental data) and s is the slope of the validated curve (27).

Area-Based Analysis of THz Spectra

The simplest approach is to describe the base line using linear regression based on a linear function as shown in Fig. 5. For this work, the absorption band between the wave numbers ~55 to ~68 cm^{-1} was selected for linear regression.

The calibration model presented in Fig. 6a indicates a positive correlation between concentrations of sucrose and the estimated peak area. The estimated R^2 was 0.99 and the RMSE of 0.3% translate to LOD of 0.9% and LOQ of 2.7%. The model was applied to partially crystalline sucrose with 'unknown' crystallinity. Figure 6b shows the estimated crystallinity of the crystalline part of this complex system.

Partial Least Square Regression

The THz dataset in the spectral region between 45 and 66 cm^{-1} was used for PLS regression analysis. The different scores for the three partitions are presented in Table IV, indicating that PC1 described the observations better than the other PCs and therefore used for further study.

The function $f(x)=a^*x+b$ was then fitted to PC1 for each partition, where x is the known concentration. The estimated parameters a, b, and RMSE for each partition are shown in Table V.

The application of the CV routine to the linear functions for each partition yields a global RMSE of ±0.2%, which translates to an LOD of 0.8% and an LOQ of 2.3%.

To test the reliability of the calibration model (Fig. 7a), it was then used to estimate the percent crystallinity in another set of samples with 'unknown' percent crystallinity. The use of the term 'unknown' is taken to mean samples (of known crystallinity) that were not included in the three partitions used for the construction of the calibration curve and can therefore be considered as unknown in relation to the developed model The unknown dataset was found to lie well within the 95% confidence interval define by the calibration model, over a wide range of actual concentrations (Fig. 7b).

Table VI. Summary for Predicted Concentrations of Polycrystalline Sucrose Incorporated in a Complex Mixture of PE and Partial Crystalline Sucrose

	Predicted concentration (%, *w/w*)	
Actual concentration (%, *w/w*)	Linear function	PLS regression
3.2	2.7	3.3
7.4	7.1	7.5
10.0	9.4	10.2
10.5	10.4	10.7
12.5	12.2	12.7
13.0	12.2	13.1

Comparison of Models (General Discussion)

The estimates of the percent crystalline content in a blend of amorphous and polycrystalline sucrose using area-based and partial least square approaches are summarised in Table VI. Comparing actual and predicted concentrations, one can conclude that the best result was obtained by using PLS regression as the minimum difference between these concentrations was obtained.

Relative error between predicted and actual concentrations for the two approaches is plotted in Fig. 8. This parameter was calculated as:

$$\text{Relative error } \% = (\text{Area predicted} - \text{Area actual})/(\text{Area actual}) \times 100\% \quad (2)$$

The area-based approach is not very precise and gives scattered results especially at lower percent (*w/w*) concentrations. This method further reveals underestimation of concentration (negative error). Moreover, the relative error increases with the decrease of the actual concentration of crystalline

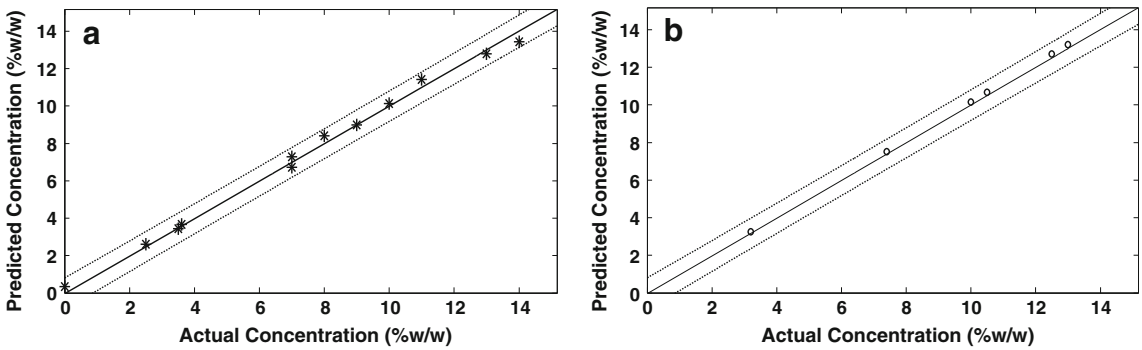

Fig. 7. **a** Linear calibration model based on partial least square regression algorithms with the concept of k-fold CV, showing the estimated 95% confidence level of the prediction bands. **b** Predicted concentrations of the "unknown" samples used to test the PLSR calibration model

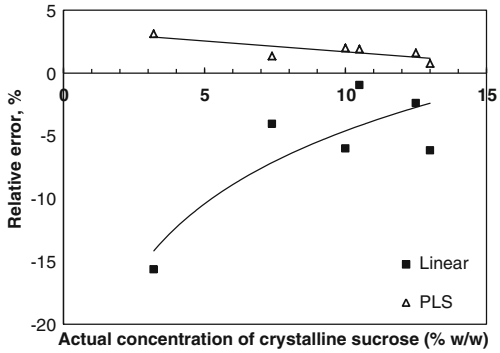

Fig. 8. Relative per cent error between predicted and actual concentrations of crystalline sucrose in a complex PE pellet of partially crystalline sucrose. *Lines* are plotted as guides

part of sucrose in semi-crystalline sugar and reaches 15% for sample with 3% of crystalline sucrose.

For PLS analysis the relative error is less than 3.1% at similar sucrose concentration. The plot also shows the PLS method is more likely to overestimate but perhaps less than 5% as shown in this work. However, this overestimation is reduced significantly as the actual concentration (percent, *w*/*w*) is increased in the samples. The relative percent error observed in area-based approach is due to the possibility that this method is a subjective decision of researcher and defined by choice of the wave number range for the peak (i.e. range of spectrum taken for analysis). In the case of PLS approach, this subjective decision is minimised as other factors such as baseline functions and effects of noise is accounted for in other principal component scorings.

Measurements of crystallinity percentage in a blend of crystalline and amorphous forms using THz spectroscopy can be performed with reasonable accuracy, as demonstrated by this study. The LOD and LOQ calculated in this work is compared with PXRD and DSC method from the literature and summarised in Table VII. The LOD estimated for the PXRD experiment for partially crystalline material (active pharmaceutical ingredient, Material A) was reported to be 2% (28). Lappalainen *et al.* also reported an LOD and LOQ (29) which was 10-fold lower than reported in both the PXRD study and this THz study. However, it is necessary to note that this work focused on the analysis of the amorphous content in crystalline sucrose from analysis of the change in heat capacity at T_g (rather than the analysis of crystallinity in an otherwise amorphous phase). Given that their standard deviations in the experimental parameter ΔC_p (i.e. change in heat capacity at the glass transition) was significantly higher at large amorphous contents then the relative error at the high amorphous content would translate to a rather higher error in the estimation of low levels of crystallinity in an otherwise amorphous phase. It is difficult to speculate on the LOD and LOQ for this DSC method at low crystallinity, but it is likely to be much higher than the LOD and LOQ that has been demonstrated here. The real question now is whether the levels of detection and quantification may be achieved under less ideal conditions of a manufacturing process, with the THz sensor integrated into the manufacturing line.

Table VII. Summary for Statistical Parameters LOD and LOQ Obtained for Different Methods

Method	LOD (%)	LOQ (%)
THz/area-based	0.89	2.7
THz/PLS	0.76	2.32
PXRD	2.0	
DSC	0.06	0.21

CONCLUSIONS

The terahertz spectra of polycrystalline, amorphous sucrose, and a binary mixture of the two forms have been measured using TPS. Comparison of the calibration models indicated that peak areas based linear regression model has higher prediction errors than the PLS model. The precision in the measurement of crystalline sucrose using the PLS method was estimated in terms of an LOD of 0.76% and an LOQ of 2.3%.

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