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Paracetamol polymorphs detection in suspension via a new *ex situ* Fourier Transform Near Infrared spectroscopy method

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Abstract

This paper addresses the solid-state and in suspension characterization of the stable monoclinic form I and the metastable orthorhombic form II of paracetamol via differential scanning calorimetry (DSC), X-Ray diffraction (XRD) and Fourier transform near infrared spectroscopy (FT-NIR) methods. For generating the metastable form II, DSC technique was used and both polymorphs were characterized via XRD. It was demonstrated that form II crystals were stable until 29 days minimum of storage and no solid-state transformation occurred (form I trace less than 5%). Additionally, the detection of this form in suspension was investigated via DSC, XRD and FT-NIR. The DSC was not a suitable method since it is highly sensitive to temperature and solution evaporation whereas XRD showed its capacity in detecting both forms in suspension but also in monitoring the solvent-mediated transformation for highly concentrated suspensions. On the other hand, *ex situ* FT-NIR method combined with partial least square discriminant analysis (PLS-DA) outperformed both methods. Indeed, it offered an outstanding distinction between paracetamol form I, form II and the mixture of the two forms, and an accurate monitoring of the solvent-mediated transformation. This approach showed good statistical performances with a low root mean square error of calibration (RMSEC), root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP) values (RMSEC=20%; RMSECV=25%; RMSEP=21%), low classification errors (less than 3.2%) and high specificity and sensitivity (higher than 93%).

Keywords: paracetamol, polymorphism, *ex situ* monitoring, near infrared spectroscopy, principal component analysis (PCA), partial least square discriminant analysis (PLS-DA).

1. Introduction

Crystallization from solution of active pharmaceutical ingredients (APIs) is well established as an essential production, purification, and separation technique throughout the pharmaceutical industries [1–3]. This process is also widely used for tuning the properties of pharmaceutical crystals such as crystal size, crystal size distribution, and final crystal shape [4–6]. However, the preferential crystallization of a desired form is complex especially if the API exhibits many crystalline forms, known as polymorphs. These crystalline forms exhibit distinct thermodynamic properties (solubility, sublimation, enthalpy, entropy, melting point, and vapor pressure), kinetic properties (dissolution rate), spectroscopic properties (vibration state transition), mechanical properties (compressibility) and packing properties (refractive index, hygroscopicity, molar volume and density) [7,8]. Thus the

emergence of different polymorphs can have a profound effect on the manufacturability, chemical stability, bioavailability and ultimately the therapeutic efficacy of the final product [9–11].

As around 50% of the APIs [12], paracetamol has three fully characterized polymorphs: stable form I (monoclinic), metastable form II (orthorhombic), and unstable form III [13]. Form II is the preferred polymorph for tableting since it can experience plastic deformation due to its crystal structure comprised of well-defined slip planes bonded with weak interplanar interactions [10, 14, 15]. Moreover, form II is more soluble in water than form I which is beneficial for paracetamol bioavailability [16,17]. However, this form is thermodynamically metastable and spontaneously undergoes solid-state transition [18–20] and solvent-mediated transformation [21–24] which limits its purification, filtration, and storage [25].

Many attempts have been made to crystallize form II with high reproducibility while also avoiding the fast solvent-mediated transformation [26–28]. For instance, a few grams of form II from ethanol solutions have been achieved by the optimization and scaling-up of a seeding technique proposed by Nichols and Frampton [26]. However, solution-grown form II is usually contaminated with crystals of the monoclinic polymorph (form I) depending on harvesting time and drying conditions. Therefore, there is an ever-growing interest in characterizing the solid-state and solvent-mediated transformation of paracetamol and eventually detecting the polymorphic form throughout the whole crystallization process.

To improve the understanding of paracetamol polymorphic transformations and better control the final product quality during crystallization operations, different technologies have been investigated over the past few years. For polymorphs solid-state characterization, DSC [19,29,30], XRD [26,31], Raman spectroscopy [31,32], FT-NIR [33,34], attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) [18] and nuclear magnetic resonance (NMR) [35] are generally used. Moreover, for polymorphs detection in suspension and solvent-mediated transformation monitoring, authors mainly tend to use *in situ* methods like Raman spectroscopy [36], ATR-FTIR [37], FT-NIR [34], focused beam reflectance measurement (FBRM) and Blaze900 (Process Analytical Technology) PAT probes [38,39].

Considering the large amount of data recorded during the crystallization process, chemometrics has been employed as an aid for accurate data handling and exploration such as assigning polymorphic identity for the solid-state crystals, establishing the content of given forms in mixtures, and studying polymorphic transformations as well as amorphization and crystallization processes [40–42]. Table 1 resumes some of the *in situ* methods and the multivariate data analysis adapted for detecting and monitoring paracetamol polymorphs in suspension. For instance, Barthe et al. monitored the nucleation of the metastable form II and the solvent-mediated transformation by FBRM probe based on the evolution of the chord length counts and the crystal shape transformation during crystallization [38]. The authors assigned the polymorphic transformation to a drop in the total chord length counts of particles (5 μm – 250 μm). Another attempt for detecting form II in suspension while using FT-NIR spectroscopy and principal component analysis (PCA) was conducted by Wang et al. [41]. The authors used PCA to determine the main variables that explain the largest variations during paracetamol cooling crystallization. It was estimated that the polymorphic transformation trend could be detected based on the principal component (PC) chosen. Furthermore, since standalone technology is not sufficient for shedding light on polymorphism in some cases, two or more *in situ* methods are usually combined. As reported in table 1, Nicoud et al. [39] associated the Blaze900 probe with an *in situ* Raman spectroscopy for establishing a polymorphism kinetic model for seeded cooling crystallization of paracetamol.

Table 1. Paracetamol polymorphs detection and monitoring in suspension

<i>in situ/ ex situ</i>	Characterization method	Process	Results	Ref.
<i>in situ</i>	ATR-FTIR	Membrane crystallization	Form II at high solvent evaporation rate and low induction time	[5]
<i>in situ</i> <i>ex situ</i>	PAT probe (Blaze900) Raman spectroscopy Raman spectroscopy	Cooling Crystallization	Establishment of a polymorphism kinetic model in seeded batch crystallizer	[37]
<i>in situ</i>	FT-NIR	Cooling crystallization	Solvent-mediated transformation monitoring through PCA	[41]
<i>in situ</i>	FBRM probe	Cooling crystallization	Solvent-mediated transformation monitoring through chord length counts	[38]

A common trait of all these *in situ* techniques is that a complex calibration model is always required. Developing an *in situ* monitoring method is complex and time-consuming (especially when combining different techniques) and is highly dependent on many events occurring during crystallization (dissolution, growth, agglomeration, breakage...) which makes it harder to detect the polymorphic form obtained whilst nucleation and subsequently monitor the solvent-mediated transformation. Furthermore, having many PAT probes introduced into the reactor could offer large surfaces for heterogeneous nucleation. Moreover, probes are often prone to fouling increasing the risk of unintentional seeding via secondary nucleation [43] and contamination from impurities and crystals can significantly affect the crystallization process [44]. *Ex situ* techniques for suspensions can help mitigate these issues by reducing contact with the crystallizing solution, thus minimizing the impact of various crystallization phenomena on measurements while providing rapid insights on solvent-mediated transformations. Additionally, these techniques can serve as reference tools for validating *in situ* calibration models without the need to isolate the polymorphic form from the mother liquor, thereby reducing operational time and improving validation accuracy.

Using a fast, simple and reliable method like FT-NIR spectroscopy is advantageous in avoiding sample preparation since this manipulation may have drastic effects on polymorph analysis [45]. When analyzing metastable samples, crystals could undergo polymorphic transformation to the stable form, giving erroneous results. For example, in the case of XRD, the polymorphic transformation may be initiated by crushing the crystalline sample to a fine powder. Solvent evaporation is also a major issue for many other *ex situ* methods such as ATR-FTIR and DSC especially if the solvent used is volatile. Crystallization by evaporation could occur leading to the formation of nuclei of the most stable form, initiating the solvent-mediated transformation. This phenomenon is hindered during FT-NIR analysis since sealed samples are tested and the method is not heat sensitive.

The primary objective of this study is to qualitatively assess the capability of FT-NIR spectroscopy to classify paracetamol polymorphic forms and to evaluate the method suitability for detecting the solvent-mediated transformation from the metastable form II to the stable form I.

In this manner, this study explores the establishment of an *ex situ* method for paracetamol suspensions characterization. Various qualitative multivariate data analysis approaches, including PCA and partial least square analysis (PLS-DA), are compared. The first part of this paper focuses on characterizing monoclinic and orthorhombic forms of paracetamol in solid-state. The second part is dedicated to

developing the prediction model and comparing the performance of PCA and PLS-DA. Finally, the experimental validity of the prediction model is investigated.

2. Materials and methods

2.1. Chemicals and ultrapure water

Form I (monoclinic) of paracetamol was purchased from Sigma Aldrich (99%, Aldrich Chemical Co. Inc., MO) as a commercial powder and was used as received. Isopropanol was also purchased from Sigma Aldrich (99%, Aldrich Chemical Co. Inc., MO) and ultrapure water was prepared using a Synergy unit system (Millipore, Molsheim, France).

2.2. Production of form II of paracetamol

Form II was prepared through DSC heating/cooling cycles. The first cycle consisted on melting 33 mg of commercial form I by heating to 190 °C at a rate of 10 °C/min in an open pan under anhydrous nitrogen atmosphere (cycle 1). After a swift cooling-down to room temperature at a rate of -50 °C/min (cycle 2), the amorphous phase formed was again heated to 120 °C at a rate of 10 °C/min in order to produce form II (cycle 3) in sealed and in open DSC pans.

2.3. Polymorph characterization

The characterization of paracetamol polymorphs was performed via DSC, XRD and FT-NIR spectroscopy.

DSC measurements were performed using a DSC Q200 differential scanning calorimeter supplied by TA instruments. The temperature calibration was performed with the melting point of indium (429.78 K) at a heating rate of 10 °C/min. The energy scale calibration was performed with the enthalpy of fusion of indium (28.45 J/g). A nitrogen purge of 50 mL/min was employed for all measurements. The weight of the samples was nearly between 15 and 33 mg for solid-state analysis and about 50 µL of 70/30% w/w water/isopropanol solution for suspensions in sealed, open, or perforated aluminium pans. The heating rate was fixed at 1°C/min; 10°C/min and 50°C/min. All measurements were done in triplicates.

The purity of form II powder was determined via XRD and compared to reference data from Florence et al. [46]. The samples were carefully prepared by spreading out form II crystals on a deep-ribbed polymethylmethacrylate (PMMA) support with a cylindrical volume of 25 mm x 12 mm.

The diffraction patterns were recorded by a Bruker D8 Advance diffractometer operating at room temperature with $K\alpha$ radiation of wavelength 1.540560 Å. The samples were measured using an X-Ray beam with voltage of 40 kV and a current of 40 mA with an angular scan 2θ ranging from 5° to 50° using a step scan mode with step width of 0.02° and scan speed of 2 s/step. The acquired data were analyzed using the DIFFRAC.EVA program. The area of all diffractogram peaks were calculated via Fityk software.

For the analysis of form II in suspension, 10 to 15 mg of form II were placed in 50 µL of 70/30% w/w water/isopropanol solution. The suspension was then rapidly introduced in a “dome-like” sealed home-made support and analyzed by Bruker D8 Advance diffractometer using Cu $K\alpha$ radiation. This support was helpful in preventing the solution evaporation but also by keeping the solution homogenized during the measurement. The recorded data were analyzed using DIFFRAC.EVA software in order to detect both polymorphs in suspension and eventually monitor the solvent-mediated transformation precisely while also preserving the measurement quality. The samples

measured were taken in a tight angular scan 2θ between 23° and 27° using a step scan mode with step width of 0.02° and scan speed of 0.2 s/step.

2.4. Prediction model development

The FT-NIR was the main method used for detecting paracetamol polymorphs in suspension. The methodology consisted of two main stages: the model development from calibration samples (training set) and its validation using test samples calibration and prediction model establishment.

2.4.1. Data collection. For data collection of calibration samples, 10 to 29 mg of form I or form II or both (50% w/w to 63% w/w of form I) were placed into a transparent HPLC vial and 100 μL solution of 70/30% w/w water/isopropanol was added. The samples concentration ranged from 100 mg/mL to 290 mg/mL and the solid content varied from 19 to 71%. The samples were agitated by hand and rapidly analyzed to reduce as much as possible the solvent-mediated transformation. The NIR diffuse reflectance analyses were carried out using a FT-NIR spectrometer Nicolet iS50 equipped with the integrating sphere accessory module. Spectra were collected with OMNIC software. All FT-NIR *ex situ* spectra were continuously collected using SOLO software (Version 9.1) from Eigenvector using MATLAB PLS-toolbox. Each spectrum was acquired by averaging 32 scans with a resolution of 8 cm^{-1} and over the range of 4 000 to 10 000 cm^{-1} . The acquisition of a full spectrum took approximately 16 s for each sample.

2.4.2. Experimental factors influence. Variations in concentration, temperature, and particle size can all affect FT-NIR spectra. Since all samples are collected at room temperature, the temperature effect is neglected. Concentration variations were intentionally added to the raw data prior to pre-processing to further reduce the effect of paracetamol concentration. Particle size variations are also accounted for in the calibration spectra. Form I sample includes commercial powder with a narrow size distribution whereas form II is formed off agglomerates along with fine powder given that it was gently recovered from DSC pans with the least amount of mechanical stress to prevent phase transformation. These variations are reduced by pre-processing raw data acquired for the calibration model.

2.4.3. Raw data pre-processing. Raw data were collected and pre-processed by second derivative and standard normal variation (SNV). Second derivative was used for reducing the drift of the baseline and linear trend as well as for highlighting some parts of the spectral information corresponding to polymorphism. On the other hand, SNV was applied to remove scatter differences between the samples for instance, light scatter effects, liquid concentration due to dilution phenomena and the size of the crystals which is not equally distributed between samples, resulting in different scatter effects. Analysis of the scores and loadings for the most important PCs, as determined from percent variance plots, were used to investigate changes in the spectral features of the FT-NIR data. The outliers were removed according to the representation of Q residuals versus Hotelling's T^2 for better classification.

2.4.4. Multivariate data analysis. The pre-processed FT-NIR spectra obtained were subjected to PCA and PLS-DA multivariate data analysis methods using SOLO software (Version 9.1) equipped with MATLAB PLS-toolbox. PCA is an unsupervised dimensionality reduction method which allows the visualization of a dataset based on the inherent spectral variances. It is mainly useful for a better understanding of high dimensional systems by projecting the data in a new system defined by Principal Components (PCs). PCs are linear combinations of the original variables maximizing the variance of the data. In this paper, PCA was performed to obtain a graphical representation of the FT-NIR spectra in a low-dimensional space, aiming at investigating the geometrical proximities of the samples in relation with their composition (form I, form II and mixture).

PLS-DA is a supervised classification technique adapted from Partial Least Squares (PLS) regression for categorical dependent variable Y (here the polymorphic form). PLS-DA models are based on latent variables (LVs) that are linear combinations of the original variables determined to have a maximum covariance with the categorical label.

3. Results and discussion

3.1. Solid-state polymorph characterization by DSC

The thermal behavior of both polymorphs was first tested by DSC scanning by applying the following heating and cooling cycles in sealed pans:

- Cycle 1: Commercial form I heating from 20 to 190 °C at a rate of 10 °C/min
- Cycle 2: Rapid cooling of the melt from 190 to 20 °C at a rate of -50 °C/min
- Cycle 3: Reheating of the amorphous phase from 20 to 200 °C at a rate of 10 °C/min

DSC curves recorded during cycle 1 and cycle 3 are shown in Figure 1. Heating commercial form I resulted in an endothermic peak at 169 °C corresponding to the melting point of the monoclinic form I (cf. Figure 1 (a)). On the other hand, Figure 1 (b) shows an exothermic peak at 82 °C referring to a cold crystallization event and an endothermic peak related to form II melting point at 157 °C (cf. Figure 1 (b)). These results tie well with previous studies wherein paracetamol thermal behavior was investigated [46–48].

The difference between the melting points is mainly due to the molecular structure and different hydrogen bindings which are crucial for the stability of each polymorph: type A (NH··O) and type B (OH··O) bindings. As reported by Wang et al., paracetamol form I exhibits stronger hydrogen bindings than form II since the distance of the NH··O and OH··O bindings present in its structure are respectively 2.91 Å (NH··O) and 2.65 Å (OH··O) for form I and 2.97 Å (NH··O) and 2.72 Å (OH··O) for form II [41].

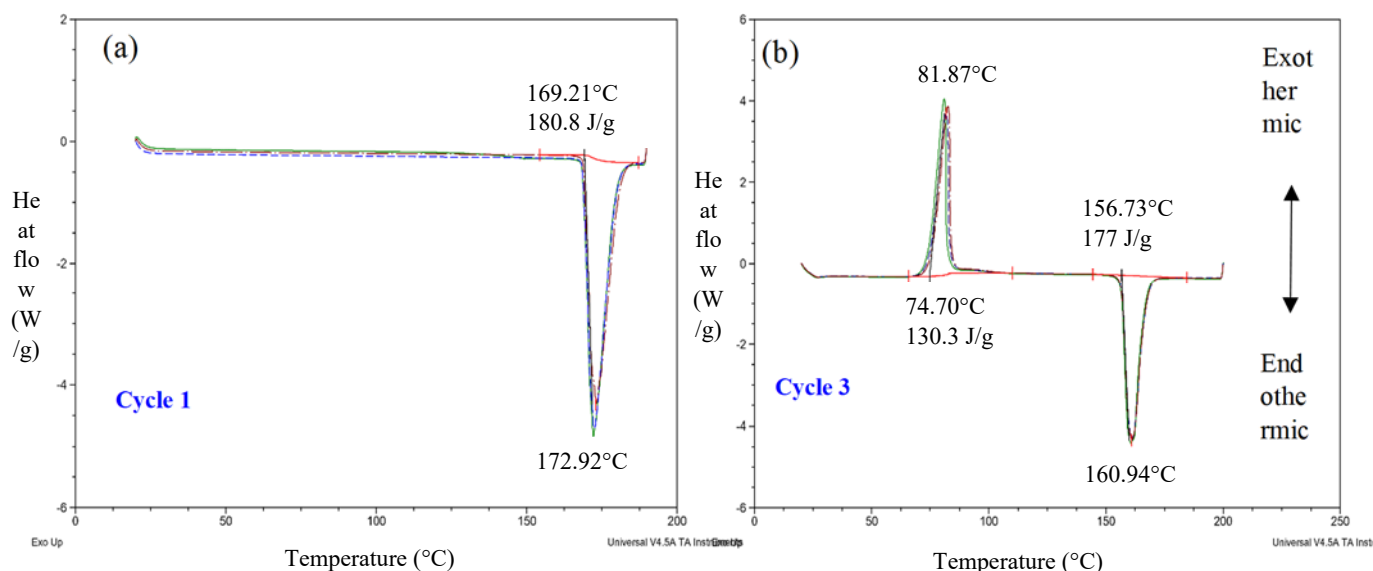


Figure 1. DSC thermogram for (a) monoclinic paracetamol (form I) and (b) orthorhombic paracetamol (form II) in sealed DSC pans

Similar cycles were performed in open DSC pans to test the possible form II recuperation for further polymorph characterization. Interestingly, a small exothermic event was observed around 132 °C during cycle 3 (cf. Figure 2 (a)). The occurrence of such a minor event (enthalpy $H = 9.142$ J/g) did not alter the general understanding of the major thermal events ($H = 120.6$ J/g) in the DSC scans and its main cause is yet unclear [50]. A potential explanation is that it is due to the presence of trace amount of a chemical impurity, but XRD measurements of the recrystallized form II revealed no evidence of impurities at the detection levels associated with this technique ($\pm 1\%$). Nevertheless, it is also possible that paracetamol samples have undergone thermal decomposition whilst DSC scans. However, the thermogravimetric analysis (TGA) results for commercial paracetamol pointed out the fact that commercial paracetamol form I degradation occurs at 317 °C (data not shown). Another potential explanation is that this event could refer to the transformation of a small amount of the highly metastable form III to form II. This statement was further investigated by Di Martino et al. [18] who previously detected a peak near 54 °C in DSC experiments while producing form II from melt-cooled paracetamol with no formation of the amorphous phase. The authors obtained a high metastable form III under these conditions and assigned this exothermic peak in the range 120-130 °C as the transition from form III to form II.

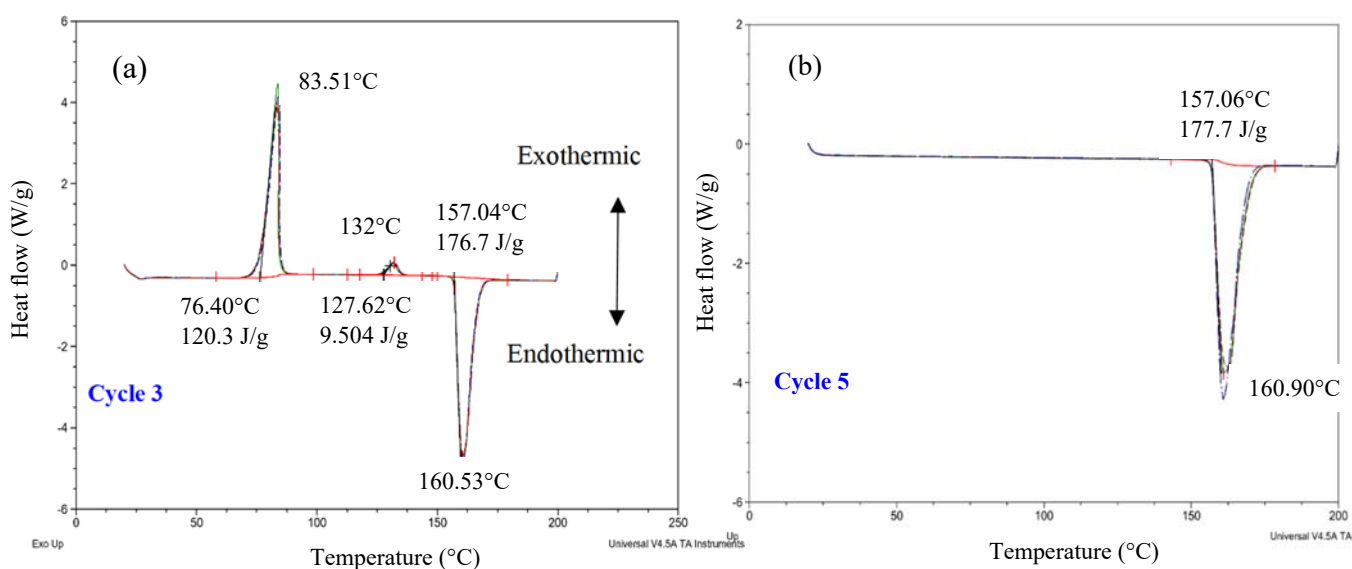


Figure 2. DSC thermogram of (a) orthorhombic paracetamol (form II) production and its (b) stability analysis in open DSC pan

The same phase transformation was also reported by Wang et al. [34]. The authors further elucidated the origin of such exothermic event by correlating the DSC thermograms with FT-NIR spectrum. It was found that the hydrogen bonding type B of form III transformed into hydrogen bonding type A of form II at 125 °C which refers to a phase transformation.

We conducted a deeper analysis about the formation of such peak by applying two additional cycles to the form II produced:

- Cycle 4: cooling from 120 to 20°C at a rate of -1 °C/min
- Cycle 5: heating from 20 to 200°C at a rate of 10°C/min

The final cycle shown in Figure 2 (b) did not reveal any major events besides form II melting point at 157 °C which clearly indicates that no solid-state transition from form III to form II or from form II to form I occurred upon heating form II. Therefore, it can be concluded that the exothermic peak detected earlier is the result of traces of form III conversion to form II. The study of Telford et al. [51] concerning the stability of form III led to identical conclusion about such transformation. The effect of

sample thermal history and encapsulation method on paracetamol polymorphic behavior was also studied by Qi et al. [52]. According to the authors, the transition of form III to form II is recorded in pinholed pans when reheating form III [52]. In our case, the use of open pans with N₂ atmosphere favors the stability of form III where solid-solid transition upon reheating form III meanwhile for hermetically sealed pans the contact with the headspace gas enhances the instability of form III making the transition fast and hard to recorded.

Furthermore, the detection of the melting point of form II in the final cycle validated this method as a form II production technique in open DSC pans which will be adopted for further analysis.

3.2. Solid state polymorph characterization by XRD

Both paracetamol polymorphs were also characterized via XRD, and their purity was investigated. The experimentally obtained XRD patterns for the characterization of the commercial form I and form II obtained by DSC are presented in Figure 3. The powder data for the monoclinic form I were in line with that given by Florence et al. [46] and with the findings of Chadwick et al. [21]. The XRD pattern for the orthorhombic form II did not perfectly match the chosen reference pattern [46]. According to Figure 4, some traces of the most stable form (form I) were observed which are probably due to sample preparation or grinding which initiated the solid-state polymorphic transition [18].

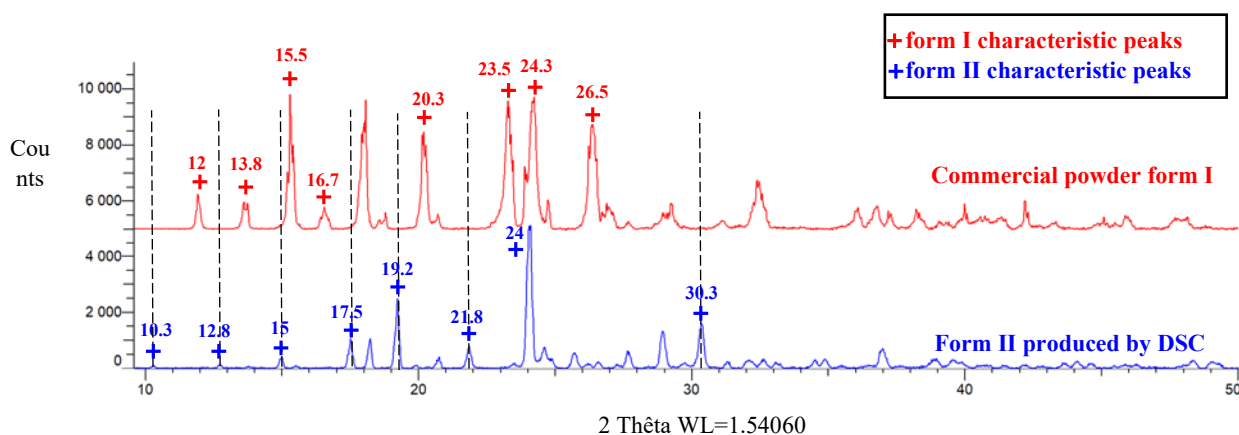


Figure 3. XRD patterns for paracetamol monoclinic (form I) and orthorhombic (form II) prepared by DSC

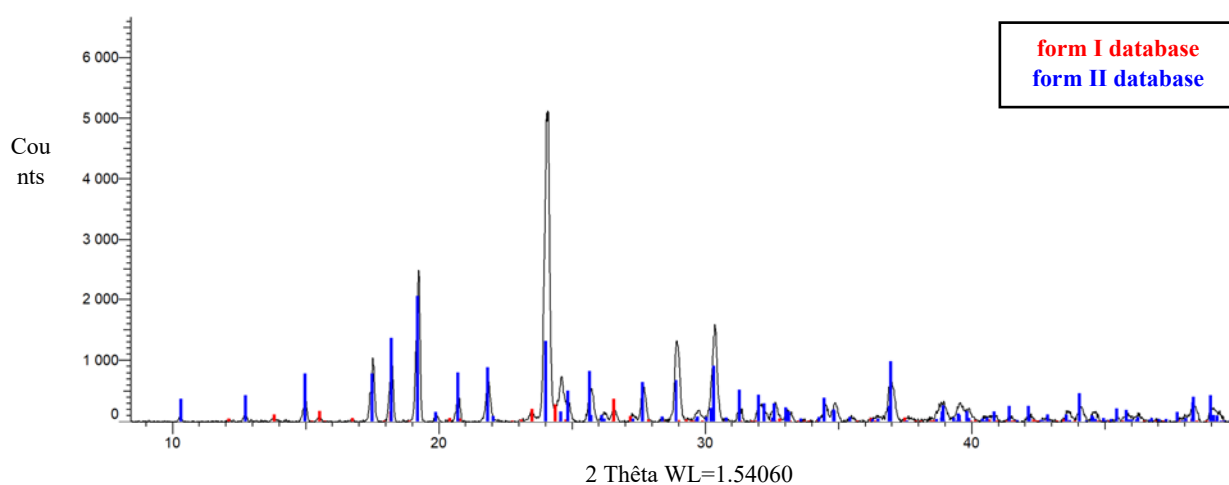


Figure 4. XRD pattern of paracetamol orthorhombic (form II) prepared by DSC

Nonetheless, characteristic peaks of form I have relative integrated intensities (expressed as the ratio of form I peak area to that of the highest peak of form II) about 3-5% according to the XRD pattern of

form II presented in Figure 4 which could enhance the phase transition into the more stable form upon storage [53]. With this in mind, the solid-state transition of form II was also investigated during storage and for potential reuse of this form during crystallization operations (i.e. seeding). As observed in Figure 5 and according to the peaks area analysis, relative intensities of the monoclinic form I peaks did not vary even after minimum 29 days of storage in a closed flask under room conditions ($T= 20 \pm 2 \text{ }^\circ\text{C}$, $P=P_{\text{atm}}$). Therefore, having traces of the stable form I did not lead to a solid-state transformation. This result is in accordance with Di Martino et al. findings [14] which demonstrated that the increase of form I content from 20 to 68% in powder mixtures did not affect the stability of form II compared to the storage of pure form II (less than 2% of form I).

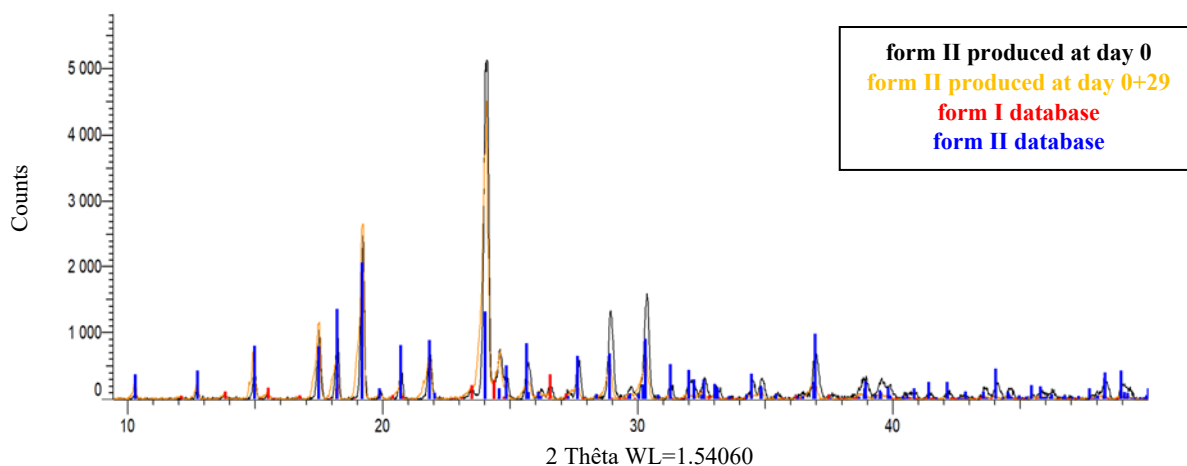


Figure 5. Investigation of the solid-state transition of orthorhombic form II of paracetamol after minimum 29 days by XRD

3.3. Polymorph characterization in suspension by DSC

The first attempt to detect paracetamol polymorphs in suspension was via DSC scanning. The suspension was heated from ambient temperature to 200°C at a rate of $1^\circ\text{C}/\text{min}$ in perforated pans to observe the melting point of both forms. Perforated pans were used for avoiding condensation phenomena on the walls of the pan.

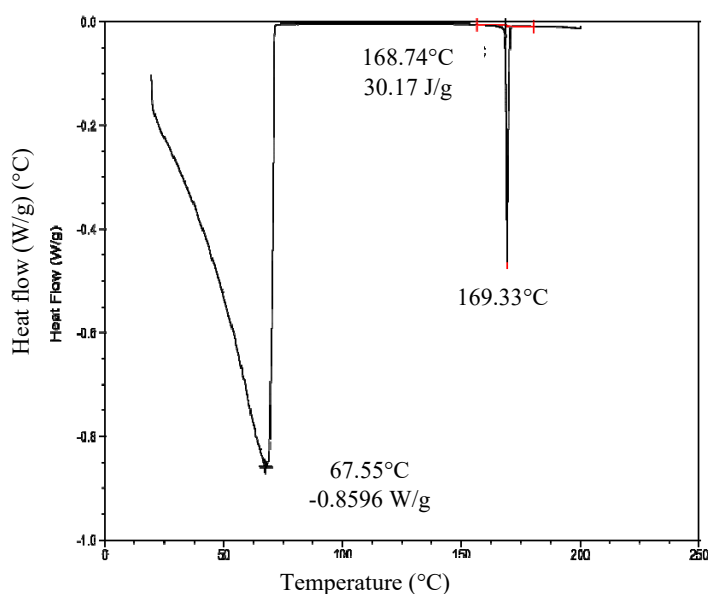


Figure 6. DSC thermogram of orthorhombic paracetamol (form II) in suspension in perforated DSC pan when heating from 20 to 200°C at a heating rate of $1^\circ\text{C}/\text{min}$

The DSC thermogram shown in Figure 6 brought out the appearance of two endothermic events. These thermally induced events of form II suspension have been interpreted as the evaporation of the solution at 67 °C followed by the melting of form I at 169 °C. Surprisingly, neither the melting point nor the phase transition of form II to form I was observed. These findings could be correlated to the evaporation phenomena occurring during the DSC scan which initiates the preferential nucleation of form I and thus the solvent-mediated transformation to the stable form I. This suggests that DSC is unlikely to be a reliable method for the detection of crystallized form II in suspension because the major thermal event is the melting of form I that results from the thermally induced conversion of form II to form I.

3.4. Polymorph characterization in suspension by XRD

Another exploratory study was carried out using XRD analysis for characterizing both polymorphs in suspension. The sample preparation method and the support used are described in section II.2.2. From the diffractograms represented in Figure 7 (a), form I was clearly detectable by XRD since its peak corresponded to the peaks of form I presented by Florence et al. [46]. Similarly, peaks of form II were detected in suspension when having a mixture of polymorphs (cf. Figure 7 (b)).

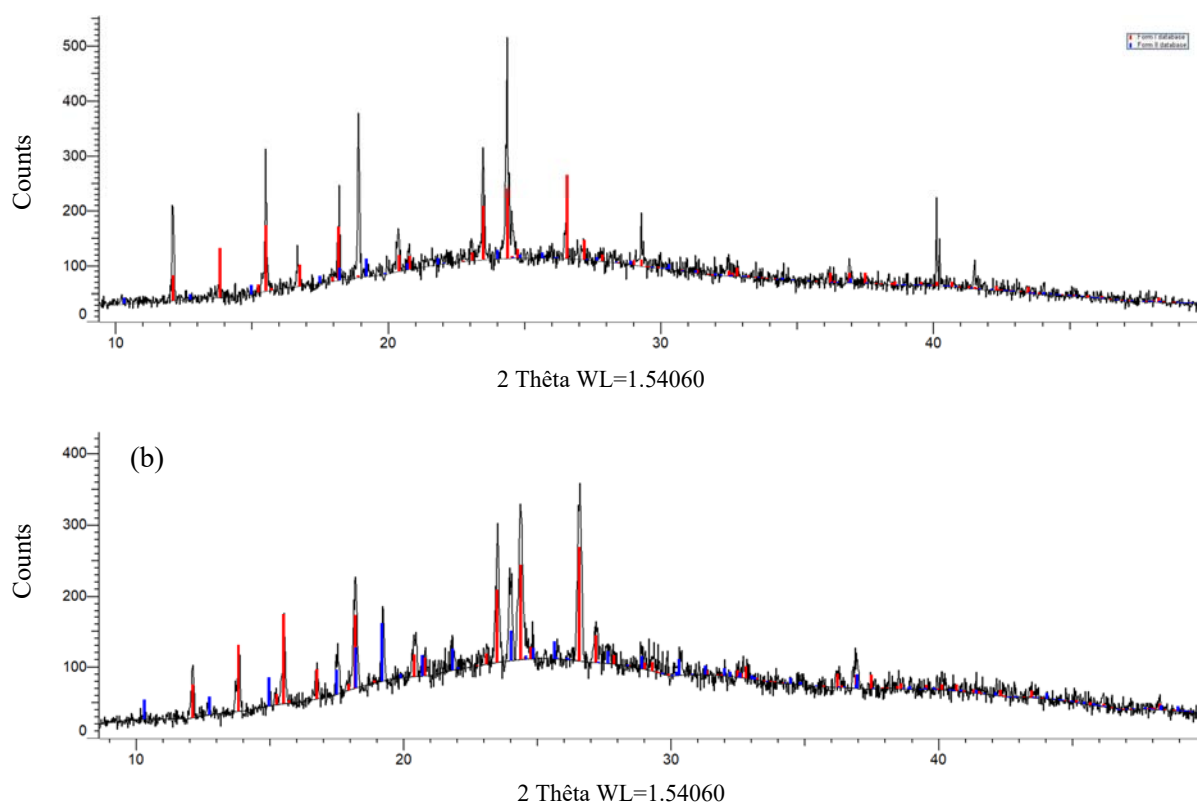


Figure 7. Detection of (a) monoclinic (form I) and (orthorhombic) form II in a solution of 70/30% w/w water /isopropanol by XRD

From these results, key findings emerge. Firstly, the detection of both polymorphs is possible via XRD in well-concentrated and homogenized samples to avoid high background noise. Second, a potential viable quantitative approach for assessing and monitoring form I content in polymorphic mixtures in suspension might be established.

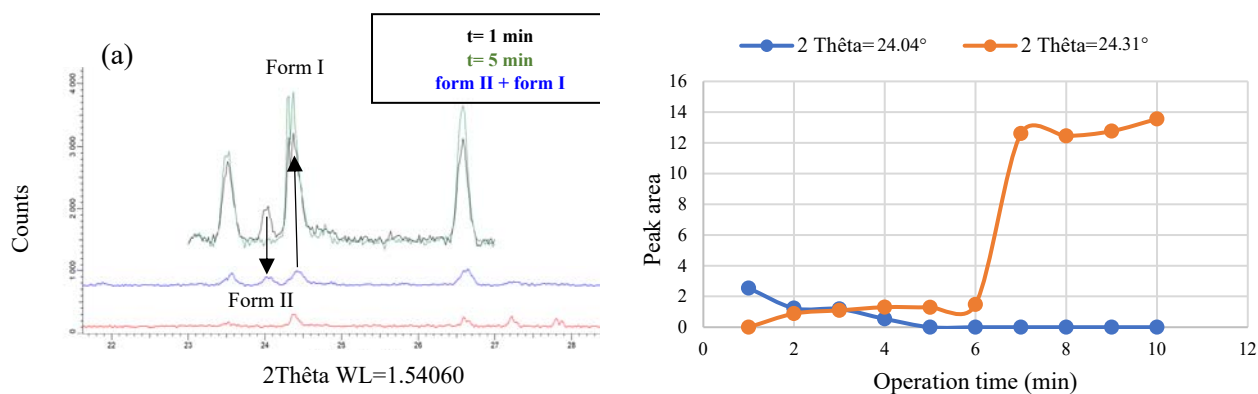


Figure 8. Investigation of the (a) solvent-mediated transformation of paracetamol in a mixture of 70/30% w/w water/isopropanol by XRD and (b) evolution of the characteristic peak height of form I and form II in suspension

The solvent-mediated transformation was also studied via XRD. The patterns recorded whilst the introduction of form II suspension were compared to the patterns of form I and the mixture of form I and form II suspension (cf. Figure 8 (a)). Throughout the series of diffractograms recorded and the investigation of the peak height evolution of form I and form II in suspension (cf. Figure 8 (b)), it has been observed that the area of the peak referring to the presence of form II ($2\theta = 24^\circ$) decreased and disappeared after 5 min of analysis. In the meantime, the peak at 24.31° referring to the presence of form I increased with respect to the analysis time. These results confirm that form II is unstable in suspension and is prone to a fast conversion to the most stable form (i.e. form I). Overall these findings are in accordance with results reported by Méndez del Rio et al. and Sudha et al. regarding paracetamol form II stability during crystallization processes [24, 51]. The stability time of form II was reported to be approximately 15 min, which is in line with our XRD results if we take into consideration the time of sample preparation and analysis estimated to be around 8 min.

The distinction between the two paracetamol polymorphs was identified using XRD analysis, which also detected a solvent-mediated transformation in concentrated samples. However, this analytical technique is not suitable for diluted samples, such as those taken at the early stages of nucleation during crystallization operations, due to high noise levels and preferential orientation issues.

3.5. Polymorph characterization in suspension by FT-NIR

FT-NIR can serve as a rapid tool for screening polymorphs and studying polymorphic transformations typically solid-state transformation and solvent-mediated transformation [34,52]. FT-NIR spectra furnishes distinct information on molecular structure and conformation from vibrations of atoms. It works on the principle that polymorphs differ in the intermolecular hydrogen bonding pattern in a crystal lattice which results in a shift of absorption bands in NIR spectra.

3.5.1. FT-NIR spectra of paracetamol polymorphs in suspension

Paracetamol polymorphs (forms I and II) are excellent examples of how NIR spectroscopy may be used to discern two crystal forms, as their NIR spectra differ significantly. For clearly identifying the difference between form I, form II and solution NIR spectra, a first derivative pre-processing (Savitzky-Golay first derivative) was applied to the raw data as observed in Figure 9. In the region located between $4\ 000$ and $7\ 500\ \text{cm}^{-1}$, no absorption peaks of the solvents were detected whereas the samples containing paracetamol exhibit absorbance peaks in this region. This paracetamol spectra region was previously reported by Wang et al. as the main zone for differentiating paracetamol

polymorphs during cooling crystallization [41]. Therefore, the development of the prediction model will be based on this spectral region.

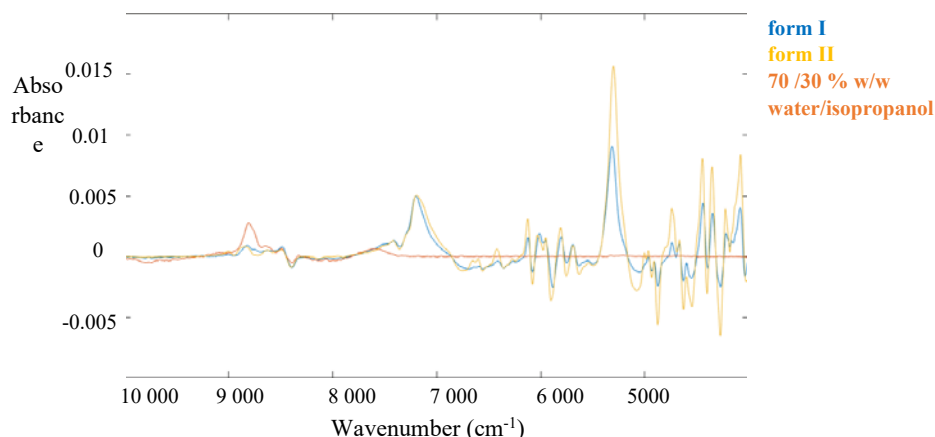


Figure 9. FT-NIR spectra of paracetamol form I and form II with a first derivative pre-processing

3.5.2. Raw data preprocessing

Light from the sample is received by the spectroscopic FT-NIR detectors as scattered light, specular reflectance, and diffuse reflectance after absorption. The latter two convey no meaningful information; only the diffuse reflectance has chemical information. Systemic noise resulting from light scattering, sample dilution, particle size variances, and instrumental drift may have an impact on the obtained spectra. Therefore, by using preprocessing techniques, the raw spectra must be corrected to obtain an accurate polymorphism prediction from spectroscopic observations. Pre-processing the collected data was therefore the initial step in creating the prediction model. Figure 10 (a) shows the raw spectra that were taken into consideration for the calibration dataset. It can be shown that scattering additive and multiplicative effects with a significant baseline have an impact on raw spectra. It is most likely because of light scatter effects, paracetamol concentration variations and crystal size variations that the scattering effect is visible. As a result, SNV was used to pre-treat NIR spectra initially. The SNV application on the acquired NIR raw data is depicted in Figure 10 (b), which distinctly reduced the scattering additive effect. Applying the SG second derivative further reduced the residual multiplicative impact, which was characterized by an increasing tendency with higher wavelengths, as well as the baseline (cf. figure 10 (c)). It is evident from a comparison of the final pre-processed data and the raw data that the noise effect was minimized although significant polymorphism variations were maintained, which made it simpler to analyze NIR spectra.

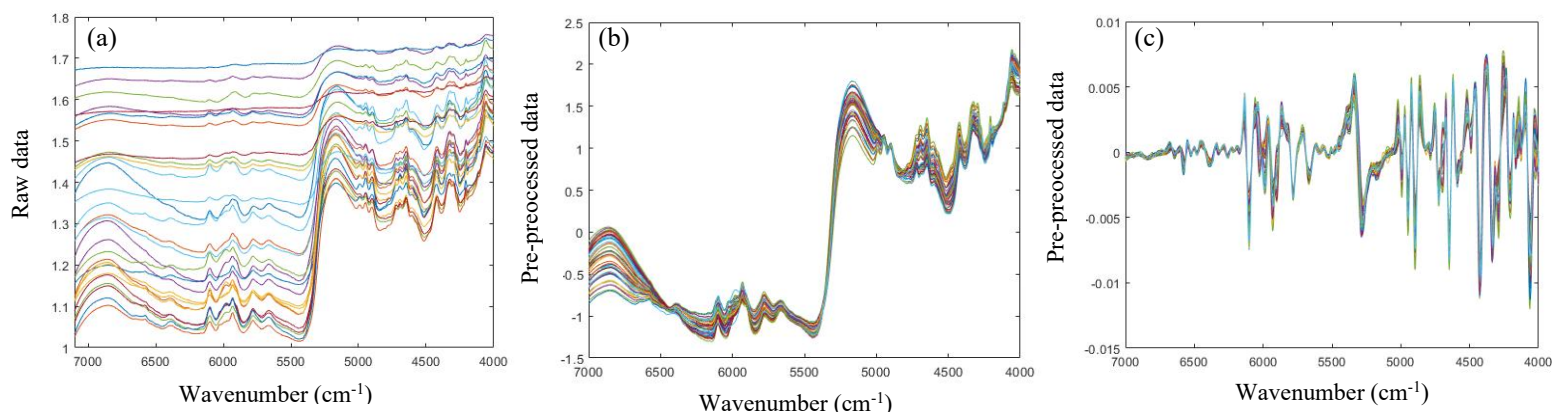


Figure 10. From right to left: (a) raw FT-NIR spectra, pre-processed data with (b) SNV and (c) SNV followed by SG second derivative

3.5.3. Principal component analysis

The homogeneity and classification of samples belonging to the same polymorphic form are essential to ensure that there are no significant differences between batches of the same polymorphic forms. Thus, an exploratory study was performed using PCA on a well-established dataset to evaluate the possibility of clearly distinguishing form I, form II and mixture samples from FT-NIR spectra. The dataset used is composed of:

- Thirty spectra of form I in suspension
- Thirty spectra of form II in suspension
- Forty spectra of mixture of form I and form II in suspension

PCA was first used to graphically represent the measured FT-NIR spectra. Figure 11 (a) represents the scores on the first two PCs which explain ~99% of the total variance. Comparative analysis evidenced that PC1 and PC2 were able to successfully discriminate the samples according to their polymorphic form (I, II or mixture).

Interestingly, the distinction was more prominent according to PC2, highlighting the fact that PC2 captures the majority of polymorphism-related variance (cf. Figure 11(b)). Furthermore, many other changes were noticed as a result of the dissolution and dilution effects.

These changes are most likely represented by PC1, specifically the kinetic size of paracetamol molecule, which reduces during the dissolving phase, and the paracetamol solid concentration, which decreases during dilution. When the sample is diluted, the dot points move to the right side of the 2D plot, while the most concentrated samples are plotted on the left side (cf. Figure 11 (a)). It is also essential to note that two polymorphs have different solvent solubility values. This implies that the large distribution of form II in the 2D plot is associated with the dissolution kinetics of form II which are generally faster than the most stable form I [13].

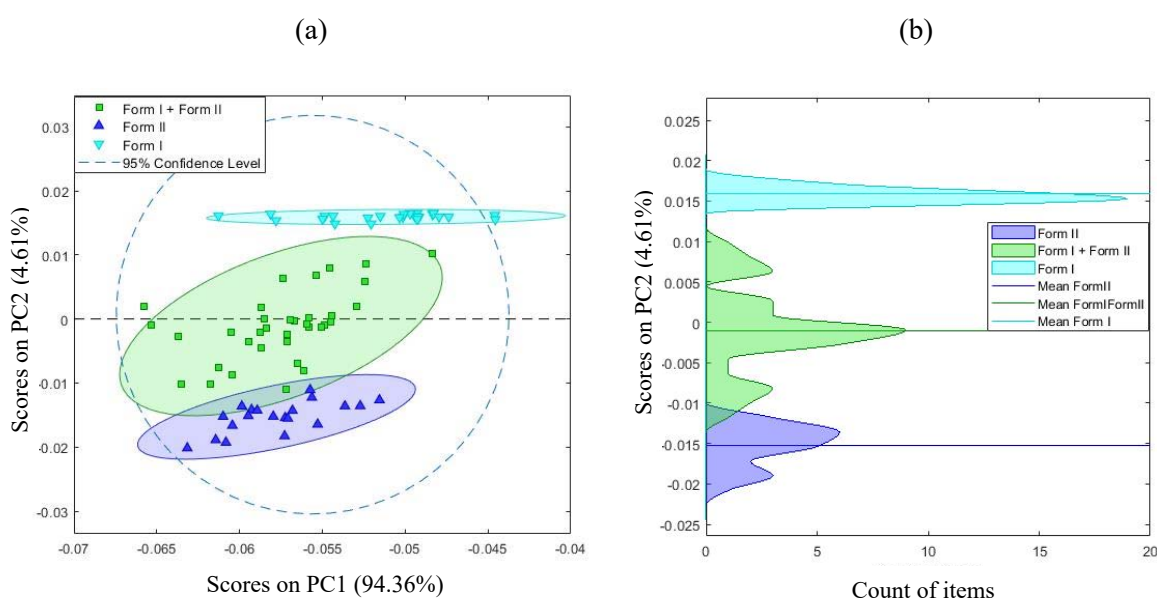


Figure 11. A typical (a) PC score plot, and (b) count of items distribution among PC2 for the different

For further understanding of the system behavior, PCA loadings (PC1 and PC2) were analyzed in comparison with the pre-processed data plot (cf. Figure 12). As depicted by Figure 12 (a), the spectral behavior of form I, form II and the mixture mainly differ in the regions in red frames. Besides, the

analysis of PC1 revealed the presence of large variances in the regions between 4 000 and 4 900 cm^{-1} , 5 000 and 5 500 cm^{-1} , but also between 5 800 and 6 200 cm^{-1} .

On the other hand, PC2 also exhibited a rich pattern where intense variations were observed around 5 800 and 6 200 cm^{-1} , 5 000 and 5 500 cm^{-1} , and between 4 000 cm^{-1} and 4 900 cm^{-1} although the variations in the region of 5 800 cm^{-1} to 6 200 cm^{-1} were higher than the variations on PC1. Following the loadings assessment, it is obvious that the two first PCs primarily describe the polymorphism phenomena.

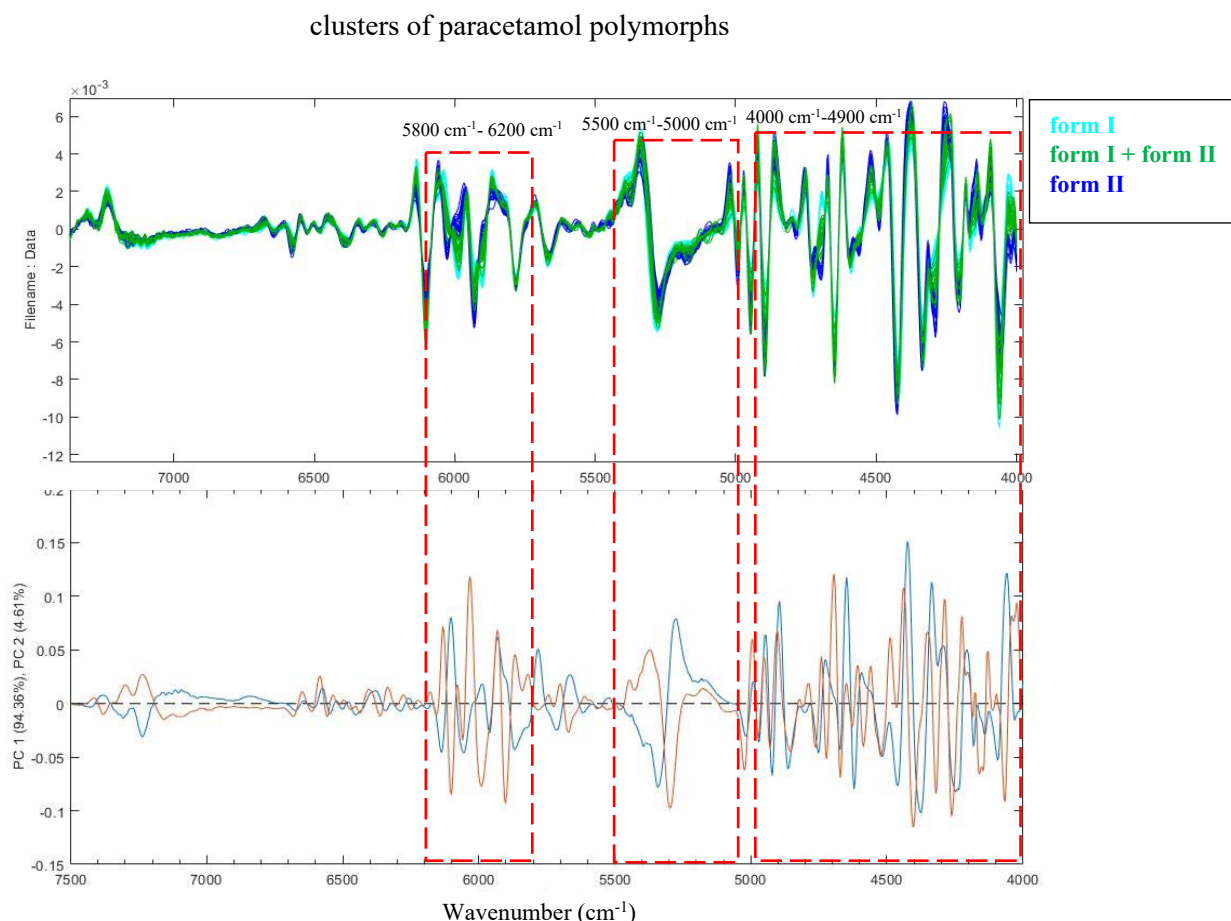


Figure 12. Plot of (a) pre-processed data with SNV and SG second derivative, (b) PC1 and PC2 loadings obtained from the training dataset

3.5.4. Development of PLS-DA model

PLS-DA was applied to the dataset used for PCA analysis to further qualitatively investigate paracetamol polymorphism. The strategy involved training the model on three different categories: form I, form II, and mixture (80% of the dataset) and then removing 20% of the initial dataset for validation of the model accuracy. Similar pre-processing methods as PCA were applied on the calibration dataset. The PLS-DA model output showed that the data were grouped into three separate clusters (cf. Figure 13 (a)) and the classification based on the first latent variable (LV1) was the most precise and similar to that given by the PCA model (data not shown). On the other hand, it was observed that the variance related to polymorphism was mainly captured by LV1 and LV2; LV1 separated form I samples from other samples whereas LV2 isolated form II from the rest of the samples. Therefore, the combination of those LVs fully describes the polymorphism phenomena with approximately 99% of the total system variations.

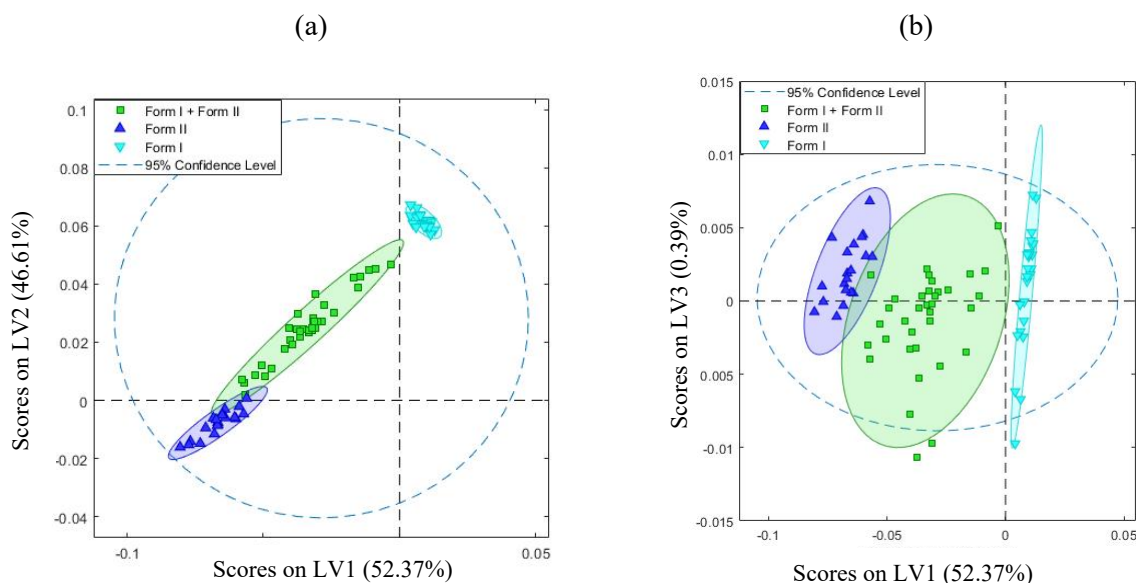


Figure 13. A typical (a) PLS-DA score plot, and (b) count of items distribution among LV1 for the different clusters of paracetamol polymorphs

Moreover, the variances related to dilution depending on polymorphs solubility values and solid content described earlier in the PCA model are not captured in LV1 and LV2. Conversely to the PCA model, those variations account for less than 1% of the total system variances. This remaining scattering effect on the model output could be mitigated by applying an external background consisting of a saturated solution for FT-NIR samples.

The choice of LV1 and LV2 latent variables for describing the system was confirmed by the investigation of the evolution of the latent variables in comparison with the FT-NIR spectra of the samples. As outlined in Figure 14, the evolution of LV1 and LV2 are almost proportional and complementary on the spectral region where the difference between form I and form II are noticeable, typically between 4 000 and 4 900 cm^{-1} ; 5 000 and 5 500 cm^{-1} ; 5 800 and 6 000 cm^{-1} .

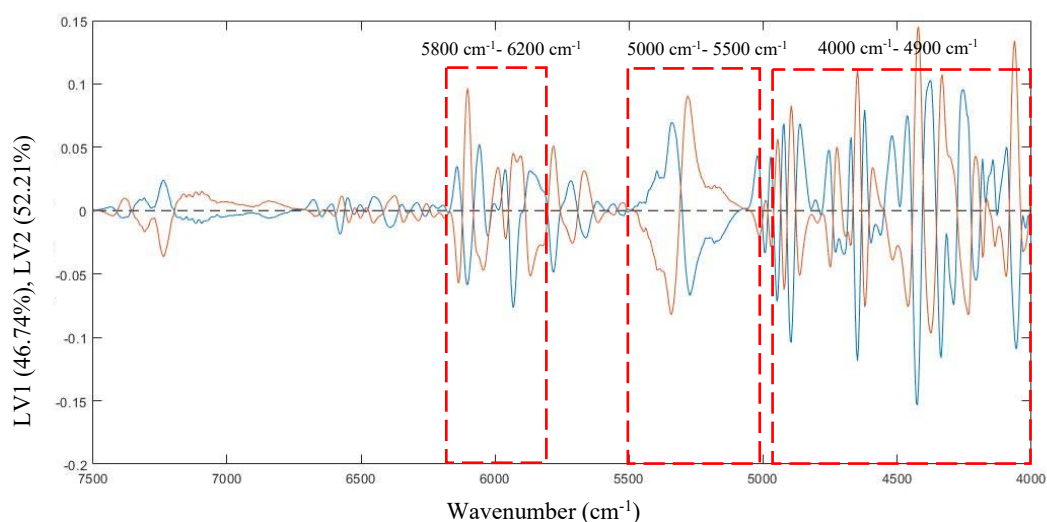


Figure 14. Plot of LV1 and LV2 loadings obtained from the training dataset

A statistical evaluation of the model was also performed for the first seven latent variables for which the calibration and cross validation classification errors average tend to stabilize. As depicted in table

2, the PLS-DA method has high sensitivity and specificity values with low classification errors and RMSEC (RMSEC=20%), RMSECV (RMSECV=25%) and RMSEP (RMSEP=21%) values.

Table 2. PLS-DA results

		Sensitivity (%)	Specificity (%)	Classification error (%)	Root mean square error (%)
LVs=7	Cross Validation (n=16)	100	93.6	3.2	25
	Calibration (n=64)	100	95.7	2.1	20
	Prediction (n=13)	100	100	0	21

The results of the two approaches were found to be suitable for qualitative analysis and prediction of paracetamol polymorphs in suspension. Furthermore, because PLS-DA is typically an ideal classification model for discriminating samples when the variability within a group is greater than the variability between several groups [56], we can deduce that having similar classification for PCA and PLS-DA results in the predominance of the polymorphism phenomenon as a major variation between FT-NIR spectra. However, the ability of PLS-DA approach to classify different groups while also decoupling other phenomena variances from polymorphism variances was demonstrated making it more suitable for polymorphism content prediction.

3.5.5. Experimental validation

The ability of the model to detect mixtures of polymorphs in suspension was first tested with a model suitability set of four samples, containing 25/75% w/w, 50/50% w/w, 60/40% w/w and 75/25% w/w of form I/form II. According to Figure 15, the model successfully placed the samples in the intermediate region between the two pure polymorph clusters. As expected, the sample location on the 2D plot was directly correlated with the amount of form I present in the samples. Typically, the samples containing 25% of commercial form I was the closest to the cluster of form II, succeeded by the samples of 50 % and 60%. The sample containing 75% of commercial form I was classified in the limit region between the mixture and pure form I.

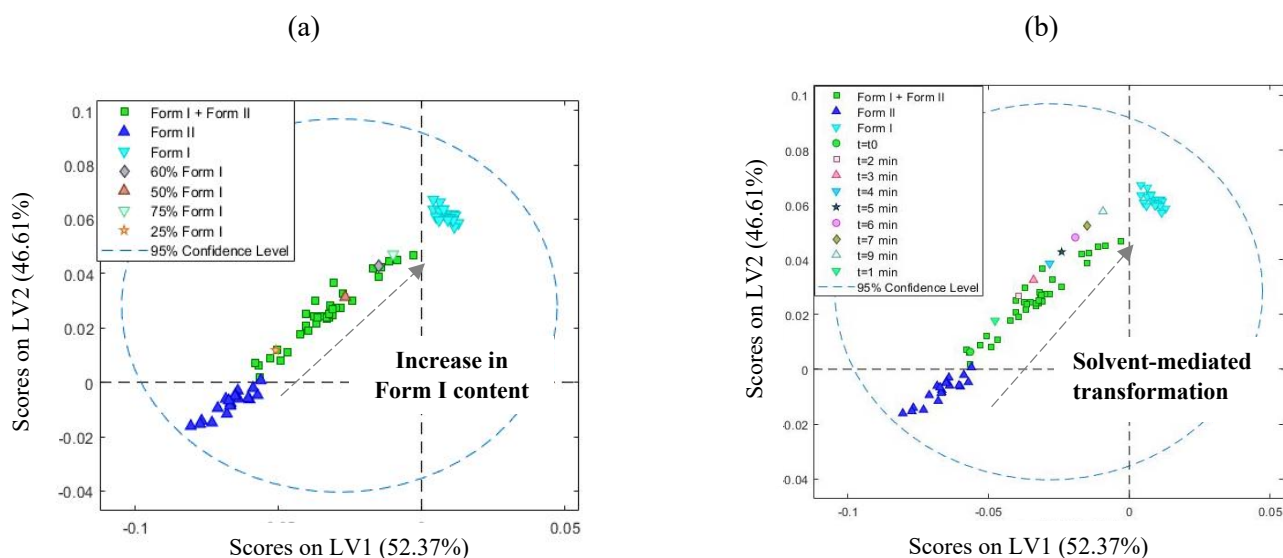


Figure 15. Experimental validation of the prediction model by monitoring (a) form I content and (b) solvent-mediated transformation

Overall, the plot points started from a polymorphic mixture with a high form II content and followed a straight line to form I cluster. As depicted by the 2D plot in Figure 15 (a), the variations described by LV1 could be attributed to the variation of form I content in suspension since the dot points shift towards form I cluster when its content increased.

The prediction model was also helpful to shed light on the polymorphic transformation of the metastable form II to the more stable form I. By applying series of measurements on a sample initially containing form II prepared by DSC in suspension, the FT-NIR spectra of the sample was recorded versus the analysis time. As shown in the 2D plot shown in Figure 15 (b) and while comparing it with the 2D plot of Figure 15 (a), the sample taken at $t=t_0$ contained more than 75% of form II. This content drastically decreased to almost 50% of form II in 2 min and to 0% in 6 min. These results are in direct correlation with the results obtained by XRD analysis which demonstrated that the solvent-mediated transformation of paracetamol is extremely fast especially in the presence of few crystals of form I.

4. Conclusion

In this work, XRD and DSC measurements were employed to characterize paracetamol forms I and II in solid state. XRD analysis suggested that form II produced by DSC scanning was stable for up to 29 days. The investigation of polymorph detection in suspension showed that accelerated solvent-mediated transformation during the DSC scan limited the detection of form I in suspension, and only its melting point was observed. In contrast, XRD analysis of polymorphs suspensions shed light on the potential identification of both polymorphs in suspension and the observation of the solvent-mediated transformation from the metastable form II to the most stable form I. However, sample preparation during XRD analysis is long and complex which may lead to erroneous results and such a method is not suitable for diluted samples. Therefore, a new methodology based on the combination of *ex situ* spectroscopic FTIR-NIR and chemometrics techniques, primarily PCA and PLS-DA, was developed as a predictive tool for qualitative classification of unknown paracetamol suspensions and detection of the solvent-mediated phase transformation. Because of its ease of use, convenience, non-destructive nature, and sensitivity, the new technique is intended to deliver a fast prediction and classification of polymorphs. Furthermore, unlike all other characterization approaches, this procedure is not heat sensitive and is not susceptible to solution evaporation. Similar classification was obtained when using PCA and PLS-DA on FT-NIR spectra data. PLS-DA approach had 99% of the system variance described with the variance related to the solvents reduced to less than 1%, implying that the model is not very sensitive to phenomena like dissolution, growth, agglomeration, breakage and so on, which is the main advantage of this method over *in situ* spectroscopic methods. This technique can be further tested on different crystallization operations to investigate potential operational factors influence, i.e. paracetamol solid content, on the model classification. Finally, unlike its stability in solid-state, paracetamol solvent-mediated transformation appears to be fast, which confirm a significant challenge in detecting its metastable pure form II during crystallization operations. Some procedures, such as seeding, and ultrasounds are needed to stabilize such form in order to produce pure form II and harvest it with the lowest stable form I content.

Author contributions

This manuscript was written with the help of the authors, who also approved the submission of the final version for publication.

Declaration of Competing Interest

The authors state that they do not have any known competing financial interests or personal ties that could appear to have influenced the work disclosed in this study.

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