

GLOBAL APPROACH FOR THE VALIDATION OF AN IN-LINE RAMAN SPECTROSCOPIC METHOD TO DETERMINE THE API CONTENT IN REAL-TIME DURING A HOT-MELT EXTRUSION PROCESS

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Abstract

Since the Food and Drug Administration (FDA) published a guidance based on the Process Analytical Technology (PAT) approach, real-time analyses during manufacturing processes are in real expansion.

In this study, in-line Raman spectroscopic analyses were performed during a Hot-Melt Extrusion (HME) process to determine the Active Pharmaceutical Ingredient (API) content in real-time. The method was validated based on a univariate and a multivariate approach and the analytical performances of the obtained models were compared. Moreover, on one hand, in-line data were correlated with the real API concentration present in the sample quantified by a previously validated off-line confocal Raman microspectroscopic method. On the other hand, in-line data were also treated in function of the concentration based on the weighing of the components in the prepared mixture. The importance of developing quantitative methods based on the use of a reference method was thus highlighted. The method was validated according to the total error approach fixing the acceptance limits at $\pm 15\%$ and the α risk at $\pm 5\%$. This method reaches the requirements of the European Pharmacopeia norms for the uniformity of content of single-dose preparations. The validation proves that future results will be in the acceptance limits with a previously defined probability.

Finally, the in-line validated method was compared with the off-line one to demonstrate its ability to be used in routine analyses.

Keywords:

In-line validation, Real-time analysis, Raman spectroscopy, Hot-melt extrusion, API content, Accuracy profile

1. Introduction

In the pharmaceutical field, more and more applications used the Process Analytical Technology (PAT) approach to monitor their manufacturing process in real-time time, as shown in Fig. 1 [1]. Indeed, since the Food and Drug Administration (FDA) published in 2004 a guidance for industry entitled [2]: “PAT – A framework for innovative pharmaceutical development, manufacturing and quality assurance”, the number of studies focused on the development of realtime analyses during a manufacturing process increased.

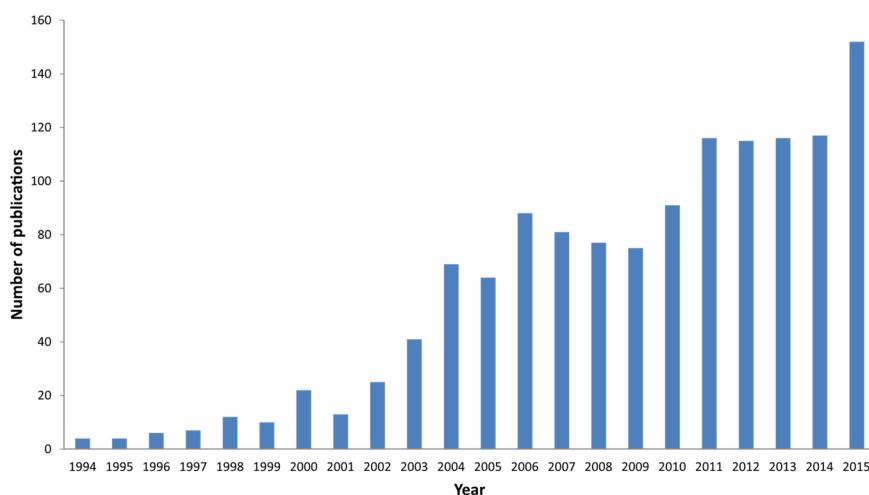


Fig. 1. Representation of the number of publications in the 1994–2015 period, illustrating the evolution of the application of Process Analytical Technology (PAT) tools. Data were obtained with the database Scopus® and using the analyze TITLE (Process Analytical Technology) AND (LIMIT TO (SUBJAREA, "PHAR")). These data were assessed on January 2017.

The objective of the PAT approach is to analyze and control the process and the product during the manufacturing by implementing specific tools leading to the development of on- or in-line methods. During the last decade, applications in the pharmaceutical domain applying this approach spread out as can be read in the review Netchacovitch et al. [1]. It is possible to notice that vibrational spectroscopy (IR, NIR and Raman) was often used as PAT tool [1– 5]. Raman spectroscopy is a non-destructive and non-invasive technique needing no or few sample preparation. The sample is irradiated by a monochromatic radiation and the scattered light is analyzed by the spectrometer. The Raman spectrum represents thus the intensity of the scattering in function of the wavelength. According to the acquired Raman spectrum, it is possible to perform qualitative and quantitative analyses determining the chemical composition of a sample, the polymorphism and solid-state, the content of the Active Pharmaceutical Ingredient by measuring the intensity of a Raman specific band, and so on [6,7]. During this work, Raman spectroscopy was implemented as PAT tool during a Hot-Melt Extrusion (HME) process. The hot-melt extrusion process is a continuous one leading to an increase of the API bioavailability. The pharmaceutical mixture goes through a die under controlled conditions (pressure, temperature, feed rate and screw speed) in order to form a solid dispersion. The API state conversion is a critical step during a HME process and can be studied by Raman spectroscopy [8]. As recent works, Monteyne et al. studied the link between rheology and continuous twin-screw melt granulation on molecular level. They used in-line Raman spectroscopy and in situ Fourier Transform Infrared during twin screw hot-melt granulation to study the polymorphic conversion of anhydrous caffeine in order to enhance the process understanding and development [9]. The research of Van Renterghem et al. was focused on a mini hot-melt extrusion process using in-line Raman spectroscopy

[10] and compared this research with the results of Saerens et al. [11,12] performed on a larger scale extruder. The trend is also to develop in-line quantitative methods based on a validation protocol. Saerens et al. [13] validated an in-line Raman spectroscopic method determining the API content during a hot-melt extrusion process. The validation protocol was based on the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) approach, the accuracy profile [14–17]. This approach is also used in this study and is exposed in the following paragraph.

In this study, the validity of the method was also demonstrated according to a risk-based protocol using the accuracy profile as decision tool. Indeed, the SFSTP commission developed a simple and visual decision tool for the validation of quantitative analytical procedures. It was based on the total error (bias and standard deviation) of the method. First, based on the calibration samples, a response function was selected. It represented the relationship between the response and the analyte quantity. Then, the bias and precision of the method were estimated. The bias represented the systematic error while the precision represented the random error. Using these estimations, it was possible to determine the accuracy of the method including limitations such as the β -expectation tolerance interval representing an area where the future results will fall and the acceptance limits fixed by the analyst taking into account the intended use of the method [14–17].

This study was focused on the development and validation of a quantitative in-line Raman spectroscopic method implemented on a hot-melt extrusion process in order to quantify the API content in realtime. The validation protocol was based on the ICH Q2 (R1) guidelines [18] and the total error approach was applied. Moreover, the importance of using a reference method to determine the accuracy of the validated method was demonstrated. This is an important step when developing HME processes. Finally, the ability to use this in-line method in routine was demonstrated by comparing the concentration of test samples estimated with the in-line method and with the off-line reference one.

2. Materials and methods

2.1. Chemicals

Itraconazole (ITZ) was purchased from Lee Pharma Ltd. (Andhra, Pradesh, India), Soluplus[®] and Croscarmellose sodium (AcDiSol[®]) were provided from BASF (Ludwigshaven, Germany) and FMC Biopolymer (Brussels, Belgium), respectively.

2.2. Hot-melt extrusion process

Mixtures containing 70%, 85%, 100% and 110% of itraconazole (ITZ) targeted concentration were prepared as shown in Table 1. They were composed of ITZ, AcDiSol[®] and Soluplus[®]. 150 g of these mixtures were extruded.

The mixtures were premixed in a TURBULA[®] Shaker-Mixer for 20 min and then put inside the volumetric feeder to be extruded. An 18 mm twin screw hot-melt extruder was used (L/D=32, Scamex[®], Crosne, France) with a common screw configuration containing two kneading zones (45° forward). The temperature gradient (70–135–145–155 °C) was applied to the four heating zones of the barrel. A volumetric feeder was used and the feeding rate was set at 4 rpm corresponding with the formulation mixture to 6g min⁻¹. The

rotational screw speed was set at 100 rpm. The melt was analyzed during the extrusion process by an in-line Raman spectroscopic probe and extrudates were collected after cooling to ambient temperature for offline analysis.

Table 1. Mixtures preparation for the validation of a quantitative in-line spectroscopic method for the determination of ITZ content during a hot-melt extrusion process.

Calibration standards	%ITZ	%ITZ inside the mixture	ITZ (g)	AcDiSol® (g)	Soluplus® (g)
	70	17.5	26.25	3.75	120.00
	100	25	37.50	3.75	108.75
	110	27.5	41.25	3.75	105.00
Validation standards	%ITZ	%ITZ inside the mixture	ITZ (g)	AcDiSol® (g)	Soluplus® (g)
	70	17.5	26.25	3.75	120.00
	85	21.25	31.88	3.75	114.37
	100	25	37.50	3.75	108.75
	110	27.5	41.25	3.75	105.00

2.3. Validation protocol

The in-line Raman spectroscopic method was validated following the ICH Q2 (R1) guidelines [18].

Calibration standards were prepared at three concentration levels (70%, 100% and 110% of the API targeted concentration) and validation standards were prepared at four levels of concentration (70%, 85%, 100% and 110% of the API targeted concentration) as presented in Table 1. These sets of calibration and validation standards were prepared independently four times (four validation series).

2.4. Equipment

2.4.1. In-line Raman analysis

Spectra were collected in real-time during the extrusion process using a dispersive Raman spectrometer, the RamanStation 400F (Perkin Elmer, MA, USA) implemented with an extruder Dynisco Raman probe (Kaiser Optical Systems Inc., MI, USA). The apparatus is equipped with a two-dimensional charge-coupled device (CCD) detector (1024×256 pixels) working at -50 °C. The used laser has an excitation wavelength of 785 nm and its power was set at 100 mW. The melt was analyzed, using the single point mode, with 1 acquisition of 15 s over a spectral range going from 250 to 3200 cm⁻¹. The spectral resolution was fixed at 4 cm⁻¹. These parameters are summarized in the first part of Table 2.

2.4.2. Off-line reference method

A previously validated off-line confocal Raman microspectroscopic method was used as reference method to determine the real percentage of ITZ in the extrudates [19]. Before analyzing the samples, their surface was prepared using a Leica EM Rapid milling system equipped with a tungsten carbide miller (Leica Microsystems GmbH, Wetzlar, Germany) in order to obtain a plane and smooth surface. The samples were then analyzed using a Labram HR Evolution (Horiba scientific, Kyoto, Japan) apparatus. It is equipped with a two-dimensional 970 front-illuminated EMCCD detector (1600×200 pixel sensor) (Andor Technology Ltd.) and a 785 nm laser (XTRA II single frequency diode laser, Toptica Photonics AG). A Leica 50x Fluotar LWD objective (NA=0.55, Wetzlar, Germany) was selected. The laser power applied was set at 100%,

corresponding to a laser power of 20 mW on samples. Samples were analyzed at 1200 cm^{-1} with a 300 gr/mm grating covering a spectral range from 464 cm^{-1} to 1852 cm^{-1} . Four accumulations of 1 s were performed. A confocal slit-hole fixed at $200\text{ }\mu\text{m}$, a binning factor of 1 and an Electron-Multiplying gain of 50 were applied. A point mapping mode was used to analyze the extrudates surface. The mapped area was $250\times 250\text{ }\mu\text{m}^2$ analyzing a map of 10 pixels on 10 pixels. These parameters are summarized in the second part of Table 2.

Table 2. Summary of the performed treatments and methods.

Approach		Univariate Intensity of ITZ band (1614 cm^{-1})		Multivariate On whole spectrum: remove spikes/Savitsky Golay derivative/Mean Center+PLS regression	
Data treatment		A	B	C	D
In-line analysis to determine API content in real-time during a Hot-Melt Extrusion process	Method	Apparatus	RamanStation 400F		
		Probe	extruder Dynisco Raman probe		
		Detector	CCD		
		Laser	785 nm		
		Power (laser)	100 mW		
		Spectral range	$250\text{--}3200\text{ cm}^{-1}$		
		Acquisition time	15 s		
		Number of acquisitions	1		
Determination of API content in the extrudates	Off-line Reference method	Spectral resolution	4 cm^{-1}		
		Apparatus	Confocal Raman microscope [19]	Theoretical value based on the weight of the components in the mixture	Confocal Raman microscope [19]
		Detector	Labram HR Evolution		Labram HR Evolution
		Objectice	EMCCD		EMCCD
		Laser	Leica 50x LWD		Leica 50x LWD
		Laser	785 nm		785 nm
		Power (laser)	20 mW		20 mW
		Spectral range	$464\text{--}1852\text{ cm}^{-1}$		$464\text{--}1852\text{ cm}^{-1}$
		Acquisition time	1 s		1 s
		Number of acquisitions	4		4
		Grating	300 gr/mm		300 gr/mm
		Confocal slit hole	200 μm		200 μm
		Binning factor	1		1
		EM Gain	20		20
		Map	$250\times 250\text{ }\mu\text{m}^2$		$250\times 250\text{ }\mu\text{m}^2$
			$10\times 10\text{ pixels}$		$10\times 10\text{ pixels}$

2.5. Univariate and multivariate approaches

The in-line data were treated based on two different approaches, a univariate and a multivariate one. For both, two analyses were realized as presented in Table 2. Firstly, one considers the real sample concentration based on an off-line Raman validated reference method. Indeed, for each mixture, six mappings realized by the off-line Raman reference method were compared with the in-line corresponding spectrum (data treatment A and C in Table 2). As often used in the literature, the second data treatment (B and D in Table 2) was based on the calculation of the sample concentration using the weighing of each component in the mixture. Indeed, in several publications, scientists considered that the concentration of their samples corresponded to the weighing of each component. But it is important to keep in mind that it

is only possible if no loss of any component appears during the manufacturing process or if no filling issue occurs impacting on the mixture homogeneity. This is the reason why this study compares the results obtained from the two data treatment, on the one hand by considering the weighing of the components and on the other hand by using a reference method, previously validated, to quantify the real API concentration in the analyzed sample.

2.5.1. Univariate approach

The univariate approach was performed on raw spectra by integrating the ITZ specific band (situated at 1614 cm^{-1}) using the PLS toolbox (EigenVector Research) version 7.5.2. The intensity of this band was calculated and linked to the concentration by a linear regression. Based on these values, the validation was carried out using the e.noval 3.0b software (Arlenda, Liege, Belgium) to build the accuracy profile.

2.5.2. Multivariate approach

The multivariate approach of the validation samples was performed on the whole spectrum to improve the accuracy of the predictive model. By using multivariate analysis, it is possible to include all wavelengths and their interactions leading to a more robust model for future real samples analysis. A preprocessing step was applied on raw spectra. Spikes were first manually removed followed by a Savitsky Golay derivative (filter window=15, polynomial order=2, derivative order=1) and a mean center pre-treatment using Matlab R2016a (The Mathworks, Natick, MA, USA). A Partial Least Squares (PLS) regression was then performed, based on five latent variables, on the pretreated data using the PLS Toolbox (EigenVector Research, Wenatchee, WA, USA) version 7.5.2. The validation based on the PLS regression was realized using the e.noval v3.0b software (Arlenda, Liege, Belgium).

3. Results and discussion

During this study, in-line data were treated according to two approaches, a univariate and a multivariate one. A comparison was made in order to highlight the analytical performances of one approach versus the other. Moreover, the use of a reference method was also tested.

3.1. Univariate analysis

In-line data were first treated using a univariate approach. The validation protocol was analyzed according to the accuracy profile approach based on the total error of the method.

To validate this method, the data obtained during the in-line Raman acquisitions were compared to the real API concentration of the sample using an off-line analysis (as presented in Table 2: data treatment A).

A response function was first selected based on the calibration standards, which was the linear regression model being a simple model for routine analyses. The ICH Q2 (R1) criteria were estimated to build the accuracy profile. These criteria are presented in Table 3.

The trueness or relative bias gives information on the systematic error by calculating the closeness of agreement between a reference value and a mean experimental value. As shown in Table 3 and Fig. 2a, the trueness of this method is close to $\pm 0\%$ except for the 100% API targeted concentration level which has a

trueness of 1.87%. The precision of an analytical method, based on the repeatability and the intermediate precision, indicates on the results dispersion and gives thus information on the random errors of the method. In this study, these estimations are situated around 5%. This value can be explained due to uncontrollable parameters such as the humidity and room temperature. These parameters influence the physicochemical properties of the powders which induce electrostatic interactions with the metallic screw and thus random variations. This variability can be reduced if room humidity and temperature are controlled in the extrusion laboratory. Moreover, when a filling issue occurs, it induces screw speed and melt filling variations in the barrel impacting the Raman focus and thus the Raman spectrum. The uncertainty of the analytical method characterizes the dispersion of the results attributed to the measurand. It estimates an interval around the results where the unknown true value can occur with a confidence level of 95%. For this method, the unknown true value is situated at a maximum of 10.56% around the measured result with a confidence level of 95%.

The acceptance limits were fixed at $\pm 15\%$ in accordance with the uniformity of content of single-dose preparation norm of the European Pharmacopeia [20]. The risk that the future results will fall outside the tolerance interval was set at $\pm 5\%$. The obtained accuracy profile is presented in Fig. 2a. It is possible to conclude that the in-line Raman spectroscopic method quantifying the API content in real-time during a hot-melt extrusion process is valid, for the tested concentration range, by using the univariate approach, with acceptance limits fixed at $\pm 15\%$ and α at $\pm 5\%$, inducing that 95% of future results will fall inside the acceptance limits.

By comparison with the previously validated method, the in-line data were treated identically but compared with the concentration determined according to the weighing in the prepared mixtures (as presented in Table 2: data treatment B). The accuracy profile is presented in Fig. 2b. It is possible to note that the same analytical method is not valid, based on the univariate approach, by considering the weighing of the mixtures as real concentrations. Moreover, the bias and precision are higher showing systematic and random errors coming from the theoretical concentration values.

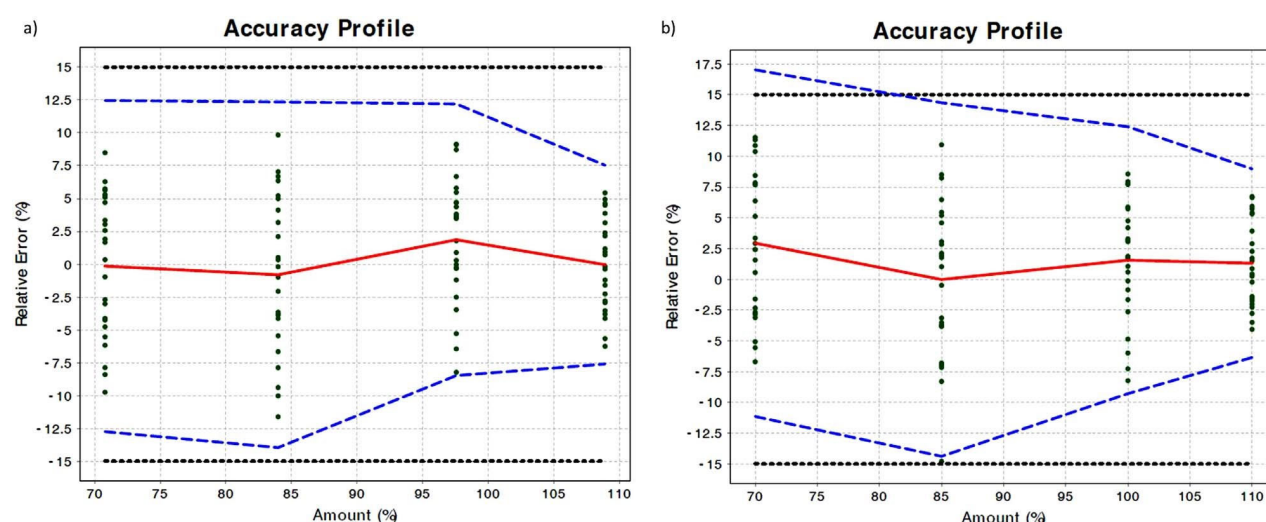


Fig. 2. Accuracy profiles for the validation of the in-line Raman spectroscopic method quantifying the API content in real-time during a hot-melt extrusion process by a univariate approach, a) based on a reference method; b) based on the weighing. The red plain line represents the relative bias, the dashed blue lines are the β -expectation tolerance limits and the black dotted lines represent the acceptance limits. The dots are the relative error of the back-calculated results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This comparison highlights the importance of using a reference method to determine the real concentration of the produced samples.

Table 3. ICH Q2 (R1) validation criteria obtained from the validation of an in-line Raman spectroscopic method developed to determine the API content during a hot-melt extrusion process based on a univariate approach.

API concentration (%)		70	85	100	110
Trueness	Relative bias (%)	-0.14	-0.80	1.87	-0.03
	Repeatability (R.S.D. %)	4.58	5.64	4.67	3.48
Precision	Intermediate precision (R.S.D. %)	5.54	6.08	4.84	3.55
	Uncertainty of the bias (%)	1.28	1.36	1.12	0.88
Uncertainty	Uncertainty (%)	4.12	5.28	4.85	3.96
	Expanded uncertainty (%)	8.25	10.56	9.70	7.92
	Relative expanded uncertainty (%)	11.65	12.57	9.95	7.28
Accuracy	β -expectation tolerance limits (%)	[61.77; 79.61]	[72.27; 94.36]	[89.32; 109.5]	[100.6; 117.1]
Linearity	Range	70–110%			
	Slope	1.024			
	Intercept	-1.868			
	r^2	0.9178			
	LOD	21.45%			

3.2. Multivariate analysis

The same in-line data were secondly treated with a multivariate approach based on a PLS model. This model was obtained by comparing the in-line Raman spectra with the extrudates concentration calculated according to a previously validated reference method (as presented in Table 2: data treatment C).

The PLS model was first evaluated regarding the root mean square errors. They were evaluated around 4%. A linear relationship between the introduced and predicted concentrations was presented in Fig. 3.

According to this PLS model, the ICH Q2 (R1) criteria were estimated and are presented in Table 4.

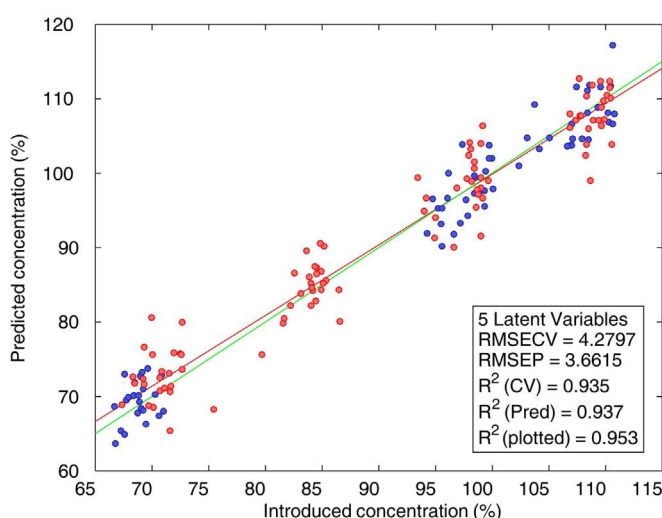


Fig. 3. PLS regression between the introduced and the predicted concentrations (%) for the calibration standards (blue circles) and the validation standards (red circles) for the validation of the in-line Raman spectroscopic method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The trueness of the method has a relative bias lower than 1% except for the 70% API targeted concentration level which has a relative bias of 2.21%. The precision of the method is situated under 5% due to random

variations as explained previously. The uncertainty has a maximum value of 8.00% meaning that the unknown value is situated at a maximum of this value around the measured result.

Table 4. ICH Q2 (R1) validation criteria obtained from the validation of an in-line Raman spectroscopic method developed to determine the API content during a hot-melt extrusion process based on a multivariate approach.

API concentration (%)		70	85	100	110
Trueness	Relative bias (%)	2.21	0.82	0.82	-0.95
Precision	Repeatability (R.S.D. %)	4.96	3.48	3.68	2.54
	Intermediate precision (R.S.D. %)	4.96	3.53	3.96	3.17
Uncertainty	Uncertainty of the bias (%)	0.73	0.64	1.03	1.18
	Uncertainty (%)	3.59	3.03	4.00	3.65
	Expanded uncertainty (%)	7.18	6.06	8.00	7.30
	Relative expanded uncertainty (%)	10.14	7.21	8.20	6.71
Accuracy	β -expectation tolerance limits (%)	[64.90; 79.80]	[78.40; 90.95]	[90.00; 106.7]	[99.89; 115.9]
Linearity	Range	70 – 110%			
	Slope	0.9411			
	Intercept	5.821			
	r^2	0.9407			

According to these estimations and the objective of the method, the accuracy profile was built as shown in Fig. 4a. The acceptance limits were fixed at $\pm 15\%$ in accordance with the uniformity of content of single-dose preparation norm of the European Pharmacopeia. The risk to have measurements falling outside the acceptance limits was fixed at $\pm 5\%$ maximum.

The accuracy profile, presented in Fig. 4a, illustrates that the 95% β -expectation tolerance limits are included inside the acceptance limits over the studied concentration range. It is thus possible to conclude that the in-line Raman spectroscopic method quantifying the API content in real-time during a hot-melt extrusion process is valid, by using the multivariate approach, with acceptance limits fixed at $\pm 15\%$ and α at $\pm 5\%$, proving that each future result will fall within the $\pm 15\%$ acceptance limits with a probability of at least 95%.

As for the univariate approach, a comparison with the previously validated method was made with considering the concentration of the samples based on the weighing of the components in the mixture (as presented in Table 2: data treatment D). The accuracy profile is presented in Fig. 4b. It is possible to note that the same analytical procedure is not valid below 75% of API targeted concentration, based on the multivariate approach, by considering the weighing of the mixtures as concentrations. Once again, this comparison highlights the importance to use a reference method to determine the real concentrations of samples.

3.3. Univariate versus multivariate approach

The analytical performance of the validated method based on the two approaches was performed. It is possible to note that the model of the validated method according to the multivariate approach is more accurate. For both approaches the bias has the same order of magnitude. But the precision has better estimated values for the model based on the multivariate approach proving that the results dispersion is lower. This can be explained by the fact that the data treatment for the multivariate approach includes the whole analyzed spectrum in contrast with the ITZ specific band for the univariate one. The multivariate approach includes thus more variability in its model reducing the results dispersion. These findings are demonstrated in the respective accuracy profiles.

Moreover, in order to support these results, a study was performed and presented in the following paragraph.

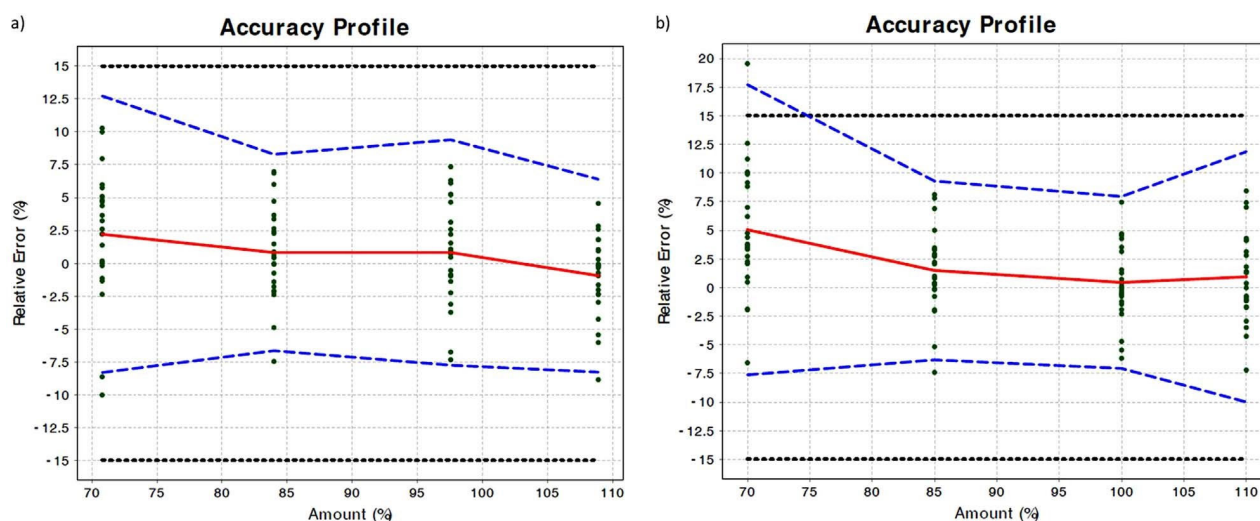


Fig. 4. Accuracy profiles for the validation of the in-line Raman spectroscopic method quantifying the API content in real-time during a hot-melt extrusion process by a multivariate approach, a) based on the reference method concentrations; b) based on the weighing of the components in the mixtures as concentrations. The red plain line represents the relative bias, the dashed blue lines are the β -expectation tolerance limits and the black dotted lines represent the acceptance limits. The dots are the relative error of the back-calculated results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Use of the validated method in routine analysis

The use of the validated method was demonstrated for routine analysis. Seven batches were extruded at 155 °C with a screw speed fixed at 100 rpm and analyzed in-line with a Raman probe with the same acquisition parameters applied for the validation step. Two batches were prepared at 70% of the API targeted concentration, one at 85%, two at 100% and two batches were prepared at 110% of the API targeted concentration. The in-line Raman acquisitions were performed during the extrusion of the whole mixture. Then six extrudates per batch were analyzed off-line with the confocal Raman microspectroscopic validated reference method in order to determine the real ITZ concentration. These concentrations were correlated with the corresponding in-line Raman spectrum. The in-line concentrations were estimated according to the univariate and multivariate models.

As shown in Fig. 5a, it is possible to note that the curve representing the API content determined by the in-line Raman method (red curve) based on the univariate approach is close to the curve of the API concentration evaluated according to the off-line reference method (blue curve). This proves that the model based on the univariate approach is applicable for its intended use. The same ascertainment is made for the multivariate approach as shown in Fig. 5b. Moreover, the closeness is higher for the multivariate approach demonstrating that the latter model is more accurate than the univariate one. Indeed, this model takes into account whole spectra and applies preprocessing treatments on raw data in contrast with the univariate one which is only based on the ITZ specific band. The multivariate approach includes thus more variability in its predictive model.

After evaluation of these results, it is possible to conclude that the in-line Raman spectroscopic method, based on both approaches, determining the API content in real-time is an accurate validated method and may be used for routine analysis.

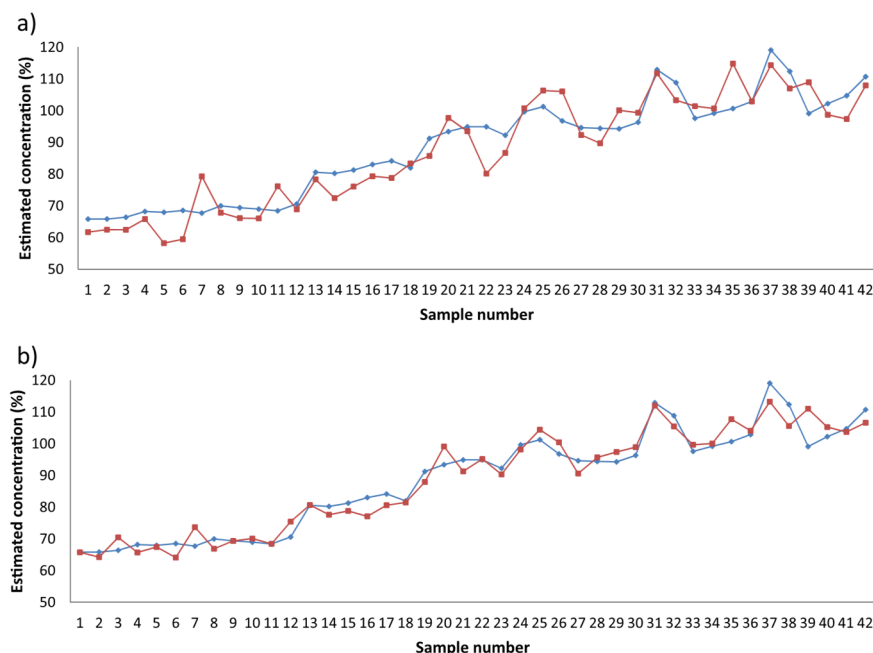


Fig. 5. Determination of ITZ content in real samples using the in-line and off-line methods based on a) the univariate approach and b) the multivariate approach. The blue diamond curve represents the estimated concentration based on the off-line reference method and the red squared curve represents the one based on the in-line validated method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

An in-line Raman spectroscopic method was validated to determine the API content in real-time during a hot-melt extrusion process. Two approaches were used to treat data, a univariate and a multivariate one. Both approaches conclude that the analytical method was valid when considering as real concentrations the ones calculated based on an offline confocal Raman microspectroscopic validated method. In contrast, when trying to validate this same method by considering the weighing of the components in the mixtures as concentrations the method was not valid on the whole dosing range. This highlights the importance of using a reference method to determine the real concentrations in samples. Indeed, losses of a component may occur during the hot-melt extrusion process changing the concentration of the melt. These changes induce a systematic error and thus a higher bias in the validation protocol as proved in the results. It is also possible to note that the validation is flawed by a random error due to dispersion in the results. This can be explained by the presence of uncontrollable parameters. Indeed, one can not control the temperature and humidity of the extrusion room impacting the physicochemical properties of powders and variations on the screw speed produced by filling issues may also induce Raman spectroscopic variations.

Moreover, by comparing the analytical performances based on the validation criteria, the validated method based on the univariate approach is less accurate than the one based on the multivariate approach. In addition, the ability to use the method in routine analyses was also evaluated with test samples on both approaches. This study was performed by comparing the estimated concentrations of real samples with the

reference method and the in-line one. It confirmed that the in-line Raman method is an accurate method quantifying the API content in real-time in comparison with a reference method.

In conclusion, an in-line Raman spectroscopic method quantifying the API content in real-time during a hot-melt extrusion process based on the accuracy profile approach was validated according to the uniformity of content of single-dose preparations norm of the European Pharmacopeia.

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