New Insights into the Amorphous State and Related Solid-State Transformations

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ACADEMIC DISSERTATION

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Abstract


There is a growing interest in the amorphous state in the field of pharmaceutics. The amorphous state is a high energy state and thus, it offers one possible solution to overcome the poor water solubility problem related to many drug molecules. Amorphous material can be created during several pharmaceutical processing steps both unintentionally as well as intentionally. Therefore, there is a need for techniques that can be used to obtain more information about the amorphous state as well as to monitor process induced changes associated with it.

The aim of this thesis was to combine spectroscopic techniques with multivariate data analysis tools to gain molecular level understanding of the differences in the amorphous state caused by preparation method and the initial polymorphic form used to prepare the amorphous sample. The same techniques were used to obtain information about the solid-state transformations that could occur during the lifecycle of an amorphous drug, from preparation with different processing steps to storage and dissolution testing.

Vibrational spectroscopy techniques combined with multivariate data analysis were well suited for the analysis of amorphous or partly amorphous systems. They also enabled quantification of the amorphous form in presence of several different solid-state forms. However, the major source of error was related to sampling and, thus, systems where the sampling area is increased for example by rotating the sample, are of great benefit.

The preparative technique as well as the original polymorphic form of the drug used to prepare the amorphous sample influenced the amorphous state. Molecular level differences were noted in the amorphous state. In particular, amorphous material obtained through a solid-state transition resembled the original crystalline form more closely. Principal component analysis could be used to screen for these molecular level differences and give an indication of stability differences already on the day of preparation.

The solid-state transitions related to amorphous state that occur during processing, storage or dissolution testing, were monitored and quantified successfully with both NIR and Raman spectroscopy using multivariate data analysis tools. In situ Raman spectroscopy offered a valuable tool for a better understanding of the phenomena that occur during dissolution.
Acknowledgements

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I would like to thank all the people who have been involved in this work.

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Helsinki, June 2008

[Signature]
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List of original publications

This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (I-IV).


This thesis also contains unpublished data related to using multivariate visualization to screen for differences in the stability of amorphous drugs in reference to paper I.

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Abbreviations

API  Active pharmaceutical ingredient
CBZ  Carbamazepine
Cp   Heat capacity
Ctr  Mean centred
DSC  Differential scanning calorimetry
HPLC High performance liquid chromatography
IMC  Indomethacin
IR   Infrared
MC   Microcalorimetry
MIR  Mid infrared
MSC  Multiple scatter correction
MTDSC Modulated temperature DSC
NIR  Near infrared
PC   Principal component
PCA  Principal component analysis
PIT  Process induced transformation
PLM  Polarized light microscopy
PLS  Partial least squares
PLS-DA Partial least squares discriminant analysis
RH   Relative humidity
RMSEC/P Root mean square error of calibration / prediction
RSD  Relative standard deviation
SC   Solution calorimetry
SNV  Standard normal variate
ss-NMR Solid state nuclear magnetic resonance
Tc   Crystallization temperature
Tg   Glass transition temperature
TGA  Thermogravimetric analysis
Tk   Kauzmann temperature
Tm   Melting temperature
TPS  Terahertz pulsed spectroscopy
UV   Mean centred and scaled to unit variance
XRPD X-ray powder diffraction
1 Introduction

The activity of a drug molecule in a patient is determined by its chemical structure in solution and how it interacts with its target, i.e. receptor [1]. However, in order to get the active drug molecule into the body it is necessary to formulate it as a drug product, typically as an oral solid dosage form. Thus, the efficacy and safety of a drug product, depends largely also on the physicochemical and material properties of the drug in the solid state.

Currently most of the drug products are still formulated from crystalline active pharmaceutical ingredients (API). However, the size of the candidate molecules is constantly increasing due to techniques used to discover new candidate molecules [2]. For example, the use of high throughput screening techniques often leads to lipophilic candidate molecules. This combined with the increase in size results often into molecules with poor aqueous solubility. Intellectual property issues play also a role in size increase as most of the therapeutically useful small molecules have already been patented. Therefore, many of the new drug candidates are poorly water soluble, which decreases their bioavailability. Several formulation approaches can be taken to improve the solubility and dissolution of poorly water soluble compounds, such as formulating the API in an amorphous form [3, 4], crystal engineering [5], decreasing particles size, such as micronization or nanosizing [6] as well as using prodrugs [7], salt formation [8], cyclodextrins [9], solid dispersions [10, 11] or lipid based formulations [12].

The amorphous state has been studied in detail during the past years and there are a few drug products that have reached the market [13]. Examples include an asthma medicine Accolate® (zafirlukast) [14, 15], an antibiotic Zinnat®/Ceftin® (cefuroxime axetil) [16, 17], and an ACE-inhibitor Accupro®/Accupril® (quinapril hydrochloride) [18, 19]. Also the first inhalable insulin (Exubera®) to reach the market, although it has already been withdrawn from the market, consisted of amorphous insulin [20, 21]. However, poor stability of amorphous solids both during manufacturing and storage often hinders the development of amorphous formulations. A solid dosage form has to generally be stable for a few years, before production is considered feasible.

Traditionally in pharmaceutical industry the quality of the product has been ensured by analyzing the end product. However, the current regulatory thinking encourages towards characterization and control of the solid form of the API throughout all of the processing steps and not just in the final dosage form [22, 23]. Thus, the aim in the pharmaceutical industry is to implement process analytical technologies (PAT) to increase science based process and product understanding in development, manufacturing and quality control [24, 25]. Spectroscopic techniques have gained a lot of interest as PAT tools as they enable the real time and continuous monitoring of a process and, thus, the determination of possible process induced transformations (PITs) [26, 27].

In this thesis, spectroscopic techniques were combined with multivariate data analysis tools to gain molecular level understanding of the differences in the amorphous state caused by preparation method or the initial polymorphic form used to prepare the amorphous sample. The same techniques were used to obtain information about the solid-state transformations that could occur during the lifecycle of an amorphous API, from the preparation through different processing steps to storage and dissolution testing.
2 Review of the literature

2.1 Different solid-state forms

Solid materials can exist as either crystalline or amorphous subphases [28]. In the crystalline state the molecules are arranged in a definite 3D-structure in the crystal lattice, whereas in amorphous state no long-range order exists. However some short-range order may exist. Crystalline solids can be further divided into polymorphs and solvates (including hydrates). In some recent reviews also co-crystal have been included as a separate subset [29, 30]. Different polymorphs have the same molecular structure but the molecules are arranged differently in the crystal lattice. Solvates, hydrates, and co-crystals are similar in that their crystal lattices are made up of more than one type of molecule. In the pharmaceutical environment the crystal is composed of the API and a guest molecule; solvates contain a solvent molecule, in hydrates water is included and in co-crystals the guest molecule included in the crystal structure is a solid under ambient conditions.

The solid phases have different physicochemical as well as mechanical properties (Table 1). The differences stem from differences in molecular mobility, intermolecular distances and order of packing, which lead to differences in potential energy [29]. From a pharmaceutical point of view, the differences in the physicochemical and mechanical properties are significant as they can affect the drug product performance, bioavailability, stability, and processability [28, 31]. Therefore, there is an increasing emphasis by the authorities towards sufficient characterization of solid-state properties of drug products during all manufacturing stages [22, 23, 32-34]. In this review the emphasis is on the amorphous state.

Table 1. List of physical properties that vary between different solid-state forms. Modified from [28]

<table>
<thead>
<tr>
<th>Packing properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density, refractive index, conductivity, hygroscopicity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thermodynamic properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting/glass transition temperature, enthalpy, entropy, heat capacity, chemical potential, thermodynamic activity, solubility</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spectroscopic properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electronic (UV-Vis spectra), vibrational (MIR, NIR and Raman spectra), rotational (Far-IR, microwave spectra) and nuclear spin (NMR spectra) transitions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kinetic properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolution rate, physical and chemical stability</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surface properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface energy, interfacial tension, habit</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mechanical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardness, tensile strength, compressibility, flowability</td>
</tr>
</tbody>
</table>
2.2 Amorphous state

2.2.1 Significance of the amorphous state

Due to the lack of long-distance order, the molecular mobility and intermolecular distances are greater in the amorphous state compared to the crystalline [4, 29]. Thus, the amorphous state is a high energy state compared to the crystalline state. The interest towards the use of drugs in the amorphous state stems from these properties because they lead to a higher solubility and dissolution rate.

Important factors that affect the drug dissolution rate can be expressed by the modified Noyes-Whitney equation:

\[
dC/dt = AD(C_s - C) / h
\]  

(1)

where

- \(dC/dt\) is the dissolution rate
- \(A\) is the surface area available for dissolution
- \(D\) is the diffusion coefficient
- \(C_s\) is the saturation solubility of the drug
- \(C\) is the concentration of the drug in the dissolution medium at time \(t\)
- \(h\) is the thickness of the diffusion layer

The higher saturation solubility of the amorphous form compared to the crystalline counterpart is, thus, the driving force that leads to the higher dissolution rate from the amorphous form.

A 10 to 1600 -fold increase in the predicted solubility can be obtained from the amorphous state, whereas only one to four fold increase from metastable polymorphs is obtained compared to the stable crystalline counterpart [35]. On the other hand, due to the higher potential energy the amorphous samples are more physically unstable than the crystalline forms and they tend to crystallize to a more stable crystalline form [4, 29, 36]. It has also been shown that the increase in molecular mobility decreases chemical stability [37]. This creates a problem during manufacturing and storage. Therefore, the storage and processing conditions have to be controlled carefully [38]. An increase in either temperature or humidity increases the molecular mobility of the sample. Amorphous samples are hygroscopic and, therefore, the absorbed moisture acts as a plasticizer. An increase in molecular mobility can lead to crystallization of the sample. A well known example of the stability problems related to amorphous formulations is the case of the cholesterol-lowering drug product Lipitor® [1]. During the development phase the API (atorvastatin) was formulated as an amorphous salt. However, during Phase III studies the API crystallized. This increased the costs of product development, since new studies had to be conducted before the drug could be formulated in a crystalline form. Even though the drug remains amorphous during storage and processing, there is still the possibility that the amorphous drug crystallizes in the gastrointestinal tract when in contact with the fluids [35, 39]. If the amorphous drug crystallizes during the dissolution, the dissolution rate will gradually change...
towards that of the crystalline form, and the solubility and dissolution rate advantage is partly or completely lost.

### 2.2.2 Thermal behaviour of the amorphous material

The phase transitions that occur upon heating or cooling of an amorphous sample can be classified into first and second order transitions. First order phase transitions involve latent heat, i.e. release or absorption of energy. Such transitions occur during crystallization ($T_c$) of an amorphous sample or melting ($T_m$) of crystalline material. The release of energy due to crystallization or absorption of energy due to melting is detected in a differential scanning calorimetry (DSC) thermogram as an exothermic or endothermic peak, respectively (Fig. 1a).

In the second order phase transitions on the other hand, such as that at the glass transition temperature ($T_g$), there is no release or absorption of energy. However, a step change in heat flow occurs in the DSC thermogram.

The $T_g$ is the most important property used to describe amorphous solids. When the sample is heated, several properties, such as volume, heat capacity, viscosity, and dielectric relaxation, change at that temperature (Fig. 1b) [4]. At the $T_g$, the sample undergoes a change in heat capacity ($C_p$) due to changes in physical properties. This can be seen in the DSC thermogram as the step change in the heat flow (Fig. 1a). At the $T_g$ the molecular mobility increase and the sample changes from a glass to a supercooled liquid, i.e. from the glassy to the rubbery state. However, there are studies showing that significant molecular mobility exists also below the $T_g$ allowing the amorphous sample to crystallize [40, 41]. Samples may have to be cooled at least 50 ºC below the $T_g$ for the molecular motions to be negligible during the product life-time [40]. This region corresponds to the Kauzmann temperature ($T_k$), where the configurational entropy of the amorphous sample approaches zero.

![Figure 1.](image.png)

**Figure 1.** a) A schematic presentation of a typical DSC trace obtained, when heating amorphous materials. A first order phase transition can be seen at the crystallization temperature ($T_c$) and the melting temperature ($T_m$) and a second order phase transition at the glass transition temperature ($T_g$). b) Variation of thermodynamic properties with temperature. Modified from [3, 38].
2.2.3 Differences in the amorphous state

The existence of polymorphism in the crystalline state has raised the question of whether different solid-state forms could also exist in the amorphous state. This proposed phenomenon is called polyamorphism. In order for polyamorphism to exist, a first order phase transition has to separate two amorphous phases [29, 42-44]. Polyamorphism was first found to exist in water [45] and after that in some inorganic materials [46, 47]. So far there seems to be little evidence that small organic materials exist as polyamorphs [29, 42].

In the field of pharmacy, there are studies where preparation technique, process parameters used or the solid form of the starting material can generate differences in the amorphous state [41, 48-51]. These different relaxational stages can differ for example in stability, as was the case of indomethacin [49, 51], or hygroscopicity, as was the case with two different spray dried amorphous cefditoren pivoxil samples [48]. However, these amorphous states are not true polyamorphic forms, since no clear first order transition occurs between the forms.

Theoretical models of amorphous materials have been proposed in the literature based on molecular packing, intermolecular bonding network, or amorphous material having local domains of different densities [52]. These regions with different packing densities differ in potential energy [29, 52]. Thus, these differences in local structure can have a significant effect on the physical and chemical properties of an amorphous material. The other attribute that is typical for amorphous materials is structural relaxation, i.e. aging. The amorphous material changes gradually towards a more thermodynamically favourable state. The heterogeneity in the amorphous state combined with the structural relaxation could explain some of the reported differences in the differently prepared amorphous samples of the same API.

2.3 Preparation of amorphous forms

Amorphous materials can be prepared through different pathways, through a solution, a liquid, a vapour, and a solid state [3, 38] (Fig. 2). Amorphous solid is obtained, if the transition from the liquid state (molten or solution) through the melting point to a solid is fast enough for the molecules to instantly “freeze” in a random order. Crystallization requires time to overcome the energy barrier between the crystal-liquid interface for the molecules to rearrange themselves before nuclei formation and crystal growth can begin [29]. Thus, the most typical way to prepare amorphous material is through a liquid transition by quench cooling a melt. This method is also called vitrification.
Amorphous materials are prepared through a solution state by spray or freeze drying as well as by salting-out or antisolvent addition methods, where the formation of amorphous material is based on rapid precipitation [3, 38]. In spray drying, solution is sprayed into hot air and the solvent is evaporated so fast that the molecules remain unorganized. In freeze drying the solution is rapidly frozen and the solvent is sublimed in low temperature and pressure. As the solvent is removed the solute molecules remain in the unordered structure they were frozen in. The same phenomenon is also involved in preparation of amorphous material through vapour state, if the condensation of the material from vapour state to solid state occurs fast enough, the molecules remain unorganized.

The fourth pathway to prepare amorphous material is through mechanical activation. The process varies from the two previous methods. In solid-state transition, the amorphous state is formed through the disruption of the crystal lattice for example during milling and dehydration [53]. The amorphous solid is formed, when the amount of crystal defects accumulates gradually above a critical level. Therefore, the amorphous form is more likely to possess some “memory” of the long-range order of the original polymorph and to have some seeds or nuclei of the original polymorph left [50, 54, 55]. Cryogrinding of piroxicam forms I and II led to X-ray amorphous materials that had similar short-range orders but differed in the residual long-range order [54]. This “memory” of the original polymorph can have an influence on the stability as well as dissolution behaviour of the amorphous sample. This was observed, when amorphous indomethacin (IMC) was prepared by cryogrinding from both α- and γ-IMC [55, 56].

2.4 Process induced changes in amorphicity or crystallinity

In addition to the methods used to deliberately prepare amorphous materials, amorphous material is often created unintentionally during several pharmaceutical processes, such as milling, compression and drying (Fig. 3) [38, 57, 58]. However, the change in crystallinity can
also happen in the other direction. Amorphous material can crystallize to either anhydrate or hydrate forms due to process induced transformations (PITs). Crystallization of amorphous material can happen during processes such as granulation or coating, where the sample could be heated above the $T_g$ or water is present to plasticize the amorphous sample.

The underlying mechanism behind the crystallization or amorphization is either a solid-state, solution or solution-mediated transformation [38]. In the solid-state mechanism the transformation occurs in the solid state without going through any intermediate liquid or vapour phases. Such a mechanism could occur during dehydration or compression. Amorphous CBZ can be produced through a solid-state transition mechanism by dehydrating CBZ dihydrate [59, 60]. In solution and solution-mediated transformations a solvent is present [38]. Transformation via solution occurs during drying and is caused by subsequent removal of the solvent. The final solid can be either crystalline, amorphous or a mixture of several forms. This type of transformation is seen for example during drying of theophylline [61] and carbamazepine [62] granules, freeze-drying of mannitol [63, 64] or spray drying of lactose [65, 66]. A solution-mediated transformation, on the other hand, is caused by the solubility difference between the solid-state forms. It can, thus, happen only from the metastable amorphous phase towards the stable crystalline phase. This type of transformation is possible during dissolution and solubility experiments [35, 67, 68].

Changes in the solid-state forms during manufacturing can change the performance of the product, since the transformations can affect both the physical and chemical stability of the product as well as the bioavailability. Abbot laboratories found that their prototype of the Abbot-232 drug product was chemically unstable [69]. The instability was caused by a PIT through a solution mechanism from anhydrate to amorphous form during wet granulation. In another case, the dissolution rate of theophylline anhydrate tablets was affected by a PIT during wet granulation, which lead to a decrease in crystallinity of theophylline anhydrate granules [61]. The amorphous part converted faster to the monohydrate during dissolution, and therefore a decrease in the dissolution rates was observed. Thus, there is a need for techniques that can be used to monitor these PITs and to determine the changes in the crystallinity.
Figure 3. A simplified presentation of a lifecycle of an oral solid dosage form. The typical steps involved in the development of an oral solid dosage forms as well as the factors that can induce solid-state transformations: a) crystallization of amorphous material and b) amorphization of crystalline material. The manufacturing process might involve all or only some of the unit operations presented as well as multiple solid-state transformations.
2.5 Determination of amorphous content

The determination of amorphous or crystalline content is based on the various differences in physical properties noted between the solid-state forms presented in Table 1. Thus, the methods can be divided into techniques that probe the sample on the molecular level (properties associated with individual molecules), on the particulate level (properties related to individual solid particles) and on the bulk level (properties linked to a mass of particles) (Table 2) [98]. There are several review articles and studies presented in the literature, where different techniques are compared as analysis methods [99-108].

Table 2. Comparison of different methods used to quantify amorphous content

<table>
<thead>
<tr>
<th>Method</th>
<th>Destructive</th>
<th>Process analysis*</th>
<th>Time scale</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectroscopic techniques</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIR</td>
<td>No</td>
<td>Yes</td>
<td>&gt; 30 s</td>
<td>1-2% [107]</td>
</tr>
<tr>
<td>NIR</td>
<td>No</td>
<td>Yes</td>
<td>&gt; 10 s</td>
<td>1% [100, 109, 110]</td>
</tr>
<tr>
<td>Raman</td>
<td>No</td>
<td>Yes</td>
<td>&gt; 10 s</td>
<td>1% [111, 112]</td>
</tr>
<tr>
<td>TPS(^a)</td>
<td>No</td>
<td>Yes</td>
<td>~1 min</td>
<td>1-5%[113]</td>
</tr>
<tr>
<td>ss-NMR</td>
<td>No</td>
<td>No</td>
<td>0.5-10 h</td>
<td>0.5% [114]</td>
</tr>
<tr>
<td><strong>Powder level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRPD</td>
<td>No</td>
<td>No</td>
<td>10-60 min</td>
<td>10% [115]</td>
</tr>
<tr>
<td><strong>Thermal methods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSC</td>
<td>Yes</td>
<td>No</td>
<td>10-30 min</td>
<td>10% [115]</td>
</tr>
<tr>
<td>MTDSC</td>
<td>Yes</td>
<td>No</td>
<td>1-2 h</td>
<td>&lt;1% [116]</td>
</tr>
<tr>
<td>High speed DSC</td>
<td>Yes</td>
<td>No</td>
<td>1 min - 5 h(^c)</td>
<td>0.2-1% [117-119]</td>
</tr>
<tr>
<td>SC</td>
<td>Yes</td>
<td>No</td>
<td>0.5-1 min</td>
<td>1% [120, 121]</td>
</tr>
<tr>
<td>MC</td>
<td>Yes</td>
<td>No</td>
<td>0.5-4 h</td>
<td>0.5% [114, 122-124]</td>
</tr>
<tr>
<td><strong>Bulk level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravimetry</td>
<td>Yes</td>
<td>No</td>
<td>24-48 h</td>
<td>0.2-1% [100, 115, 125]</td>
</tr>
<tr>
<td>Density</td>
<td>No</td>
<td>No</td>
<td>10-30 min</td>
<td>10% [115, 126]</td>
</tr>
</tbody>
</table>

*Possibility for in line or on line measurement; \(^a\)Mainly looking at intermolecular interactions; \(^c\)Measurement time depends on the annealing time

The method of choice depends for example on the amount of sample available, amount of amorphous or crystalline content necessary to determine, whether the determination has to be done during processing or whether the technique has to be surface sensitive. Even though detection limits as low as 1% have been determined for vibrational spectroscopy techniques, these limits might be difficult to obtain in formulations where several substances are present or when the analysis is done in a process environment. Most techniques do not differentiate between surface and bulk amorphicity. However, PIPs often create only low levels of amorphous material and the amorphous regions are often present only on the powder surface [108]. When there is a need for surface sensitive methods or detection of very low amorphous content, methods based on vapour sorption, such as dynamic vapour sorption (DVS), or isothermal microcalorimetry, are preferred [104]. In isothermal microcalorimetry, the heat change caused by crystallization due to sorption of water in a specific relative humidity is measured. Amorphous levels as low as 0.2% could be measured with these techniques. Also high speed DSC (Hyper DSC) has proven successful in detecting very small amounts of amorphous material [117]. Even though the methods based on vapour sorption or thermal analysis (high speed DSC or isothermal MC) have low detection limits,
they cannot be used during process analysis. PAT tools require techniques that are fast, non-destructive, and preferably non-invasive to enable in-line or on-line monitoring.

Vibrational spectroscopy, such as mid infrared (MIR), near infrared (NIR), and Raman spectroscopy can be used to obtain molecular level information of the sample [127, 128]. They are sensitive to changes in the intramolecular interactions and can be used to obtain complementary information about the molecular interactions in the solid state. The main difference between these techniques is that MIR and NIR spectroscopy are absorption techniques, whereas the Raman spectroscopy is a scattering technique. For quantitative purposes, MIR and NIR spectroscopy follow the Lambert-Beer law

\[
\frac{\log I_0}{I} = A = abc
\]

where
- \( I_0 \) is the intensity of the intensity of the incident light
- \( I \) is the intensity after passing through the sample
- \( A \) is absorbance
- \( a \) is the absorption coefficient or the molar absorptivity
- \( b \) is the path length
- \( c \) is the concentration of the absorbing material.

Whereas in Raman spectroscopy, as long as the sample is not significantly absorbing the incident light, the intensity of the peak due to Raman scattering (\( I_{\text{Raman}} \)) is directly proportional to concentration (\( c \))

\[
I_{\text{Raman}} \sim c
\]

The MIR and NIR spectrometers have a polychromatic light source. The light is directed to the sample and the sample absorbs specific frequencies corresponding to its molecular vibrational transitions (Fig. 4) [128]. In Raman spectroscopy, on the other hand, the sample is irradiated with monochromatic laser light. This radiation excites the molecules to a higher virtual potential energy state. Most of the molecules return from this excited energy state to the ground state and the light is elastically scattered to different directions. This is called Rayleigh scattering. However, a small number of molecules may return to the first excited vibrational state instead of the ground state and the light is inelastically scattered. This phenomenon is referred to as Stokes scattering and the frequency difference corresponds to the vibrational energy of the MIR absorption. In anti-Stokes scattering the molecule starts from the first excited vibrational state and returns to the ground state by inelastic scattering. However, in Raman spectroscopy the Stokes scattering is more commonly measured.

For a vibration to be active in MIR spectroscopy, a change in the dipole moment is needed [127]. MIR spectroscopy is sensitive to changes in both harmonic and anharmonic vibrations. Polar groups, such as O-H, N-H, S-H and C=O bonds, are MIR active. NIR spectroscopy detects the anharmonic overtones and combinations of these vibrations. Raman spectroscopy, on the other hand, is a complementary technique to MIR and NIR spectroscopy. For a molecular vibration to be Raman active, a change in the polarizability of the molecule is required. Thus, symmetric vibrations of nonpolar groups are Raman active. In addition to
intramolecular bonding, changes in molecular conformation or intermolecular bonding associated with solid-state differences can affect vibrational modes.

![Virtual energy state diagram for MIR, NIR and Raman spectroscopy.](image)

**Figure 4.** Vibrational energy state diagram for MIR, NIR and Raman spectroscopy. \(v=0\) is ground state, \(v=1\) is first excited vibrational energy state, \(v=2\) is second excited vibrational energy state etc. Modified from [129].

### 2.6 Process analysis using spectroscopic techniques

The use of spectroscopic techniques in a process environment, NIR and Raman spectroscopy in particular, have been studied extensively, since they are fast, non-destructive, and no sample preparation is needed. Spectroscopic techniques and various processes can also be interfaced with fibre optic probes. NIR spectroscopy has been used as a PAT tool for various applications, such as to determine solid-state transformations during crystallization [130], granulation [131, 132], drying [62, 93], pelletization [133] and tableting [132] as well as blending of powder mixtures [134, 135] and assay of the end product [136, 137]. Raman spectroscopy has been used to monitor the process steps during manufacturing of gels and emulsions [138], and to analyze PATs during drying [62, 93, 139], transformations during dissolution testing [140], conversion kinetics of APIs in slurries [141-143], and homogenization [144] and assay of APIs in suspensions [145, 146]. The wide use of Raman spectroscopy in the aqueous environment demonstrates an important benefit of Raman spectroscopy compared to NIR spectroscopy. Water does not saturate the Raman signal, since OH vibrations are not Raman active.

Often hyphenated techniques, such as a combination of the complementary spectroscopic techniques [147] or a combination of spectroscopic techniques and other techniques, such as TGA-IR [148], DVS-NIR [149, 150] and DVS-Raman [151] spectroscopy provides additional information about the studied phenomena. Most of the studies have been conducted with crystalline materials and significantly fewer studies can be found where spectroscopic techniques have been used as PAT tools for analysis of amorphous materials. Analysis of the amorphous form using spectroscopic techniques is more challenging as the spectral features are less distinct due to the lack of molecular order, and often no clear peak shifts occur and only merging of the bands is observed.
2.7 Data analysis using multivariate methods

Process analysis creates large amounts of data. Therefore, powerful tools are needed to extract relevant information from these data sets. Multivariate data analysis can be used for this purpose either to get an overview of a dataset, to classify observations or to correlate two data sets together [152, 153]. The advantage, which is specifically related to interpretation of spectroscopic data, is that a full spectrum and not just a few peaks can be used for the analysis. This makes multivariate methods an attractive option especially for the analysis of amorphous systems.

2.7.1 Principal component analysis (PCA)

Principal component analysis (PCA) of large data sets provides a method to get an overview of the data and to detect trends, groupings and outliers [154]. PCA is a multivariate projection method, which is used to extract and display systematic variation in a data matrix $X$ (e.g. spectra) [152, 153]. To do this, $X$ is decomposed as

$$X = T^*P^* + E$$

$$= t_1p_{1}^* + t_2p_{2}^* + \ldots + t_Ap_{A}^* + E$$

(3)

where
- $X$ is a $(n \times m)$ data matrix
- $T$ is object scores, a $(n \times A)$ data matrix
- $P$ is variable loadings, a $(m \times A)$ data matrix
- $E$ is error and noise, a $(n \times m)$ data matrix
- $n$ is number of samples
- $m$ is number of variables
- $A$ is number of principle components (PC) extracted

Thus, a large data matrix $X$ is reduced to two smaller matrices $T$ (scores) and $P$ (loadings) that contain all the relevant information from the original data matrix $X$ and are easier to understand. The data is organized such that the first PC captures the most variation and the second PC the second most and so on. Hence, each PC consists of two vectors, a score vector $t$ and loading vector $p$. Scores are new variables that summarize the information from the original variables, and loadings describe how the variable influences the PC. The scores and the loadings are orthogonal to each other. The noise is largely left to the matrix $E$.

PCA of spectroscopic data has been used in the field of pharmaceutics for various applications, for example to monitor different unit operations, such as API synthesis [155], crystallization [156], blending [157], granulation [158-161], drying [162], coating [158] and freeze drying [63], as well as for polymorph screening [80, 163], analysis of the amorphous samples (glassy or rubbery state) [164], or as a quality control tool to identify raw materials [165-169], different batches or faulty end products [170-172] as well as to detect counterfeit medicines [173, 174].
2.7.2 Partial least squares (PLS) regression analysis

Partial least squares (PLS) regression analysis is an extension of PCA. In PLS regression analysis the $X$ matrix (variables, e.g. spectra) is correlated with a $Y$ matrix (responses, e.g. concentrations) [152, 154, 175]. Thus, quantitative information can be obtained. PCA is performed on both matrices and then the best possible correlation between the matrices is determined using the least squares technique. Thus, the PLS regression analysis results in a model describing how the matrices $X$ and $Y$ relate to each other.

Typically PLS regression analysis of spectroscopic data has been used for quantification of either the amount of API in a product or different solid-state forms of the API both off line [169, 172, 176-188] or during processing [93, 189-191]. PLS regression analysis has been combined with both NIR [105, 191] and Raman [105, 192] spectroscopic techniques for determination of changes in amorphous content. However, the possible applications are numerous. NIR spectroscopy has also been combined with PLS regression analysis to analyze moisture content of raw materials and products [193-195] and to follow mixing kinetics [181]. Since in addition to the chemical information, physical information is also present in the NIR and Raman spectra, PLS regression analysis has been used to determine relationships between Raman spectra and coating thickness [196] as well as NIR spectra and tablet hardness [168, 197, 198], dissolution [158, 177, 199] and disintegration times [200]. NIR spectroscopy with PLS regression analysis has also been used to determine suitable coating times [158] and particle size [201].

2.7.3 Partial least squares discriminant analysis (PLS-DA)

Partial least square discriminant analysis (PLS-DA) is based on the PLS regression analysis. PLS-DA is used to qualitatively discriminate between different classes of observations based on their $X$ variables (e.g. spectra). It can have better discriminating power than PCA, since a $Y$-matrix (e.g. different solid-state forms) describing the class memberships is included. So far it has been used less than the PCA or PLS regression analysis in the field of pharmaceutics. Some studies exist where PLS-DA has been used for process monitoring [62] as well as quality control of different raw materials [167] and batches of end products [158, 177].

2.7.4 Model transfer

A common challenge related to the use of multivariate calibration models is model transfer [202]. The problem arises from two possible sources, either from the need to use the same calibration model on different instruments or differences between the calibration samples and the actual samples analyzed. Although the spectra measured on different instruments have the same profile, differences for example in peak intensities or slight peak shifts occur due to for example differences in sampling set ups, path lengths of optical probes and wavelength registration intervals [203]. Also the aging of probes, light sources or detectors can cause instability of the signal over time or nonlinearities [204, 205].
The use of calibration models in process analysis is challenging since the spectra used to build the calibration model often differ from the process data. Often it is not possible to measure the calibration samples in similar environmental conditions, such as temperature, humidity and/or sample movement, as the process is carried out. Environmental factors such as temperature or humidity can cause differences in the spectra. Sometimes unexpected compounds also appear during processing. Typically the spectra recorded in or on line are also noisier than the calibration spectra. Several approaches can be taken to account for these differences. For example, if possible, the calibration data can be created in similar condition as the process data [177] or the model can be improved by adding spectra of the process samples in the model [172, 178]. Another way of accounting for the variation is to include samples in the model that have been measured in different conditions such as in higher temperature or humidity [93]. If the model is already in use and cannot be updated with new samples, it is possible to apply standardization methods to account for new variation [202]. Spectral standardization aims to make the new spectra measured as similar as possible to the old ones [203]. The calibration model can for example be improved by careful selection of spectral range and pre-processing methods [189]. Spectral range selection can also be used if unexpected compounds that have not been taken into account when building the model appear during the analysis. Leaving out the spectral regions where the peaks of the unexpected compound appear may improve the model.
3 Aims of the study

The aim of this thesis was to use spectroscopic techniques combined with multivariate methods to study differences in the amorphous state as well as the solid-state transformations that might occur during the lifecycle of any amorphous pharmaceutical compound. The specific aims of this study were:

- to study molecular level differences in the amorphous state caused by the preparative technique and the initial polymorph using different spectroscopic techniques in combination with PCA

- to use the molecular level information to predict for stability differences in the amorphous samples

- to build quantification models based on NIR and Raman spectroscopy for determination of amorphous content in presence of one or several crystalline forms using PLS regression analysis

- to monitor and quantify solid-state transformation induced changes in amorphous content using spectroscopic techniques and multivariate analysis during milling, storage and dissolution

- to determine the effect of common sources of error for the PLS quantification models based on NIR and Raman spectroscopy.
4 Experimental

4.1 Materials

Two APIs, \(\gamma\)-indomethacin (IMC) (Hawkins, Inc., Minneapolis, MN, USA) (I, II, IV) and carbamazepine (CBZ, form III) (Hawkins, Inc., Minneapolis, MN, USA) (IV), as well as two carbohydrates, \(\alpha\)-lactose monohydrate (Pharmatose 200M, DMV International, Veghel, The Netherlands) (III) and trehalose dihydrate (SigmaAldrich Chemie GmbH, Steinheim, Germany) (III) were used as received as model substances in this study. \(\alpha\)-IMC (I, II, IV) was prepared by recrystallization from absolute ethanol. CBZ form I (IV) was prepared by heating the CBZ form III at 170 °C at atmospheric pressure for 2 h. CBZ dihydrate (IV) was recrystallized by slow cooling of 70 °C saturated aqueous solution to room temperature.

4.2 Preparation of amorphous samples

4.2.1 Transformation through solution state (I, III)

Two types of spray dryers were used for this study, a Büchi Mini Spray Dryer B-191 (III) and B-290 (I) (Büchi Labortechnik AG, Flawil, Switzerland), with the latter enabling the use of solvents during processing. Amorphous IMC was prepared by spray drying a 1% (m/V) methanol solution from both \(\alpha\)- and \(\gamma\)-IMC under nitrogen atmosphere (I). Spray dried indomethacin particles were stored at 4 °C at a close to 0% relative humidity (RH) (over \(\text{P}_2\text{O}_5\)). Amorphous lactose and trehalose were prepared by spray 15% (w/w) aqueous solutions prepared from \(\alpha\)-lactose monohydrate and trehalose dihydrate, respectively (III). The lactose and trehalose particles were dried at 40 °C for 24 hours to remove the excess moisture and stored in vacuum desiccators at about 0% RH (silica gel) at room temperature.

4.2.2 Transformation through liquid state (I, II, IV)

Amorphous IMC and CBZ samples were prepared by melting the drugs above their melting temperatures of 165 °C and 193 °C, respectively and then cooling the melt at room temperature at close to 0% RH (over \(\text{P}_2\text{O}_5\)) (I, II, IV) or by quench cooling the melt using liquid nitrogen (I).

4.2.3 Transformation through solid state (I)

Amorphous IMC was prepared from both \(\alpha\)- and \(\gamma\)- forms. IMC was milled at 4 °C in a ball mill (Retsch MM301, Germany) for six hours at a speed of 30 Hz. The ball to mass ratio was 14:1.
4.3 Stability prediction based on PCA

The amorphous samples that were prepared by different techniques were stored at 4 °C at close to 0% RH (over P₂O₅) and measured on the day of preparation and after one month of storages with XRPD, MIR, NIR and Raman spectroscopy using the methods described in paper I.

4.4 Monitoring solid-state transformations during storage, processing and dissolution testing

4.4.1 Amorphization of crystalline material by milling (III)

Mechanical activation induced changes in crystallinity were studied by milling crystalline α-lactose monohydrate in a ball mill (Pulverisette 6, Fritsch GmbH, Idar-Oberstein, Germany) for 3 hours. The rotation speed was 400 rpm and the ball to mass ratio was 10:1. The milling was suspended after 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 150, and 180 minutes to determine both Raman and NIR spectra of the ground samples directly from the milling bowl. For comparison, XRPD patterns were determined at the same time points.

4.4.2 Crystallization of amorphous material during storage (III)

Amorphous lactose and trehalose were stored in a humidity chamber at 85% RH (KCl) at ambient temperature. The crystallization of amorphous lactose and trehalose was followed in line with NIR and Raman spectroscopy. In the NIR spectroscopy experiments, the sample was stored in a glass vial and the NIR probe was immersed into the sample. The Raman signal was measured through a quartz window. Data was gathered every 15 minutes.

4.4.3 Solid-state transformations during dissolution (IV)

Dissolution tests were conducted in a channel flow intrinsic dissolution test apparatus with a quartz sight window for the Raman probe [140, 206]. The solid state of amorphous IMC and CBZ sample surfaces were analyzed in situ during dissolution testing through a quartz sight window using Raman spectroscopy. The amount of dissolved drug in the dissolution media was determined using UV-Vis spectrophotometer from a flow-through cuvette at wavelengths of 288 nm (CBZ) and 318 nm (IMC). The dissolution media was phosphate buffer solution of pH 7.2 at 25 °C and the flow rate was 8 ml/min for both IMC and CBZ. For IMC also water-ethanol (50% (w/w)) solution was used.
4.5 Analytical methods

4.5.1 Near infrared spectroscopy (NIR) (I-III)

In paper I, the NIR spectroscopy equipment consisted of a Bruker Equinox 55 FRA 106/S optical bench (Bruker Optik, Ettlingen, Germany) with a Bruker IFS-55 interferometer. The NIR spectra were gathered using an EasiDiff accessory (Pike Technologies, Madison, WI, USA), a D301 deuterated, triglyceride sulphate (DTGS) detector and a globar source (model number W548/3). In papers II and III, the process-type NIR spectrometer had an InGaAs diode array detector and a tungsten light source (Control Development Inc., South Bend, IN, USA). NIR spectra were recorded from both stationary (I-III) and rotating (II, III) samples as well as from a milling bowl (III) and a humidity chamber (III).

4.5.2 Raman spectroscopy (I-IV)

In paper I, a FT-Raman instrument was used that consisted of a Bruker FRA 106/S FT-Raman accessory (Bruker Optik, Ettlingen, Germany) with a Coherent Compass 1064-500N laser (Coherent Inc, Santa Clara, USA) attached to a Bruker IFS 55 FT-IR interferometer, and a D425 InGaAs detector. The laser wavelength was 1064 nm (Nd:YAG laser) and the laser power 120 mW. In papers II, III and IV, the Raman spectra were collected employing a process-type Raman spectrometer (Control Development Inc., South Bend, IN, USA) equipped with a thermoelectrically cooled CCD detector and a fiber optic probe (RamanProbe, InPhotonics, Norwood, MA, USA). The laser power was 500 mW with a wavelength of 785 nm (Starbright 785S, Torsana Laser Technologies, Skodsborg, Denmark). Raman spectra of samples were recorded from a stationary (I, II) as well as rotating sample holder (II, III), a milling bowl (III), a humidity chamber (III) and during dissolution testing through a quartz sight window (IV).

4.5.3 Mid infrared spectroscopy (MIR) (I)

The MIR spectroscopy equipment consisted of a Bruker Equinox 55 FRA 106/S optical bench (Bruker Optik, Ettlingen, Germany) with a Bruker IFS-55 interferometer (I). The MIR spectra were recorded using diffuse reflectance with an EasiDiff accessory (Pike Technologies, Madison, WI, USA), a D301 deuterated, triglyceride sulfate (DTGS) detector and a globar source (model number W548/3). KBr was used as a background and to dilute the samples.

4.5.4 X-ray powder diffractometry (XRPD) (I-IV)

In paper I, a PANalytical X’Pert PRO MPD system (PW3040/60, Philips, The Netherlands) using CuKα radiation with $\lambda = 1.54$ Å (40 kV and 30 mA) and a divergence slit of 1° was used. In papers II-IV, a θ-θ diffractometer (Bruker AXS D8 Advance, Bruker AXS GmbH, Karlsruhe, Germany) was used. The measurements were performed in symmetrical reflection mode with
CuK$_\alpha$ radiation (1.54 Å) at 40 mA and 40 kV using Göbel mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. Both stationary (I, II, IV) and rotating (III) sample holders were utilized.

The experimental diffraction patterns were compared to the theoretical ones, which were based on the data obtained from the Cambridge Structural Database (CSD). The data from the ref codes INDMET02, INDMET03, CBMZPN11, CBMZPN01, FEFNOT02, LACTOS10, BLACT002 and TREHAL10 was used to generate the theoretical diffraction patterns of α- and γ-IMC [207, 208] (I, II, IV), CBZ form I, form III and dihydrate [209-211] (IV) as well as α-lactose monohydrate [212], β-lactose anhydrate [213] and trehalose dihydrate [214] (III), respectively.

4.5.5 UV-Vis spectroscopy (IV)

The amount of dissolved drug in the dissolution media was determined using UV-Vis spectrophotometer (Ultrospec III, Pharmacia LKB Biotechnology, Sweden) from a flow-through cuvette at wavelengths of 288 nm (CBZ) and 318 nm (IMC). The absorbance values were collected with an interval of 10 s.

4.5.6 Thermal analysis (I, II)

Two types of differential scanning calorimeters (DSC) were used. In paper I, DSC and modulated temperature DSC (MTDSC) analyses were carried out using a TA Q100 (TA instruments, New Castle, USA). The heating rate in the DSC analysis was 10 °C/min from 25 °C to 180 °C. The MTDSC analysis was performed at 2 °C/min with amplitude of 0.212 °C and period of 40 s from 30 °C to 60 °C. Both DSC and MTDSC were performed using crimped aluminum pans and the calibration was performed with indium. In paper II, the DCS analysis was performed using a Mettler DSC823e (Mettler-Toledo AG, Greifensee, Switzerland) with a heating rate of 10 °C/min from 25 °C to 180 °C. The calibration was performed with sapphire and indium. In paper I, thermogravimetric analysis was performed using a TGA100 instrument (TA instruments, New Castle, USA) with a nitrogen purge.

4.5.7 High performance liquid chromatography (HPLC) (I)

Samples were analyzed using an HPLC system consisting of a Shimadzu LC-10ATvp pump and Shimadzu SPD-10Avp UV-Vis detector (Shimadzu corp., Kyoto, Japan) set at a wavelength of 320 nm. Chromatography was performed at ambient temperature using a Phenosphere-NEXT 5uC18 column (250 mm length, 4.6 mm diameter, 5 µm particle size; Phenomenex, Torrence, CA, USA). The mobile phase consisting of 75% methanol and 25% of 0.2% phosphoric acid was used at a flow rate of 1.5 ml/min. The injection volume was 20 µl. The retention time of IMC was 7.4 min.
4.5.8 Polarizing light microscopy (I)

Polarizing light microscopy (Nikon Optiphot microscope, Japan) was used to determine whether the samples were birefringent with different magnifications (10x-50x) (I).

4.6 Qualitative and quantitative analysis

4.6.1 Multivariate analysis (I-IV)

In paper I, PCA was used to evaluate molecular level differences between the amorphous forms of IMC prepared by different techniques. In papers II and III, PLS regression analysis was used to build quantification models for determination of amorphous content in three or two component systems, respectively. PLS model was then used in papers III and IV to quantify the amount of different solid-state forms during processing. The calibration samples, i.e. binary (III), tertiary (II) and quaternary (IV) mixtures of the different solid state forms, were prepared by geometrically mixing the components and measured using spectroscopic techniques. The data was randomly divided into two sets in order to create (2/3 of samples) and to test (1/3 of samples) the model. PLS-DA analysis was used in paper IV to monitor the solid-state transitions occurring during dissolution.

All multivariate analyses were conducted using Simca-P software (v.10.5, Umetrics ab, Umeå, Sweden). Prior to multivariate analysis, all spectroscopic data were pre-processed. The data were corrected for baseline and intensity differences using standard normal variate (SNV) transformation (I-IV) [215], multiplicative scatter correction (MSC) (II, III) [216], and 1st and 2nd derivative calculation (II, III) [217]. In addition to pre-treatment algorithms, scaling methods such as mean centering (Ctr) alone and with unit variance scaling (UV) were applied to the data to improve the model quality.

4.6.2 Quantification based on XRPD data (III)

A whole pattern fitting approach was used to estimate the crystallinities of the carbohydrate samples after processing. The intensities of the crystalline and amorphous component were fitted to the experimental XRPD intensity curve. The intensity curve from totally amorphous samples (100%) was used as the amorphous model intensity curve. The crystalline model intensity curves consisted only of the diffraction reflections of the samples. The crystallinities were calculated as the ratio of the integrals of the intensities of the crystalline component and the studied sample.
5 Results and discussion

5.1 Spectroscopic techniques combined with multivariate analysis as a tool for analyzing amorphous systems

Traditionally the analysis of spectroscopic data has been based on only a few characteristic peaks. The advantage in using multivariate techniques for spectral analysis is that intensity information over wide spectral region can be used for the analysis. This makes it an ideal technique for analyzing amorphous or partly amorphous systems. The difficulty with analyzing amorphous material is that due to the disordered structure of the amorphous material and, thus, lack of molecular arrangement, the differences on the spectra between crystalline and amorphous samples are often vague. Often only some decrease in peak intensity and band broadening and merging can be noted (Fig. 5). In all of the papers I-IV, however, spectroscopic techniques were applied successfully for qualitative and quantitative analysis.

Figure 5.  a) NIR and b) Raman spectra of lactose and trehalose. Modified from paper III.

5.1.1 Spectral pre-treatment

Both physical and chemical information are present in the spectra. Therefore, prior to multivariate analysis, the spectra have to be corrected due to e.g. baseline differences caused by sample packing, density and particle size. In papers II and III, where tertiary and binary quantification models were built, several different spectral ranges as well as pre-treatment algorithms such as SNV, MSC, as well as 1st and 2nd derivative were applied to the spectra to evaluate their influence on the quantification models. Also the influence of different scaling methods on the variables was evaluated, such as mean centring with and without scaling to
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unit variance (UV and Ctr, respectively). The results emphasize the importance of model development. All of the models could be improved significantly with a choice of appropriate spectral region and pre-treatment algorithms. For example, in paper III, when building a quantification model to determine the crystallinity of lactose samples using Raman spectroscopy, the variation in the baseline could be minimized by SNV correction and Ctr scaling as can be seen from the spectra and the weights plot (Fig. 6). Thus, the quantification model for determining the amorphicity of lactose mixtures could be improved significantly. In these studies, SNV correction proved generally to be the best options of the methods applied to correct spectroscopic data. The SNV transformation is more suitable than the MSC for process analytical purposes, where spectra are gathered continuously. This is because MSC needs to refer to other spectra, whereas in SNV the spectra are treated individually [215, 216]. Use of 1st and 2nd derivative as pre-treatment can be problematic in a process environment as they tend to emphasize the background noise present in the process data.

In Raman spectroscopy sample fluorescence might create problems, particularly when analyzing coloured samples under visible excitation. The resulting signal is often stronger than the Raman scattering and, hence, masks the Raman bands. In paper II, the effect of sample fluorescence caused by yellow amorphous IMC could be minimized by photochemically bleaching the sample. Performing a rubber band correction on the spectra to minimize the baseline effects caused by fluorescence before any other pre-treatment also improved the model.
Figure 6. PLS regression model for determining amorphous lactose concentrations using Raman spectroscopy without (I) and with (II) spectral pre-treatment (SNV correction and Ctr scaling): a) Raman spectra of the different concentrations, b) weights plot and c) theoretical vs. predicted concentrations. The spectra and weights are offset for clarity.
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5.1.2 Model transfer

A typical problem when building multivariate calibration models is that the data set used to build the calibration model differs from the measured samples. The variation in the spectra can be due to differences in the sample (particle size, density) and environmental conditions as well as instrument response [202]. In papers III and IV, the process data differed from the calibration data since e.g. milling decreased the particle size, moisture was absorbed into the samples during the experiments and the density of the samples changed. Besides spectral pre-treatment, these issues were taken into account by selection of appropriate spectral regions. When the models were transferred to process analysis, the regions where the process data resembled the calibration data most were chosen. An additional approach was taken in paper IV, where the same calibration model was used that had been used to analyze dehydration of CBZ [93]. To account for variation due to different sample morphology, the calibration model was updated by incorporating into the model spectra of the pure amorphous samples that were similar to the ones that were used in dissolution study, i.e. glassy samples that had not been ground.

5.1.3 Qualitative analysis

In paper I, PCA was combined with spectroscopic techniques to obtain information about the solid-state differences in the amorphous form prepared by different techniques and from different polymorphs. PCA aided the analysis of the spectral data, since the molecular level differences in the samples due to the lack of molecular order were small and difficult to pick up by only observing the spectra.

PLS-DA enables the classification of the calibration set into different classes. In paper IV, the spectra of different crystalline forms of IMC and CBZ were successfully used to create a PLS-DA model based on Raman spectroscopy, where each solid-state form formed its own class. This enabled the incorporation of three (IMC) or four (CBZ) solid-state forms in the model. These models were then applied to analyze solid-state transitions that occur during dissolution testing. To improve the model, the spectra had to be SNV corrected and mean centred.

5.1.4 Quantitative analysis

PLS regression analysis was successfully combined in papers II and III with both NIR and Raman spectroscopy to quantify mixtures of several solid-state forms, where one of the forms is the amorphous form. A model for quantifying binary mixtures and ternary mixtures were created in papers III and II, respectively.

If the aim is to detect small changes in crystallinity using spectroscopic techniques, such as small amounts of amorphous material in an otherwise crystalline matrix or vice versa, a more accurate quantification model is obtained if separate models are built for the far end of the concentration range. In paper III, the RMSEPs could be decreased to below 2.0% with both Raman and NIR spectroscopy when separate models were built for the high and low
amorphous contents. When analyzing the carbohydrate samples, NIR spectroscopy seemed to perform better than Raman spectroscopy for low levels of amorphous material (RMSEP <1.0%) whereas Raman spectroscopy performed better for high amorphous contents (RMSEP <1.0%).

5.1.5 Sources of error

The sources of error, when determining the amounts of different solid-state forms in ternary mixtures, were assessed for both NIR and Raman spectroscopy in paper II. The sources of error could be divided into two categories, those due to instrument variability and those due to sampling (Table 3). The largest source of error was found to be the sample positioning due to inhomogeneous mixing in both NIR and Raman spectroscopy (Table 4). This was consistent with previous findings and highlights the importance of using set ups with a larger effective sampling area, such as rotating sample holders or probes with larger spot sizes [218-221], or conducting the analysis in transmission mode [222, 223]. In paper III, the RMSEP for quantification of amorphous lactose was decreased from 4.3% to 2.4% after a 100-fold increase in the effective sampling area (Fig. 7). The same trend could be seen with the tertiary mixtures in paper II. The error due to positioning decreased significantly when the sample was rotated. This can be seen when comparing the errors caused by sample positioning and instrument reproducibility (Table 4). The inter-day variability of the instrument response was a significant source of error. However, the relative standard deviations (RSDs) for the γ-IMC and amorphous IMC were much higher than for the α-IMC. This increase in the RSDs in the inter-day variability compared to the RSDs in the instrument response and the intra-day variability was caused by the amorphous IMC partly crystallizing to the γ-form during the study [41, 55, 76].

Altogether no significant difference in the sensitivity could be found when comparing the two spectroscopic techniques. The overall method errors for NIR spectroscopy were 6.7% (α-IMC), 8.4% (γ-IMC) and 13.0% (amorphous IMC) and the respective values for Raman spectroscopy were 6.3% (α-IMC), 8.8% (γ-IMC) and 12.0% (amorphous IMC).

![Figure 7. Effect of the sampling area on the theoretical vs. predicted amorphous content of lactose determined with NIR spectroscopy. a) Lamp set up, effective sampling area with a diameter of 10-20 mm and b) probe set up, effective sampling area with a diameter of 1-2 mm. Modified from paper III.](image)
Table 3. Evaluation of different sources of error associated with quantitative analysis using NIR and Raman spectroscopy. Modified from paper II

<table>
<thead>
<tr>
<th>Source of error</th>
<th>Evaluation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument variability</td>
<td>Five consecutive measurements were performed on a sample.</td>
</tr>
<tr>
<td>Instrument variability</td>
<td>A sample was measured once every hour for a period of five hours.</td>
</tr>
<tr>
<td>Intra-day variability</td>
<td>A single measurement was performed on a sample each day over five days.</td>
</tr>
<tr>
<td>Inter-day variability</td>
<td>Five consecutive measurements were performed on a sample.</td>
</tr>
<tr>
<td>Sample effects</td>
<td></td>
</tr>
<tr>
<td>Sample mixing</td>
<td>A sample was divided into five sub-samples and each of them measured once.</td>
</tr>
<tr>
<td>Sample packing</td>
<td>A sample was measured five times and repacked after each measurement.</td>
</tr>
<tr>
<td>Sample positioning</td>
<td>Five consecutive measurements were performed on a sample.</td>
</tr>
<tr>
<td>Particle size</td>
<td>A second sample of a particle size range from 125 µm to 250 µm was prepared</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Five consecutive Raman spectra of a sample were recorded without</td>
</tr>
<tr>
<td></td>
<td>initially performing photochemical bleaching.</td>
</tr>
<tr>
<td>Overall method error</td>
<td>Four independent mixtures of the same composition (1/3 : 1/3 : 1/3) were</td>
</tr>
<tr>
<td></td>
<td>prepared and analyzed by performing five measurements on each sample over</td>
</tr>
<tr>
<td></td>
<td>one day.</td>
</tr>
</tbody>
</table>

*All errors were studied using a rotating sampling accessory and a mixture with a particle size range of <125µm except for “sample positioning”, where a stationary sample holder was used and for “particle size”, where a particle size range from 125 µm to 250 µm was used.

Table 4. The assessment of potential sources of error in NIR and Raman spectroscopy. Modified from paper II

<table>
<thead>
<tr>
<th>Source of error*</th>
<th>NIR spectroscopy (RSD%)</th>
<th>Raman spectroscopy (RSD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-IMC</td>
<td>γ-IMC</td>
</tr>
<tr>
<td>Instrument variability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Instrument reproducibility</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Intra-day variability</td>
<td>4.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Inter-day variability</td>
<td>7.8</td>
<td>12.4</td>
</tr>
<tr>
<td>Sample effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample packing</td>
<td>2.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Sample mixing</td>
<td>6.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Sample positioning</td>
<td>5.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Particle size</td>
<td>3.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Overall method error</td>
<td>6.7</td>
<td>8.4</td>
</tr>
</tbody>
</table>

*n=5, except for overall method error n=20.
5.2 Preparative techniques induced changes in amorphous state

Amorphous material can be obtained using various preparative techniques: through a solid-state transformation (milling), through a liquid state (melting), through a solution state (spray drying) or through a vapour state [3, 38]. In paper I, amorphous IMC was produced through all of these routes except via the vapour state from two different polymorphic forms \( \gamma \) and \( \alpha \)-IMC. All of the samples except those that were spray dried were XRPD amorphous after preparation and showed no birefringence under the polarized light microscope. The spray dried samples had different amounts of crystalline \( \alpha \)-form in the samples. MTDSC analysis showed no significant differences in the glass transition temperatures or heat capacities between the milled and melted samples. However, based on DSC results the milled samples crystallized at lower temperatures than the melted ones.

The differences in the spectra of the amorphous samples prepared with different techniques were small. However, with PCA of the MIR, NIR and Raman spectra, molecular level differences in the samples could be detected (Figs. 8 and 9d). The milled samples differed from the melted ones and bore more resemblance to the original crystalline forms used to prepare them. They were located closer to the scores of the crystalline samples, whereas the scores of the melted samples were all located near to each other. In this study, the polymorph used to prepare the amorphous sample affected the amorphous state only when the amorphous state was obtained by milling through a solid-state transformation. Thus, it seems that some short-range order or nuclei remain in the sample. In this study, the cooling rate of the melt had no effect on the amorphous state obtained.

When analyzing the original spectra and loadings plot and the regions which were most significant in the PCA, it could be concluded that the spectroscopic techniques revealed complementary information about the samples. The preparative technique seemed to cause differences in the hydrogen bonding. For example in the Raman spectra of the melted samples a shift to a lower frequency can be noted in the hydrogen bonded C=O stretch region compared to the other amorphous samples. Raman spectroscopy was more sensitive to the degradation products as the degraded samples formed a separate cluster (Fig 8). However, this was probably due to Raman technique being sensitive to the fluorescence caused by the degradation products.

Since there are molecular level differences in the amorphous samples caused by the preparative technique, this should be taken into account when building multivariate calibration models involving amorphous materials. It would be beneficial to prepare the amorphous samples using the same preparative technique as the samples that are to be analyzed. Also, it may be necessary to use the same polymorphic form of the drug to build the calibration model as the starting material during the actual manufacturing of an amorphous drug to avoid differences in the amorphous characteristics of the drug.
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Figure 8. The scores plot of the amorphous IMC samples following the PCA of the Raman spectra. Modified from paper I.

5.2.1 Using multivariate visualization to predict stability differences in amorphous state

A previous study has shown that there is a stability difference between melted and milled samples of amorphous IMC [49]. The increase in hydrogen bonding that was observed in paper I in the melted samples compared to the milled ones could partly explain the better stability of the melted samples. The other possible explanation is the different transformation pathways through which the amorphous state is formed. Thus it is possible that when the amorphous state is obtained through a solid-state transformation, some nuclei or short-range order remains in the sample. These regions would then function as nucleation sites and advance the onset of crystallization.

In this study, based on the XRPD analysis of the samples after one month of storage in 4 °C in close to 0% RH, it was noted that the milled samples prepared form both α- and γ-IMC had crystallized during the one month’s storage time to the γ-form regardless of the polymorph used to prepare the amorphous samples. The melted samples on the other hand remained amorphous (Fig 9a-c). Thus, it seems that some short-range order or nuclei remained in amorphous IMC samples prepared by milling. These nuclei then acted as seeds and induced crystallization. Although this study does not give any indication about the differences between the stability of amorphous samples prepared from different
polymorphic forms, the initial polymorphic form used to prepare the amorphous sample is known to affect the crystallization kinetics of amorphous IMC prepared by milling [55]. The amorphous samples that had been prepared by milling the α-IMC crystallized first to α-form and after an induction period crystals of the γ-form appeared. The amorphous samples prepared by milling the γ-IMC crystallized directly to the γ-form after an induction period. The α- and γ-IMC are monotropic, with the α-polymorph being the metastable polymorph [76]. The α-form would, thus, crystallize to the γ-IMC once the nuclei of the γ-IMC appeared.

The amorphous samples that had been prepared by milling and had crystallized were the ones that had clustered separately from the other amorphous samples in the scores plot following PCA of the NIR and MIR spectra (Fig. 9d). The milled amorphous γ-IMC samples had formed a separate cluster closer to the scores of crystalline γ-IMC. When the plot is rotated around t[3]-axis, the scores of the milled α-IMC behind the melted samples can be seen as a separate cluster. With the PCA of the Raman spectra this was not quite as clear as the scores did not form a separate cluster (Fig. 8). The milled samples were located nearer but still separate from the melted samples. Thus, the PCA of the spectra measured on the day of preparation offers a fast tool to screen for stability differences in the amorphous samples and to separate samples which could potentially have stability problems from the more stable ones. Thus, it offers a tool that can be used to predict for stability differences already on the day of preparation.
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Figure 9. XRPD patterns of the amorphous IMC prepared by different techniques: a) spray dried, b) melted, and c) milled on the day of preparation and after 1 month of storage at close to 0% RH at 4 °C, as well as, d) the scores plot following the PCA of MIR spectra. Figure 9d modified from paper I.
5.3 Monitoring solid-state transformations during storage, processing and dissolution testing

5.3.1 Amorphization of crystalline material

Mechanical stress, such as milling [39, 49, 51, 53, 61, 80-89] or compression [90, 94-97] can cause phase transformations in solid materials. The process induced changes can vary depending on the material, e.g. one polymorph can change to another or it can become amorphous, or hydrates can dehydrate, which can lead to an anhydrate or amorphous form.

In paper III, crystalline α-lactose monohydrate was milled in a ball mill. To get a better insight into the phase transitions the milling was stopped at different time points and Raman and NIR spectra of the milled samples were measured directly from the milling bowl. For reference, the samples were also analyzed off line using XRPD. The spectroscopic analysis could be combined with PLS regression analysis to quantify the amount of amorphous material in the sample (Fig. 10). The results were in agreement with the XRPD analysis and both spectroscopic techniques could be used to monitor the process induced changes. However, due to the differences in the calibration data and process data, such as noise and baseline, in order for the PLS quantification approach to work, it was necessary to develop the model further. The spectral region where the process spectra and the calibration data resembled each other most was chosen for the analysis. Therefore, NIR spectral regions, where water was noted, were left out from the model.

Figure 10. a) NIR spectra of the milled α-lactose monohydrate. b) Amount of amorphous lactose in the milled samples determined with NIR and Raman spectroscopy as well as XRPD. Modified from paper III.
The importance of the measuring set up could be noted. There was more variation in the Raman results compared to the NIR results due to differences in the measuring techniques and effective sampling areas. The Raman probe had a diameter of about 0.1 mm, whereas with the lamp set up used in NIR analysis, information was gathered from an area that had a diameter of 10 mm. Therefore the results obtained with NIR spectroscopy resembled more of the XRPD results. The samples for the XRPD analysis where gathered from several locations in the milling bowl and resembled, thus, the average crystallinity at that time point. The amorphous content of the ground material varied in different locations of the milling bowl and this caused more variation in the Raman data due to the smaller sampling area. Thus, spectroscopic techniques combined with PLS regression analysis not only offered a method to quantify the amount of amorphous material but the decrease in the data variation could also be used to determine the end point of a milling process.

5.3.2 Crystallization of amorphous material

Storage of amorphous samples in high relative humidity or at temperatures close to or above the $T_g$ can lead to crystallization of an amorphous product [76, 77]. In paper III, storage of amorphous samples in high relative humidity (85 % RH) was used as a model process to monitor changes due to environmental conditions. Both amorphous lactose and trehalose were stored in high relative humidity. The changes in the solid state were monitored with both Raman and NIR spectroscopy. For reference, the end-point crystallinity of the samples was determined using XRPD. Spectroscopic analysis showed that both amorphous lactose and trehalose crystallized during storage at 85% RH (Figs. 11a and 12a). However, PLS regression analysis combined with the spectroscopic data could only be used to quantify the changes in the solid state of trehalose, since it crystallized directly to the dihydrate (Figs. 11b and 12b). The crystallization pathway of amorphous lactose on the other hand is complicated. It is known to crystallize to various physical forms and their mixtures, when stored at a higher relative humidity than 50% [123, 224-226]. In paper III, amorphous lactose crystallized to a mixture of $\alpha$-lactose monohydrate and anhydrate $\beta$-lactose. In order for the multivariate approach to have worked also for the lactose samples, a tertiary quantification model, with all the possible solid-state forms, should have been built.

Ideally the data for the calibration set should be measured in similar environment as the process is carried out. Otherwise this will lead to differences in the calibration and process data and worsen the model performance. NIR spectroscopy is sensitive to water, whereas Raman spectroscopy is not. Amorphous samples are hygroscopic. Thus, measuring NIR spectra in high relative humidity created problems as the amorphous samples absorbed moisture. This changes the spectral features of the amorphous samples especially in the region where water is noted (1400-1460 nm and 1900-2000 nm). This sorption of water had to be taken into account when creating the model. To improve the model performance these spectral regions, where water is detected were left out of the model. However, these regions are also the ones where the spectral differences are most distinct. Therefore, the quantification model based on NIR spectroscopy (RMSEP = 6.4%) was not as good as the model based on Raman spectra (RMSEP = 2.6%).
A difference in the crystallization kinetics could be noted between the NIR and Raman experiments (Figs. 11 and 12). During the experiments, the amorphous samples measured with Raman spectroscopy started to crystallize within half the time than the ones measured with NIR spectroscopy. This was attributed to the different experimental set ups, as the size of the humidity chambers was different.

**Figure 11.** a) NIR spectra of amorphous trehalose stored in 85% RH and b) the change in amorphous content determined with the PLS model. Modified from paper III.

**Figure 12.** a) Raman spectra of amorphous trehalose stored in 85% RH and b) the change in amorphous content determined with the PLS model. Modified from paper III.
RESULTS AND DISCUSSION

5.3.3 Dissolution

The amorphous form is often presented as a possibility to improve the solubility and dissolution rate of poorly water soluble drugs, since several fold increase in solubility can be obtained when using amorphous forms compared to crystalline counterparts [35]. In order for the amorphous formulation approach to succeed, the drug has to remain amorphous throughout the entire storage time as well as during the dissolution. Thus, the conversion rate of the metastable form to the stable form has to be slower than the dissolution rate of the metastable form [39]. Otherwise the dissolution rate will gradually change to that of the stable crystalline form.

In paper IV, the dissolution behaviour of both amorphous IMC and CBZ was evaluated. To get a better insight to the phenomena that occur during dissolution the surface of the dissolving sample was analyzed using in situ Raman spectroscopy combined with PLS-DA. Amorphous IMC has slow crystallization kinetics [39] and, thus, a several fold increase in the dissolution rate could be noted from the amorphous form compared to the crystalline counterparts in both dissolution media (50% water-ethanol solution and phosphate buffer pH 7.2) (Fig. 13a). However, the dissolution started to slow down during the experiment. PLS-DA analysis of the Raman spectra showed that at that time point the scores of the amorphous sample started to move from the amorphous region towards the crystalline α-IMC in both dissolution media (Fig. 13b). Thus, the surface of the amorphous sample started to crystallize at that time. XRPD analysis of the sample at the end of the dissolution test verified the crystallization of the sample in water-ethanol mixture to the α-IMC. However, the crystallization of the sample surface in phosphate buffer could not be detected with XRPD as the crystalline layer was so thin. However, Raman spectroscopy combined with PLS-DA was more sensitive to the surface crystallization and was able to detect the onset of crystallization.

Figure 13. a) Dissolution of IMC from the amorphous samples as well as α-IMC and γ-IMC tablets in phosphate buffer pH 7.2. b) Respective scores vs. time plot following the PLS-DA of Raman spectra. The maximum standard deviation in concentrations is 2 µg/ml. Modified from paper IV.
Amorphous CBZ on the other hand crystallized instantly upon contact with the dissolution medium (phosphate buffer pH 7.2). No significant increase in the dissolution rate was noted (Fig. 14). In general, the dissolution rate was even lower (marked with A in Fig. 14) than from the dihydrate tablets. However, in two samples the dissolution rate was higher (marked with B in Fig. 14). The scores following the PLS-DA of the Raman spectra started to move instantly from the amorphous region first towards the form I and then towards the dihydrate. XRPD analysis at the end of the dissolution experiment confirmed the crystallization of the samples to the dihydrate during dissolution. Thus, it seems that the crystallization pathway of amorphous CBZ in aqueous solution is through the anhydrate form I to the dihydrate. PLS regression analysis could be used to extract quantitative information of the Raman spectra (Fig. 15). Based on the analysis, the amount of crystalline CBZ appearing during the dissolution experiment was different. More of both CBZ form I and dihydrate crystallized in the samples that had higher dissolution rate (marked with B in Fig. 14). The higher dissolution rate from these samples was most likely due to an increase in the specific surface area due to crystal growth.

**Figure 14.** Dissolution of CBZ from the amorphous samples and CBZ dihydrate tablets in phosphate buffer pH 7.2. The maximum standard deviation is 0.2 µg/ml. Modified from paper IV.
**Figure 15.** Quantification of different CBZ forms present in the solid sample during dissolution of CBZ from the originally amorphous samples determined by PLS analysis of Raman spectra. a) Samples with slower dissolution (marked as A in Fig. 14) and b) samples with faster dissolution (marked with B in Fig. 14). Modified form paper IV.
6 Conclusions

Spectroscopic techniques combined with multivariate analysis methods could be used for the analysis of amorphous or partly amorphous samples. Multivariate techniques offer possibilities for both qualitative and quantitative analysis even in the presence of several polymorphic forms. Multivariate methods provide the advantage compared to uni- and bivariate methods that a wider spectral region can be used for the analysis. This is a major benefit when analyzing spectra of amorphous samples. To minimize the effects of unwanted physical information on the model, the models could be significantly improved by undertaking several model development stages, such as selection of appropriate variable range, spectral pre-treatment to remove baseline effects and scaling of the variables. Multivariate calibration models could be improved further by adding spectra into the calibration data that resembled process data. However the major source of error in these quantification models came from the sampling. Thus, it is advantageous to use techniques with a large effective sampling area or rotate the sample during analysis.

Amorphous material could be prepared through different transformation routes. The selected preparation method and polymorphic form used to prepare the samples had an effect on the amorphous state obtained as some molecular level differences could be noted. PCA of the spectra could be used to visualize these differences. Therefore, when building multivariate calibration models involving amorphous materials, the amorphous samples should be prepared if possible using the same preparative technique and from the same polymorphic form as the samples that are to be analyzed. When the amorphous material was produced through a solid-state transformation by mechanical activation, it bore more resemblance to the original polymorph. Therefore, it was also less stable and crystallized back to the original polymorph faster. PCA combined with spectroscopic techniques could be used to screen for these molecular level differences and separate the less stable samples from the more stable. Thus, PCA combined with spectroscopic techniques offer a tool that can be used to predict stability differences already on the day of preparation.

Amorphous materials are often formed both intentionally as well as unintentionally during pharmaceutical manufacturing. NIR and Raman spectroscopy with multivariate methods could be used to monitor the solid-state transformations as well as to quantify the amounts of different solid-state forms present during processing and storage. However, quantification could be only carried out if all solid-state forms present are taken into account when creating the model.

The solubility advantage from amorphous materials is obtained only if the amorphous material remains amorphous during dissolution in the body. If the amorphous form transforms to a crystalline form during dissolution, the advantage is lost as the dissolution rate will gradually change to that of the crystalline form. In situ Raman spectroscopy of the sample surface combined with either PLS-DA or PLS regression analysis during dissolution testing could be used to determine both qualitative and quantitative information of the solid-state phenomena that are ongoing during the dissolution. When this information was combined with the dissolution data, a more complete picture of the dissolution behaviour was obtained.
References


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