*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

# DETECTION OF LOW DOSE OF PIROXICAM POLYMORPH IN PHARMACEUTICAL TABLETS BY SURFACE-ENHANCED RAMAN CHEMICAL IMAGING (SER-CI) AND MULTIVARIATE ANALYSIS

Johan Cailletaud[a], Charlotte De Bleye[a], Elodie Dumont[a], Pierre-Yves Sacré[a], Yoann Gut[b], Nicolas Leblanc[b], Philippe Letellier[b], Yves-Michel Ginot[b], Philippe Hubert[a], Eric Ziemons[a]

[a] University of Liege (ULiege), CIRM, Vibra-Sante Hub, Department of Pharmacy, Laboratory of Pharmaceutical Analytical Chemistry, CHU, B36, B-4000 Liege, Belgium.

[b] Technologie Servier, 27 rue Eugène Vignat, 45000 Orléans, France.

**Keywords:**

Surface-enhanced Raman scattering (SERS), Chemical imaging, Silver nanoparticles, Piroxicam, Polymorphism, Multivariate analysis.

**Abstract**

This study demonstrates, for the first time, the ability of surface-enhanced Raman chemical imaging (SER-CI) combined with multivariate analysis to detect low levels (0.1% (w/w)) of a polymorphic form in a pharmaceutical mixture. In the studied formulation, piroxicam was used as a model molecule to develop this approach. Piroxicam is a widely available nonsteroidal anti-inflammatory drug, exhibiting an interesting case of polymorphism, with two most commonly observed forms (β and α2). In this work, the SERS spectra of piroxicam polymorphic forms β and α2 are presented. These forms showed clear spectral differences in terms of band position and intensity. From a crystallographic point of view, the difference of exaltation between both forms was correlated with a preferred orientation of crystallites of form α2 making its SERS detection difficult compared to form β. A preferred orientation of the (1 $k$ 0) crystallographic planes of α2 was demonstrated in samples, not promoting an appropriate molecular orientation onto the metallic surface. Additionally, a semi-quantitative approach using SER-CI combined with chemometric tools was developed enabling to detect crystallites of form β below the detection limit of conventional Raman microscopy. The exaltation of the Raman signal in the presence of silver nanoparticles allowed a higher sensitivity and a reduction of the acquisition time by a factor of 6.

## Introduction

In the pharmaceutical industry, the detection and identification of polymorphic forms of an active pharmaceutical ingredient (API) are important concerns during the drug development and the manufacturing process. This is an area of particular interest in pharmaceutical applications due to the

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

difference of physico-chemical properties exhibited by the polymorphs such as solubility, dissolution rate and bioavailability (Hennigan and Ryder, 2013). Indeed, the polymorphic behavior of drugs can affect the therapeutic efficacy of the API and can also have an impact from an intellectual property (IP) point of view (Griffen et al., 2016). In this respect, much effort is expended by the pharmaceutical industries to determine if the polymorph present in the final product is covered by a patent for IP rights aspects.

Various analytical techniques can be used for polymorph detection and characterization such as X-ray powder diffraction (XRPD) (Qiu et al., 2015; Kang et al., 2018), solid-state nuclear magnetic resonance spectroscopy (ssNMR) (Tinmanee et al., 2017; Quiñones et al., 2018), differential scanning calorimetry (DSC) (Bellur Atici and Karliga, 2015) and vibrational spectroscopy (Bunaciu et al., 2015). Among the different techniques of vibrational spectroscopy, Raman confocal microscopy is well suited to the analysis of polymorphic forms since it enables the acquisition of spectral and spatial information at the same time with minimal sample preparation (Paiva et al., 2018). While this technique suffers from a low sensitivity, it can be combined with surface-enhanced Raman scattering (SERS) which enables to increase the intensity of the Raman scattering from molecules in direct contact or very close to metallic surfaces, resulting in surface-enhanced Raman chemical imaging (SERCI). SER-CI is a promising tool for the detection and the visualization of low dose compounds in tablets taking into account its high sensitivity while reducing image acquisition time due to the enhancement of the signal intensity. Some papers refer to the application of SER-CI for the investigation of active components or impurities in pharmaceutical products (Firkala et al., 2013; De Bleye et al., 2014; Firkala et al., 2015) but none refers to the detection of low dose of polymorphic forms. In this context, this technique was considered for the detection of different polymorphs in a pharmaceutical model.

In the present work, we reported the characterization and the development of a semiquantitative SERS approach to detect low doses of polymorph (0.1% w/w). The study was based on piroxicam as a model molecule, a non-steroidal anti-inflammatory and analgesic drug used as API in various pharmaceuticals. Piroxicam, from which it structure is shown in Fig. 1, presents a pyridyl functional group conferring to the molecule a native affinity for metallic surfaces and rendering it very attractive for SERS analyses. Moreover, at least three polymorphic forms ($\beta$, $\alpha_1$ and $\alpha_2$) and one monohydrate form are reported in literature, obtained by crystallization from various solvents (Vrecer et al., 2003). Forms $\beta$ and $\alpha_2$, existing in $P2_1/c$ space group, are the most commonly observed, while the other polymorphs are very unstable (Lipiäinen et al., 2018). In our study, SERS was used as an analytical tool to demonstrate the feasibility to characterize both forms in the same way as conventional Raman spectroscopy. In this framework, multivariate curve resolution-alternating least squares (MCR-ALS) was applied on SER-CI data in order to provide the distribution maps of a low level of one polymorphic form in pharmaceutical mixtures.

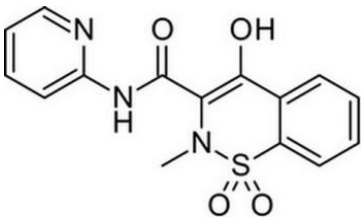


**Fig. 1.** Chemical structure of piroxicam.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

# Materials and methods

## CHEMICALS AND REAGENTS

Silver nitrate (extra pure) was obtained from Merck (Darmstadt, Germany). Trisodium citrate (anhydrous, 98%) was purchased from Acros Organics (Morris Plains, USA). Lactose monohydrate, piroxicam (purity > 98%) characterized as form β and absolute ethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA).

## PREPARATION OF SILVER NANOPARTICLES

The synthesis of silver nanoparticles (Ag Nps) was based on the method described by Lee and Meisel (Lee and Meisel, 1982). Forty-five milligrams of silver nitrate were dissolved in 250.0 mL of ultrapure water generated from a Milli-Q system (Millipore, Bellirica, MA, USA). The solution was heated to boil and 5.0 mL of a solution of 1% trisodium citrate was added dropwise under stirring with a Teflon-coated magnetic stir bar at 750 rpm. The resulting suspension was kept on stirring at reflux for one hour and turned into a greenish grey color after a few minutes.

The colloidal suspension was then cooled down to room temperature and stored in a fridge until it was used. Before each SER-CI measurement, the suspension was centrifuged for 20 minutes at 6000 rpm. The supernatant was removed in order to concentrate the nanoparticles by a factor of 20.

## PREPARATION OF PIROXICAM POLYMORPHS

The polymorphic form $\alpha_2$ was prepared by dissolving 1.5 g of form β in 380 mL of absolute ethanol at 60°C with continuous stirring until fully dissolved as described by Lipiäinen et al. (2018). The solution was then slowly cooled down to room temperature, held overnight and followed by vacuum filtration. The resulting powder was finally dried under vacuum during one hour and then characterized through the techniques of XRPD, scanning electron microscopy (SEM) and Raman spectroscopy.

In addition, the solid-state forms of two tablets solely composed of forms β or $\alpha_2$, were confirmed by XRPD in order to ensure that there was no modification of their crystal structure after compression.

## PREPARATION OF PHARMACEUTICAL TABLETS

One set of powder mixtures was used in this work in order to investigate low concentrations of piroxicam polymorphs. The set, consisting of five blends, was prepared based on a design containing 10% (w/w) of a binary mixture of polymorphic forms β and $\alpha_2$ and 90% (w/w) of lactose monohydrate. In this way, the crystalline form β content were 0.1%, 0.5%, 1%, 1.5% and 2% (w/w). To ensure a clear understanding, the composition of each tablet is summarized in Table 1.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

**Table 1.** Composition of the five samples prepared from 10% (w/w) of a binary mixture of the two polymorphs and 90% (w/w) of lactose monohydrate.

| Sample | Form β (w/w) | Form $\alpha_2$ (w/w) | Lactose monohydrate (w/w) |
|---|---|---|---|
| 1 | 2% | 8% | 90% |
| 2 | 1.5% | 8.5% | 90% |
| 3 | 1% | 9% | 90% |
| 4 | 0.5% | 9.5% | 90% |
| 5 | 0.1% | 9.9% | 90% |

The mixtures were mixed in a shaker mixer (Turbula WAB T2F, Switzerland) to ensure homogeneity. Then, tablets with a total mass of 200 mg, a diameter of 10 mm and a thickness of 2 mm were prepared with a SPECAC (Slough, England) press system under 1 t compression force and analyzed by XRPD, SEM, Raman and SER-CI analyses.

For SER-CI acquisitions, the concentrated Ag Nps were sprayed on top of the tablet surfaces in a homogeneous way as described in a previous work of our research group (Cailletaud et al., 2018).

## INSTRUMENTATION

### XRPD INSTRUMENTATION

XRPD diffractograms of piroxicam polymorphs, before and after compression, were recorded at room temperature with a BRUKER D8 Advance diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) system in the reflection mode. The measurement was carried out at 36 kV, 36 mA with a Cu $K\alpha 1$ radiation source ($\lambda$ = 1.5406 Å) between 7.5°/8.5° and 55° with a 0.014° $2\theta$. The measurement time was 1 second per step. The data collection was performed using the EVA software (Bruker AXS GmbH, Karlsruhe, Germany) and the data were processed using HighscorePlus v3.0e (Malvern Panalytical, Malvern, England).

Precautions have been taken in order to keep a constant diffraction volume between the powder and the compressed tablet. In addition, simulated XRPD diffractograms of forms $\beta$ and $\alpha_2$ were generated with the Cambridge Structural Database (CSD) files, BIYSEH03 (Sheth et al., 2004) and BIYSEH06 (Vrecer et al., 2003), respectively, from the Cambridge Crystallographic Data Centre (CCDC).

### SEM INSTRUMENTATION

The morphology of the two polymorphic forms, both in powder and in tablet, was examined by SEM (Hitachi TM-1000, Hitachi Scientific Ltd., Tokyo, Japan). The samples were scanned by an electron beam with an acceleration potential of 15 kV under a vacuum of approximately 30 – 50 Pa.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

## RAMAN INSTRUMENTATION

The tablet images were collected using a LabRAM HR Evolution system (Horiba JobinYvon, Lyon, France) and the LabSpec 6 software (Horiba Jobin-Yvon, Lyon, France). The microscope was equipped with a two-dimensional Newton 970 front-illuminated EMCCD detector (1600 x 200 pixel sensor). An objective of 10x magnification and a 785 nm laser
(Oxxius, Lannion, France) with a power of 50 mW were used for the mapping measurements. The confocal slit-hole was fixed at 200 µm and a 300 gr/mm grating fixed at 1100 $cm^{-1}$ allowing to cover the spectral range from 348 to 1761 $cm^{-1}$.

Before each SER-CI measurement, the tablet surfaces without silver colloids were investigated with a high mapping acquisition time. This was intended to obtain the distribution maps of the polymorphic forms with a sufficient signal to noise ratio (SNR) and to verify if no signal of the compound was detectable without SERS. To achieve this, 3 scans of 2 seconds were accumulated for each spectrum. The step size between each spectrum was set at 200 µm. An image of 53 x 53 pixels, corresponding to 2809 spectra, was acquired and the acquisition time for each map was approximately 315 minutes.

For SER-CI analyses, the laser power in the sample compartment was decreased to 10% of its original value with an intensity filter to avoid damages on the sample surfaces covered by Ag Nps. A single spectrum acquisition of one second exposure time was measure at each point. The step size between two consecutives pixels was set at 200 $\mu m$ with a total area of 53 × 53 pixels corresponding to 2809 spectra and the acquisition of each image was about 50 minutes.

# DATA ANALYSIS

All data treatment was realized with the Matlab R2015a software (The Mathworks, Natick, MA, USA). Raman and SERS maps were processed in the same way.

## DATA PRE-PROCESSING

Considering the spherical geometry of the tablets which did not cover the entire scanned square image, the selection of a region of interest (ROI) was applied to the data (Vidal and Amigo, 2012). The ROI was selected manually in order to remove the area around the sample composed of highly noisy spectra.

Once the background removed, each map was transformed into a bi-dimensional matrix and was corrected by principal component analysis (PCA) in order to eliminate the spectral noise (Sacré et al., 2014). The data were then pre-processed using an automatic Whittaker filter to correct baseline variations with parameters of value of $10\boldsymbol{\lambda}^{5}$ and value of 0.001. Next, the $\boldsymbol{p}$ spectral range was reduced in order to focus only on the spectral ROI corresponding to a Raman shift from 600 $cm^{-1}$ to 1600 $cm^{-1}$. Finally, the reduced spectra were normalized to unit area to mitigate the influence of the silver colloids coating process on the sample surface by reducing the high variation of the SERS response from pixel to pixel and therefore to improve the quality of the SER-CI results.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

## MULTIVARIATE CURVE RESOLUTION-ALTERNATING LEAST SQUARES (MCR-ALS)

In order to extract the relevant information from the Raman and SERS maps, the MCR-ALS method which is the most frequently used algorithm in the framework of SER-CI, was applied (Cailletaud et al., 2017).

The MCR-ALS algorithm decomposes the bi-dimensional matrix $\boldsymbol{D}$ of spectral data in a bilinear model given below:

$$\boldsymbol{D} = \boldsymbol{C}\boldsymbol{S}^{\boldsymbol{T}} + \boldsymbol{E} \quad (1)$$

Where $\boldsymbol{C}$ is the matrix of concentration profiles, $\boldsymbol{S}^{\boldsymbol{T}}$ is the transposed matrix of pure spectra and $\boldsymbol{E}$ is the residual error. The advantage of the MCR-ALS method is the possibility to apply constraints properly chosen to reduce rotational ambiguities during the iterative process. Since the concentrations of the compounds in a pharmaceutical sample should not be negative, only non-negativity constraints were applied in this study. However, a non-exhaustive list of constraints such as closure, unimodality or local-rank can also be used to improve the resolution of the system and are described in more detail in literature (Hugelier et al., 2015; Zhang et al., 2016). In return, the MCR-ALS must be initialized by a first estimate of the $\boldsymbol{C}$ or $\boldsymbol{S}^{\boldsymbol{T}}$ matrix (Boiret et al., 2015). The matrix $\boldsymbol{C}$ is generally unknown and therefore, the matrix $\boldsymbol{S}$ is initialized by pure spectra acquisition or by mathematical approaches. SIMPLISMA (SIMPLe-to-use Interactive Self-modeling Mixture Analysis) (Windig and Guilment, 1991), OPA (Orthogonal Projection Approach) (Gourvénec et al., 2003), ICA (Independent Component Analysis) (Boiret et al., 2014) and VARCLUS (VARiable CLUStering) (Farkas et al., 2017) for example, can be used on the dataset to identify pure signals. In this work, the MCR-ALS was initialized with the reference Raman and SERS spectra of the two polymorphic forms and lactose monohydrate acquired with the same equipment and the same acquisition parameters as the maps.

During the iterative process, the quality of the MCR-ALS model is estimated by two criteria which are the lack of fit (lof) and the explained variance ($R^2$) calculated as follow:

$$\boldsymbol{lof}(\%) = 100 \times \sqrt{\frac{\sum_{i,j} \boldsymbol{e}_{i,j}^2}{\sum_{i,j} \boldsymbol{D}_{i,j}^2}} \quad (2)$$

$$\boldsymbol{R}^2 = \frac{\sum_{i,j} \boldsymbol{D}_{i,j}^2 - \sum_{i,j} \boldsymbol{e}_{i,j}^2}{\sum_{i,j} \boldsymbol{D}_{i,j}^2} \quad (3)$$

where $\boldsymbol{D}_{i,j}$ is the input element of the initial matrix $\boldsymbol{D}$ and $\boldsymbol{e}_{i,j}$ is the related residual element after iterations of the algorithm.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

# Results and discussions

## CHARACTERIZATION OF PIROXICAM POLYMORPHS

The two crystalline forms of piroxicam were characterized by XRPD, SEM and Raman microscopy. The appearance and shape, the XRPD patterns and the Raman spectra of the two forms showed clear differences.

### XRPD ANALYSIS

To confirm the identity of the polymorphs, the XRPD patterns of powders (blue) form β and $\alpha_2$ were acquired. It can be seen that there were distinct differences between these two forms. Indeed, the main diffraction peaks of crystalline form β are observed at $2\theta$ = 8.7°, 11.7°, 12.5°, 14.5°, 17.7°, 21.8° and 26.8°, as shown in Fig. 2, while those of the form $\alpha_2$ were observed at $2\theta$ = 9.0°, 10.1°, 15.1°, 15.7°, 20.3° and 25.8°, as displayed in Fig. 3. The XRPD profiles are unique to each form and are in good agreement with those found in literature (Vrecer et al., 2003; Fortunato de Carvalho Rocha et al., 2011).

The XRPD profiles of pure tablets (red) of piroxicam forms β and $\alpha_2$ are also presented in Fig. 2 and Fig. 3, respectively. These experimental XRPD diffractograms were compared to their theoretical patterns (green). The peak positions in the experimental XPRD patterns, before and after compression, of form β and $\alpha_2$ were the same as those in the theoretical data generated with the CSD files of BIYSEH03 and BIYSEH06, respectively. This confirms that the crystal structures of both forms are maintained after compression.

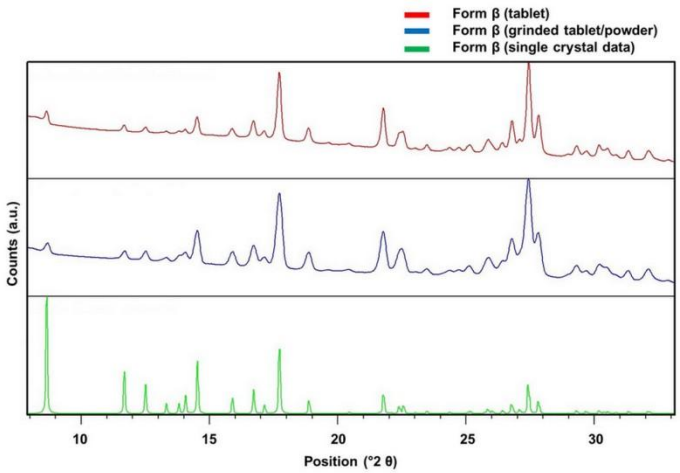


**Fig. 2.** Overlay of the XRPD patterns of the tablet (red) and the grinded tablet (blue) of the polymorphic form β including the corresponding theoretical pattern (green) calculated from the single crystal data.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

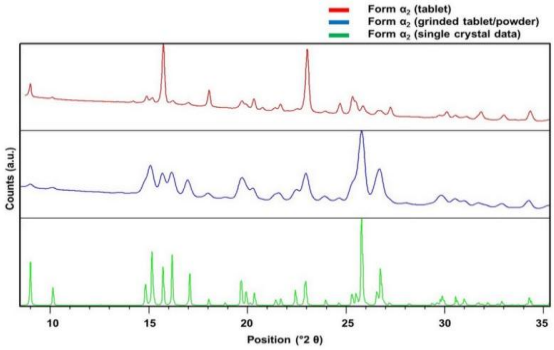


**Fig. 3.** Overlay of the XRPD patterns of the tablet (red) and the grinded tablet (blue) of the polymorphic form $\alpha_2$ including the corresponding theoretical pattern (green) calculated from the single crystal data.

## SEM ANALYSIS

The two forms of piroxicam were observed by SEM with 2000 times magnification. The SEM images revealed that the crystals of the polymorphs present differences in size, morphology and shape, as shown in the supplementary material (Fig. S-1). The form β exhibits a prism shape (Fig. S-1a), whereas the form $\alpha_2$ has a needle morphology (Fig. S-1b) which confirms the results reported in literature (Fortunato de Carvalho Rocha et al., 2011). After compression, all the prisms of the crystallites β are distributed in a heterogeneous way on the sample surface (Fig. S-1c). In the case of $\alpha_2$ polymorph, the tablet is composed of needles which are mainly aligned with the sample surface along their growth axis, as displayed in Fig. S-1d. The observation of the needle shape morphology is consistent with the presence of preferred orientation of the $\alpha_2$ crystallites from an optical point of view.

## RAMAN ANALYSIS

The Raman spectra of crystalline forms β and $\alpha_2$ of piroxicam are depicted in Fig. 4. Form β exposed a characteristic Raman band at 1523 $cm^{-1}$ while a distinct band of the form $\alpha_2$ was observed at 1543 $cm^{-1}$. The obtained Raman spectra of the two forms were consistent with the reports published by other research groups (Lipiäinen et al., 2018; Redenti et al., 1999). Despite the same intramolecular structure and conformation, these spectral differences characteristic of each form are related to different intra and intermolecular hydrogen bond interactions (Lipiäinen et al., 2018) but also to the orientation of the crystals (different selection rules due to the 2/m symmetry).

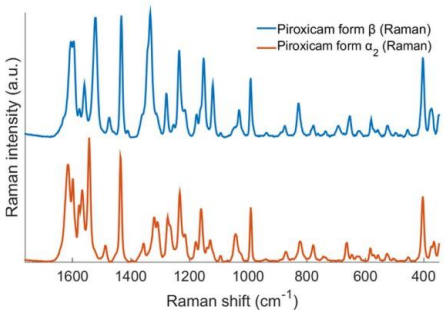


**Fig. 4.** Raman spectra of the form β (top blue) and the form α2 (bottom red) of piroxicam (acquisition parameters: 3 scans of 2 s).

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

## SERS INVESTIGATION OF PIROXICAM POLYMORPHS

The SERS characterization of piroxicam has already been studied by Hernandez et al. (2016) in colloidal solutions at different pH conditions. They reported the SERS spectra of the anionic form of piroxicam adsorbed on the Ag Nps surface in DMSO/water solution. In accordance with their results, well-resolved bands in the SERS spectrum obtained from the solid form β were observed, as shown in Fig. 5. It presents bands similar to the SERS spectra of piroxicam in solution reported in literature (Hernandez et al., 2016), notably in the 1420 – 1480 $cm^{-1}$ and 1540 – 1600 $cm^{-1}$ spectral range. This indicates that the interaction of the compound in solution with metallic surfaces is similar to the interaction of Ag Nps on the solid sample.

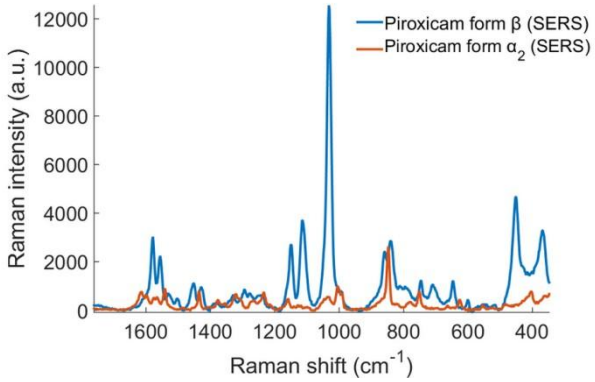


**Fig. 5.** SERS spectra of the form β (top blue) and the form $\alpha_2$ (bottom red) of piroxicam (acquisition parameters: 1 scan of 1 s).

As illustrated in Fig. 5, the SERS spectra can be used to discriminate the different polymorphs of piroxicam. Indeed, the interaction of crystalline forms with the metallic surface highlighted spectral differences between both forms. Form β was characterized by the presence of one predominant band located at 1032 $cm^{-1}$ assigned to the pyridyl group. The SERS spectrum of the β crystallite present also bands in the 1120 – 1160 $cm^{-1}$ and 1590 – 1600 $cm^{-1}$ region, associated to the benzothiazene ring and sulfonamide group, respectively, according to the table of band assignments given in literature (Hernandez et al., 2016; Long, 2004). However, the SERS spectrum of the form $\alpha_2$ was similar to its own Raman spectrum, especially in the 1400 – 1600 $cm^{-1}$ range, except that the SERS signal exhibited a decrease of the signal intensity. The differences of exaltation between the Raman and SERS spectra of both forms are displayed in Fig. 6.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

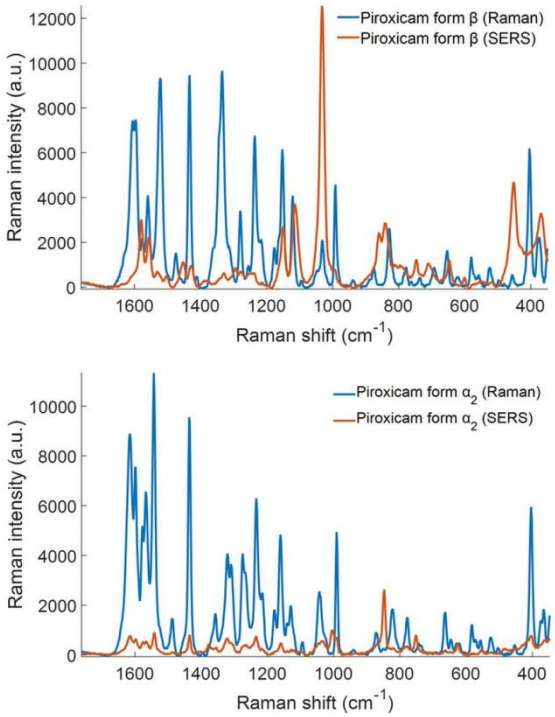


**Fig. 6.** Comparison of Raman (blue) and SERS (red) spectra of piroxicam form β (top) and form $\alpha_2$ (bottom).

The results highlight a great intensification of the SERS band situated at 1032 $cm^{-1}$ characteristic of the form β, as discussed previously, compared to the conventional Raman spectrum. In case of polymorph $\alpha_2$, the SERS spectrum has a lower signal intensity making its detection difficult due to the low SNR upon addition of silver colloids. Different adsorption orientation of the molecule to the metallic surface can explain these different SERS enhancement between both crystalline forms. Starting from this consideration, an analysis of preferred orientation from a crystallographic point of view was performed.

## PREFERRED ORIENTATION OF CRYSTALLINE FORM $A_2$ IN TABLETS

As reported in section 3.1.2, the piroxicam β and $\alpha_2$ polymorphs have different crystal shapes. The more anisotropic morphology of the $\alpha_2$ crystallites might enhance preferred orientation (also called texture) of crystals during the preparation of tablets. Preferred orientation occurs when the crystallites are not truly randomly oriented within the samples (Tenho et al., 2007). This phenomenon is described when the crystallographic planes associated to the Miller indices $(h\ k\ l)$ comprising the sample, are orientated in the same direction (Koivisto et al., 2004). The more isotropic shape of the β crystallites could prevent, or at least reduce, a preferred orientation leading to a better random orientation of the β crystallites. A comparison of the relative intensities between the experimental diffractograms and the theoretical values (single crystal), exposed in section 3.1.1, suggests a preferred orientation of the crystallites in the samples of forms β and $\alpha_2$. Regarding the polymorph $\alpha_2$, preferred orientation of the crystallites mostly occurred with the compaction because the XRPD profile of the tablet compared to

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

the theoretical profile showed clear differences of intensity ratios. In the case of the polymorph β, the XRPD profiles, before and after compression were similar to each other but different in intensity ratios from the theoretical data. This indicates that no preferred orientation appeared upon compression, the latter being rather intrinsic of the batch.

In this way, XRPD studies were performed on tablets in the studied concentration range in order to ensure that preferred orientation of form $\alpha_2$ is maintained with the blending effect but also to correlate the low SERS signal intensity with texture. The XRPD diffractograms of the different samples, displayed in Fig. S-2, were compared to the XRPD pattern of a tablet solely composed of forms $\alpha_2$ and presenting a strong texture. The results of the XRPD study could thus provide information about the orientation of the molecules in tablets.

The variations in the intensity of the diffraction maximum can be explained by a texture phenomenon (Riippi et al., 2000). Thus, in a textured XRPD pattern, the intensities of the peaks corresponding to preferred orientations of crystallographic planes are disproportionally higher than those of the peaks which are not involved in texture. In the case of the polymorphic form $\alpha_2$, a qualitative way to detect a preferred orientation in tablets is to compare the difference of relative intensities extracted from the XRPD data of the different tablets (i.e. supposed to be textured) with those extracted from the XRPD theoretical data (i.e. by principle not textured). The relative intensities are calculated according to different reference peaks of form $\alpha_2$ (23 in total) which correspond to each peak in the range 8° - 27° $(2\theta)$. A plot of the difference of the averaged relative intensity between the XRPD patterns is displayed in Fig. 7. From sample 1 to 5, only relevant peaks have been taken into account, the other peaks being irrelevant due to the strong overlap with lactose monohydrate.

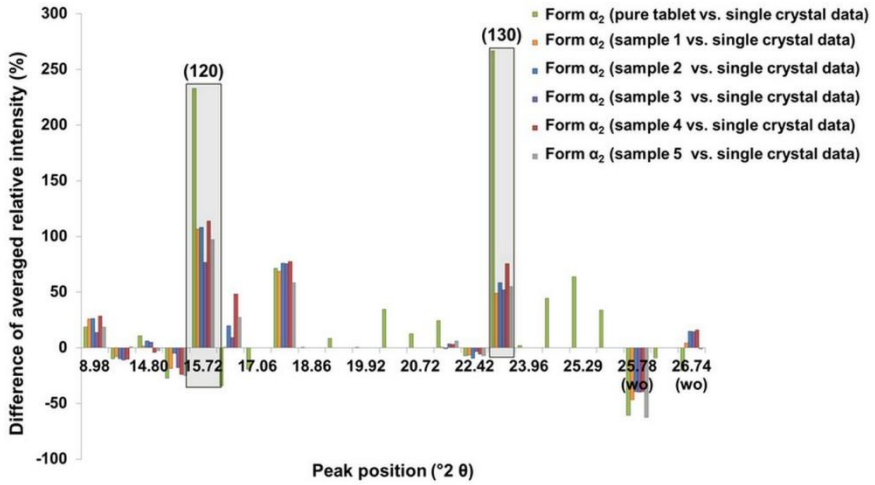


**Fig. 7.** Difference of averaged relative intensity of the $\alpha_2$ polymorph between tablets at different compositions and single crystal theoretical values (wo: weak overlap with the peaks of lactose monohydrate).

In the case of the pure form $\alpha_2$, two peaks located approximately at 15.72° and 22.96° $(2\theta)$ exhibit a clear cut difference of relative intensity (higher than 200%) corresponding to the crystallographic planes $(1\ 2\ 0)$ and $(1\ 3\ 0)$, respectively. Therefore, faces mostly oriented mainly belong to the family of planes $(1\ k\ 0)$. Concerning the five samples of the studied concentration range, the relative

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

intensities decrease by dilution effect on all the crystallographic planes $(h\ k\ l)$. However, a preferred orientation is maintained, mainly of the planes $(1\ k\ 0)$ according to the significant relative intensity (around 100%) even by strong dilution down to 8% (w/w) of the polymorphic form $\alpha_2$, corresponding to sample 1.

In addition, the SEM images of the different samples confirmed a preservation of a preferred orientation. Some clusters of form $\alpha_2$ are presented on the sample surface and are mainly lying flat, as displayed in Fig. 8a for sample 1 composed of 2% (w/w) of form $\beta$ and 8% (w/w) of form $\alpha_2$. The SEM images of the remaining samples of the studied concentration range are presented in the supplementary material (Fig. S-3).

The results are in line with a preferred orientation of the $(1\ k\ 0)$ crystal planes in the samples, even after mixing, the molecule being vertically oriented to the sample surface, as displayed in Fig. 8b. Consequently, texture and vertical orientation of the molecules are probably correlated to the low SERS signal enhancement. However, it is important to note that this approach based on XRPD data is an average result of the sample surface and remains
qualitative.

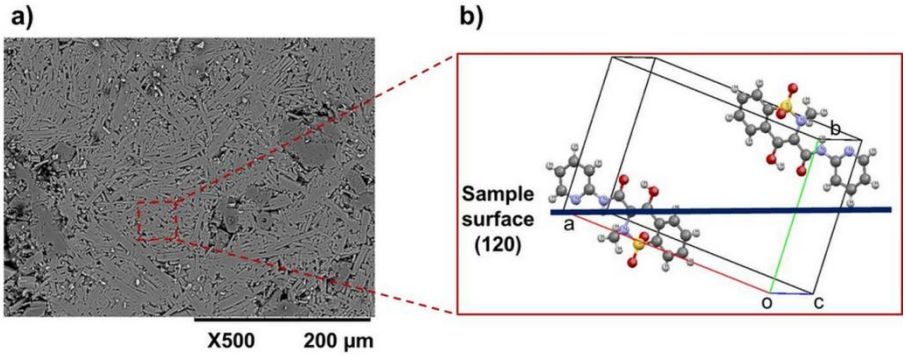


**Fig. 8.** a) SEM images of sample 1 surface containing 2% (w/w) of form $\beta$, 8% (w/w) of form $\alpha_2$ and 90% (w/w) of lactose monohydrate. b) Molecular orientation of piroxicam in $\alpha_2$ polymorph for the crystallographic planes $(1\ 2\ 0)$ which is vertically oriented to the sample surface. Atom color code: carbon (grey), nitrogen (magenta), sulphur (yellow), oxygen (red), hydrogen (white).

Regarding form $\beta$, a plot of the difference of the averaged relative intensity between the XRPD of a pure tablet compared to the theoretical values is displayed in Fig. S-4. A single peak located at 27.41° $(2\theta)$ exhibits a clear cut difference of relative intensity (200%) which corresponds to the crystallographic planes (-2 1 2), the molecule being horizontally oriented to the sample surface, as shown in Fig. S-5. The preservation of texture in the concentration range could not be studied due to the XRPD limit of detection of form $\beta$. Consequently, no correlation between the molecular orientation of form $\beta$ and the enhancement of the SERS signal could be confirmed.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

## SEMI-QUANTITATIVE APPROACH TO DETECT LOW DOSES OF THE POLYMORPH FORM B IN TABLETS USING MCR-ALS

A suitable method to extract semi-quantitative information from chemical mixtures is the MCR-ALS technique. In this study, an initial estimate that contains the individual responses of the pure compounds was required. This condition was fulfilled by acquiring reference spectra of the three different tablet constituents. The spectra were acquired with the same equipment and with the same parameters as the images. The same pre-processing was also applied on the reference spectra (see section 2.6.1). To reduce rotational ambiguities, nonnegativity constraints of spectral concentrations and intensities were applied during the optimization process.

Raman hyperspectral images without silver nanoparticles were first investigated in order to ensure that no signal of piroxicam polymorphic form β was detectable below the Raman spectroscopy limit of detection. The MCR-ALS results presented in this section are mainly focused on the form β which is the compound of interest presents at low concentrations whereas the other constituents are present in large amount and have thus no interest to be analyzed in SERS. Fig. S-6 in the supplementary material shows the calculated MCR-ALS signals of form β compared to its reference Raman spectrum. The distribution maps associated with the low dose crystalline form are shown on the top of Fig. 9. The quality of the calculated MCR-ALS spectra was characterized by the correlation coefficients between the resolved profiles and the reference spectrum in Table S-1 (Supplementary material). For the analysis, it was considered that the polymorph was not detected when the resolved spectra had a correlation coefficient lower than 0.80 which was the case when the polymorph form β content was below 0.1% (w/w).

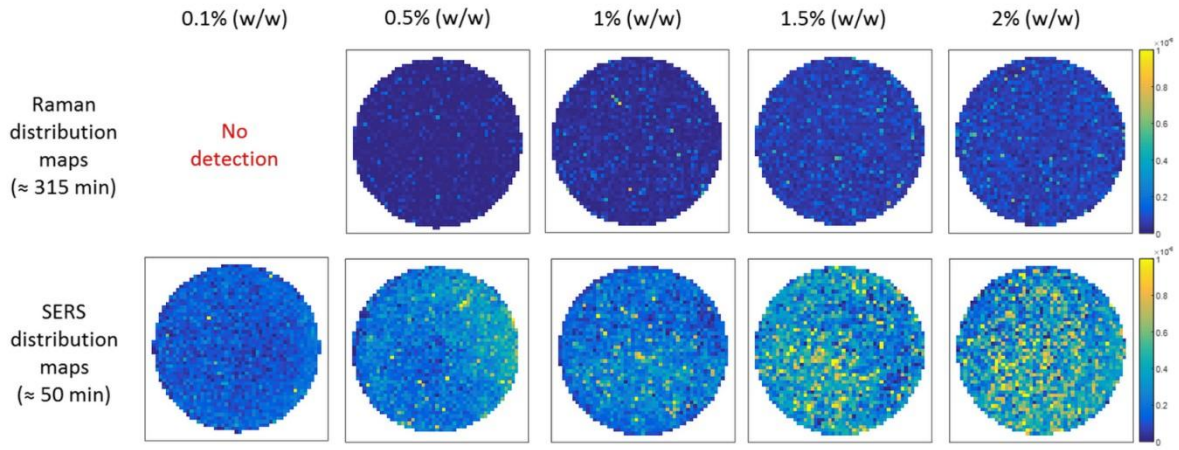


**Fig. 9.** Raman and SERS distribution maps of the polymorphic form β obtained by MCR-ALS for the five pharmaceutical mixtures.

The distribution maps associated to the SER-CI results are presented on the bottom of Fig. 9. This latter, showed a visual comparison of the relative concentration SER-CI images compared to the Raman concentration obtained with much longer measurement time. We can distinguish that the location of

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

the agglomerates of piroxicam form β were relatively similar between the corresponding distribution maps. The difference between varying polymorphic form β concentrations can be very easily recognized visually based on the SERS maps which demonstrate the better sensitivity of the SER-CI investigations. Indeed, the SERS signal of the crystalline form β was detected from the pharmaceutical mixture of 0.1% (w/w) which is not the case for the Raman signal. The pure spectrum of the polymorphic form β and the resolved spectra obtained by MCR-ALS are displayed in Fig. 10. The correlations between calculated spectra and reference SERS spectrum of form β were respectively equal to 0.98, 0.97, 0.98, 0.97 and 0.98, as shown in Table 2. A good correlation has been achieved between the spectra ensuring an appropriate resolution of the studied system.

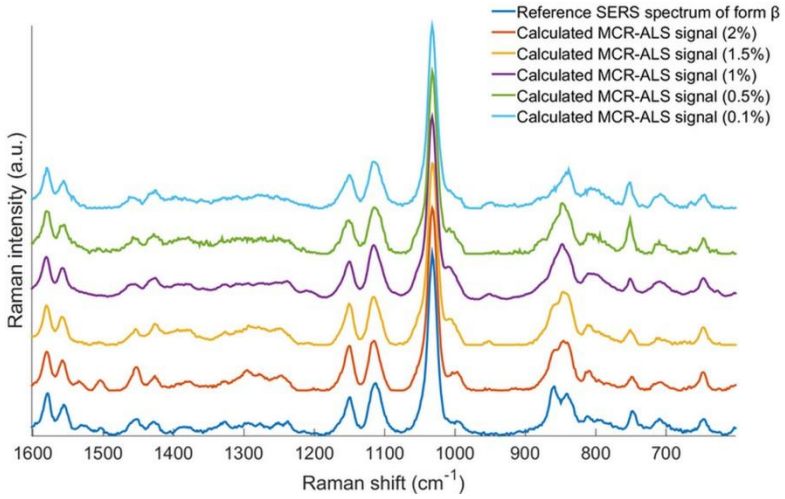


**Fig. 10.** Calculated MCR-ALS signals of piroxicam form β from the different pharmaceutical mixtures versus reference SERS spectrum of polymorphic form β.

**Table 2.** Correlation coefficients of calculated MCR-ALS signals and reference Raman spectrum of piroxicam form β.

| Calculated spectra | Reference SERS spectrum Piroxicam form β |
|---|---|
| Sample 1 | 0.98 |
| Sample 2 | 0.97 |
| Sample 3 | 0.98 |
| Sample 4 | 0.97 |
| Sample 5 | 0.98 |

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

In addition to the better sensitivity of SER-CI compared to conventional Raman spectroscopy, it also has the advantage to be less time-consuming. This has already been established in other studies (Firkala et al., 2013; De Bleye et al., 2014; Firkala et al., 2015). Due the exaltation of the signal, the SERS spectra can be acquired with short exposure time while delivering more information about the compound of interest. Considering the acquisition parameters used for the study, each Raman map acquisition took approximately 315 minutes while each SER-CI measurement lasted only 50 minutes for the same number of pixels with a spatial resolution set at 200 µm for both methods. This is 6 times faster than conventional Raman acquisitions, which is a significant advantage to obtain the relevant information rapidly and to solve urgent industrial issues in an effective way. The significant advantage of SER-CI can also enable to increase the spatial resolution of the images in order to facilitate the identification of low dose compounds which can be difficult due to the insufficient spectral contribution or lack of pure pixels on the map (Boiret et al., 2015). For example, by increasing the spatial resolution of SER-CI maps from 200 µm to 90 µm, the mapping acquisition time of SER-CI maps takes about 300 minutes which is roughly equal of the acquisition time for one Raman map with a spatial resolution set at 200 µm.

# Conclusion

For the first time, SERS was used in a polymorphism study on pharmaceutical tablets. This publication reported the investigation and the SERS characterization of two polymorphic forms of piroxicam, used as model molecule. The SERS spectra of both forms showed spectral differences in terms of bands position and also in intensity allowing the discrimination of the different polymorphs. The study of both crystalline forms suggested different orientations of the molecules to the metal surface which can explain the SERS exaltation differences. The XRPD studies demonstrated a preferred orientation of the $(1\ k\ 0)$ and $(-2\ 1\ 2)$ crystallographic planes in the tablet of form $\alpha_2$ and $\beta$, respectively. Based on these results, it was established that the degree of texture in tablets could be correlated with the SERS signal exaltation of the analyte. The polymorph form $\beta$, which presents a higher SERS signal exaltation compared to the form $\alpha_2$, may involve a better orientation of the molecules in the sample favoring the SERS detection of the crystallite. The present study proved that a preferred orientation of the sample can have an impact on the SERS activity of the target analyte. The close relationship between polymorphism, preferred orientation and SERS effect has never been mentioned in literature to date.

Moreover, a semi-quantitative SERS approach using an MCR-ALS algorithm was applied on

Raman and SERS images in order to study the distribution of low doses of the crystalline form $\beta$ within a pharmaceutical drug mixture. While conventional Raman measurement took over 315 minutes without giving semi-quantitative information, SER-CI combined with multivariate data analysis was found to be suitable for the detection of the crystallite $\beta$ at 0.1% (w/w) content. By offering the possibility to acquire images with a high sensitivity and a short mapping acquisition time, SER-CI coupled with appropriate chemometric methods appears as a promising analytical tool to detect low doses of polymorphic forms within a pharmaceutical formulation.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

To conclude, this paper demonstrates the feasibility of SERS to differentiate two polymorphs and to extract the contribution of a low dose of piroxicam form β below the Raman detection limit in a solid drug product. It is also obvious that the molecular orientation plays a role in the SERS activity. This is an additional parameter to consider in future work combining polymorphism studies and SERS.

## Acknowledgements

The authors would like to thank the National Fund for Scientific Research (FNRS-F.R.S.) (1.A030.17 - FC6921) for the research grant accorded to E. Dumont.

The Walloon Region is also gratefully acknowledged for the funding of P.Y. Sacré.

## References

Bellur Atici, E., Karliga, B., 2015. Quantitative determination of two polymorphic forms of imatinib mesylate in a drug substance and tablet formulation by X-ray powder diffraction, differential scanning calorimetry and attenuated total reflectance Fourier transform infrared spectroscopy, J. Pharm. Biomed. Anal. 114, 330-340.

Boiret, M., Rutledge, D.N., Gorretta, N., Ginot, Y.M., Roger, J.M., 2014. Application of independent component analysis on Raman images of a pharmaceutical drug product: Pure spectra determination and spatial distribution of constituents, J. Pharm. Biomed. Anal. 90, 78–84.

Boiret, M., De Juan, A., Gorretta, N., Ginot, Y.M., Roger, J.M., 2015. Distribution of a low dose compound within pharmaceutical tablet by using multivariate curve resolution on Raman hyperspectral images, J. Pharm. Biomed. Anal. 103, 35–43.

Bunaciu, A.A., Aboul-Enein, H.Y., Hoang, V.D., 2015. Vibrational spectroscopy used in polymorphic analysis, Trends Anal. Chem. 69, 14–22.

Cailletaud, J., De Bleye, C., Dumont, E., Sacré, P.Y., Netchacovitch, L., Gut, Y., Boiret, M., Ginot, Y.M., Hubert, Ph., Ziemons, E., 2017. Critical review of surface-enhanced Raman spectroscopy applications in the pharmaceutical field, J. Pharm. Biomed. Anal. 147, 458-472.

Cailletaud, J., De Bleye, C., Dumont, E., Sacré, P.Y., Gut, Y., Bultel, L., Ginot, Y.M., Hubert, Ph., Ziemons, E., 2018. Towards a spray-coating method for the detection of low-dose compounds in pharmaceutical tablets using surface-enhanced Raman chemical imaging (SER-CI), Talanta 188, 584-592.

De Bleye, C., Sacré, P.Y., Dumont, E., Netchacovitch, L., Chavez, P.F., Piel, G., Lebrun, P., Hubert, Ph., Ziemons, E., 2014. Development of a quantitative approach using surfaceenhanced Raman chemical imaging: First step for the determination of an impurity in a pharmaceutical model, J. Pharm. Biomed. Anal. 90, 111-118.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

Farkas, A., Nagy, B., Démuth, B., Balogh, A., Pataki, H., Nagy, Z.K., Marosi, G., Variable clustering and spectral angle mapper-orthogonal projection method for Raman mapping of compound detection in tablets, J. Chemom. 31, e2861.

Firkala, T., Farkas, A., Vajna, B., Farkas, I., Marosi, G., 2013. Investigation of drug distribution in tablets using surface enhanced Raman chemical imaging, J. Pharm. Biomed. Anal. 76, 145-151.

Firkala, T., Farkas, A., Vajna, B., Nagy, Z.K., Pokol, G., Marosi, G., Szilagyi, I.M., 2015. Quantification of low drug concentration in model formulations with multivariate analysis using surface enhanced Raman chemical imaging, J. Pharm. Biomed. Anal. 107, 318-324.

Fortunato de Carvalho Rocha, W., Sabin, G.P., Março, P.H., Poppi, R.J., 2011. Quantitative analysis of piroxicam polymorphs pharmaceutical mixtures by hyperspectral imaging and chemometrics, Chemom. Intell. Lab. Syst. 106, 198–204.

Gourvénec, S., Lamotte, C., Pestiaux, P., Massart, D.L., 2003. Use of the orthogonal projection approach (OPA) to monitor batch processes, Appl. Spectrosc. 57, 80-87.

Griffen, J., Owen, A., Burley, J., Taresco, V., Matousek, P., 2016. Rapid quantification of low level polymorph content in a solid dose form using transmission Raman spectroscopy, J. Pharm. Biomed. Anal. 128, 35–45.

Hennigan, M.C., Ryder, A.G., 2013. Quantitative polymorph contaminant analysis in tablets using Raman and near infra-red spectroscopies, J. Pharm. Biomed. Anal. 72, 163-171.

Hernandez, M., Corda, E., Garcia-Ramos, J.V., Domingo, C., Sevilla, P., 2016. SERS of the anti-inflammatory drug piroxicam adsorbed on the surface of silver or gold colloids as nanocarrier model, J. Raman Spectrosc. 47, 402-407.

Hugelier, S., Devos, O., Ruckebusch, C., 2015. On the implementation of spatial constraints in multivariate curve resolution alternating least squares for hyperspectral image analysis, J. Chemometr. 29, 557-561.

Kang, Y., Shao, Z., Wang, Q., Hu, X., Yu, D., 2018. Quantitation of polymorphic impurity in entecavir polymorphic mixtures using powder X-ray diffractometry and Raman spectroscopy, J. Pharm. Biomed. Anal. 158, 28–37.

Koivisto, M., Virjonen, T., Heikkilä, T., Lehto, V.P., 2004. Characterization of the preferred orientation of d-mannitol crystallites in tablets, J. Pharm. Biomed. Anal. 36, 559-564.

Lee, P.C., Meisel, D., 1982. Adsorption and surface-enhanced Raman of dyes on silver and gold sols, J. Phys. Chem. 86, 3391-3395.

Lipiäinen, T., Fraser-Miller, S.J., Gordon, K.C., Strachan, C.J., 2018. Direct comparison of low- and mid-frequency Raman spectroscopy for quantitative solid-state pharmaceutical analysis, J. Pharm. Biomed. Anal. 149, 343-350.

Long, D.A., 2004. Infrared and Raman characteristic group frequencies. Tables and charts George Socrates John Wiley and Sons, Ltd, Chichester, Third Edition, 2001, J. Raman Spectrosc. 35, 905.

*Published in : International Journal of Pharmaceutics (2020), 574, p. 118913*
*DOI: 10.1016/j.ijpharm.2019.118913*
*Status : Postprint (Author's version)*

Paiva, E.M., da Silva, V.H., Poppi, R.J., Pereira, C.F., Rohwedder, J.J.R., 2018. Comparison of macro and micro Raman measurement for reliable quantitative analysis of pharmaceutical polymorphs, J. Pharm. Biomed. Anal. 157, 107-115.

Qiu, J.b., Li, G., Sheng, Y., Zhu, M.r., 2015. Quantification of febuxostat polymorphs using powder X-ray diffraction technique, J. Pharm. Biomed. Anal. 107, 298–303.

Quiñones, R., Iuliucci, R.J., Behnke, G., Brown, R., Shoup, D., Riedel, T.M., Plavchak, C., Lininger, B.E., Spehar, J.M., 2018. Moving towards fast characterization of polymorphic drugs by solid-state NMR spectroscopy, J. Pharm. Biomed. Anal. 148, 163-169.

Redenti, E., Zanol, M., Ventura, P., Fronza, G., Comotti, A., Taddei, P., Bertoluzza, A., 1999. Raman and solid state 13C-NMR investigation of the structure of the 1: 1 amorphous piroxicam : β-cyclodextrin inclusion compound, Biospectroscopy 5, 243-251.

Riippi, M., Tanninen, V.P., Yliruusi, J., 2000. Effect of compression force on the crystal properties of erythromycin acistrate tablets, Eur. J. Pharm. Biopharm. 50, 365-371.

Sacré, P.Y., De Bleye, C., Chavez, P.F., Netchacovitch, L., Hubert, Ph., Ziemons, E., 2014. Data processing of vibrational chemical imaging for pharmaceutical applications, J. Pharm. Biomed. Anal. 101, 123-140.

Sheth, A.R., Bates, S., Muller, F.X., Grant, D.J.W., 2004. Polymorphism in piroxicam, Cryst. Growth Des. 4, 1091-1098.

Tenho, M., Heinänen, P., Tanninen, V.P., Lehto, V.P., 2007. Does the preferred orientation of crystallites in tablets affect the intrinsic dissolution?, J. Pharm. Biomed. Anal. 43, 1315-1323.

Tinmanee, R., Larsen, S.C., Morris, K.R., Kirsch, L.E., 2017. Quantification of gabapentin polymorphs in gabapentin/excipient mixtures using solid state 13C NMR spectroscopy and X-ray powder diffraction, J. Pharm. Biomed. Anal. 146, 29–36.

Vidal, M., Amigo, J.M., 2012. Pre-processing of hyperspectral images. Essential steps before image analysis, Chemom. Intell. Lab. Syst. 117, 138-148.

Vrecer, F., Vrbinc, M., Meden, A., 2003. Characterization of piroxicam crystal modifications, Int. J. Pharm. 256, 3–15.

Windig, W., Guilment, J., 1991. Interactive self-modeling mixture analysis, Anal. Chem. 63, 1425-1432.

Zhang, X., De Juan, A., Tauler, R., 2016. Local rank-based spatial information for improvement of remote sensing hyperspectral imaging resolution, Talanta 146, 1–9.