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Use of Factorial Design for Evaluation of Factors Affecting the Chemical Stability of Sirolimus (Rapamycin) in Solid Dosage Form

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Abstract

Effects of four process and formulation parameters (spraying rate of ethanol solution, drying and tablet hardness and hydroxypropyl methyl cellulose (HPMC) content) were evaluated in terms of initial quality of tablets using factorial design approach. For determination of stability of final drug product, the tablets were exposed to stress testing conditions and the three most significant factors were investigated (spraying rate of ethanol solution, drying and HPMC content). Considering the chemical stability of Sirolimus, the following responses were found to be most important: total sum of degradation products, levels of impurity I and assay of isomer C. Investigated factors and their interactions most significantly affected the assay of isomer C in initial and in stressed stability testing samples.

The factorial design approach is a very economic way of obtaining the maximum amount of information in a short period of time, which is especially important in studies that include a variety of different factors and their interactions.

Keywords: Stability testing; Factorial design; Experimental design; Quality by design; Critical parameters.

1. Introduction

Traditionally, quality of pharmaceutical products was established with control of predefined parameters of final drug products. The specification limits of chosen parameters were usually set on the basis of regulatory requirements or guidelines. Sometimes additional studies (toxicological studies etc.) were performed, but their aim was to justify the quality of already developed drug product. Such approach that was established by FDA (Food and Drug Administration) and named quality by testing approach (QbT), lead to less flexible manufacturing processes with fixed process and formulation parameters and included tight specifications and testing results of raw materials, in-process materials and final drug products.[1]

In order to optimize the quality of drug products (and reduce the number of recalls from the market), in 2002 FDA started with a new initiative. This initiative was based on quality by design (QbD), risk management and quality systems. QbD presents a systematic approach to pharmaceutical development and requires understanding of how formulation and process parameters influence product quality. QbD leads to a quality, that is built into the product and is not established only by testing the final drug product.[1,2,3,4]

Today, QbD has become a regulatory (FDA) requirement. In the part of registration documentation describing the pharmaceutical development, an enhanced knowledge of product performance over a range of formulation parameters and manufacturing process parameters should be demonstrated by the applicant. This understanding can be gained by application of formal experimental designs.[1,2,5] Experimental design, also called design of experiments (DoE), is a concept for planning and execution of informative experiments. It is a structured, organized method for determining relationship between factors affecting a process and the output of that process.[5,6,7]

DoE helps in identification and classification of formulation and process parameters affecting the drug product quality. The first selection of factors and responses to constitute a platform for experimental design can be done

by reviewing the data from previous experiments, considering the equipment capacities, analytical methods etc. Using factorial designs, which form a basis for all experimental designs, it is further possible to distinguish between critical and non-critical parameters. Critical quality parameters are properties that need to be controlled as they impact either product efficacy or patient safety.[1,2,5]

Factorial designs are one of the groups of experimental designs. They are commonly adopted in pharmaceutical research that is concerned with the effects of formulation and process parameters and their interactions on different responses, because it yields the most information from the fewest experiments. A full factorial design is one in which all possible combinations of the factors at all levels involved in the experiments are utilized.[8,9]

Two important reasons for using a factorial design in experiments which test whether the response depends on factor level, are (a) the factorial experiment detects and estimates any interaction, which one factor at a time experiment cannot do, and (b) if the effects of the factors are additive, then the factorial design needs fewer measurements than the one factor at a time experiment in order to give the same precision.[10]

The efficient use of experimental design in preformulation compatibility studies has already been confirmed.[11] So was the use of this approach in development, optimization and validation of analytical methods.[12,13,14,15] However, with the presented study we show that experimental design, namely the factorial design can also be used for determination of those process and formulation parameters that are critical in terms of initial quality as well as further stability of drug product.

Considering the stability study presented in the article, together with the application of experimental design, another optimization of pharmaceutical development is emphasized. Namely, application of more severe storage conditions in stability studies, which gives the results in days instead of in months can lead to reduction of time and costs. Most stability studies in development phase are performed as stress testing experiments at elevated temperature and sometimes also different humidity with a short time exposure.[16] Especially for stability studies it is very important to precisely define all the experiments and experimental conditions at the beginning, otherwise a lot of time can be spent before the optimal stability is achieved and determined.

In the present work, full factorial designs were used for systematic study of different factors. The objective of our work was to emphasize the benefits of experimental design approach in comparison to one factor at a time experiments in light of gaining more information from fewer experiments. Nevertheless, our experiments show a good example how in one experimental design both process and formulation parameters can be included, leading to optimization of formulation, technological process and final manipulation of drug product.

2. Experimental

2. 1. Materials

The tablets containing 2 mg of sirolimus (rapamycin) were produced. The active substance was obtained from Biocon (India). Besides the active substance also the following excipients were used: Hydroxypropyl cellulose (Klucel EF, Hercules, USA), starch pregelatinized (Colorcon, USA), hydroxypropyl methyl cellulose (DOW, USA), glyceryl behenate (Gattefosse, France), low substituted hydroxypropyl cellulose (JRS Pharma, Germany), silicified microcrystalline cellulose (ShinEtsu, Japan) and Ethanol 96% (Sasol solvents, Germany).

For the analyses of the samples, formic acid and methanol were obtained from Merck (Darmstadt, Germany), acetonitrile from J. T. Baker (Philipsburg, NJ, USA) and purified water for chromatography from a Milli-Q purification unit (Millipore, Milford, MA, USA). Volumetric flasks (10, 20, 50, 200 and 1000 ml) were provided by Brand (Wertheim, Germany). The samples used for stability evaluation were stored in glass vials (Nuova Ompi, Padova, Italy).

2. 2. Instrumentation

Granulate, comprising active substance and excipients, was prepared in fluid bed apparatus (Glatt GPCG 3, Glatt, Germany). The apparatus was assembled in top spray setup with the 1.2 mm nozzle (Düsen-Schlick, Germany) in the upper position. Tablets were compressed from the final mixture on a rotary tablet press (Kilian LX 10, Kilian, Germany) and dried in a hot air tray dryer (Kambič, Slovenia).

For evaluation of stability, the samples were stored in closed glass vials for seven days in thermostatic chamber (Sutjeska, former Yugoslavia) at 60 °C.

For related substances and degradation products determination, Acquity^{TM} Ultra Performance Liquid Chromatogprahy (UPLC) system (Waters, Milford, Massachusetts, USA) was used. The analyses were performed using chromatographic column Zorbax Eclipse XBD-C18, 1.8 μm, 100 × 4.6 mm (Agilent Technologies, California, US). For the preparation of sample solution analytical (Mettler Toledo XP 205; Greifense, Switzerland) and micro balance (Mettler Toledo MX5/M; Greifense, Switzerland) were used together with ultrasonic bath (Branson 8510, Danbury, USA). Statistical evaluation of factorial design was performed with computer program EFFECTS.[18]

2. 3. Methods

2. 3. 1. Tablets Preparation

All samples were prepared from the same active substance according to the procedure described below. Only variations between samples were those originating from the experimental design. First, the active substance and

hydroxypropyl cellulose (HPC) were mixed in ethanol on a magnetic stirrer until dissolved. Hydroxypropyl methyl cellulose (HPMC) was mixed in water on a magnetic stirrer until dissolved. Pregelatinized starch was sieved and transferred to a fluid bed apparatus and heated to 30 °C with the inlet air. The temperature and the velocity of the inlet air were 42 °C and 1.5 m/s, respectively, and were constant throughout the experiment. The apparatus was assembled in top spray setup with the 1.2 mm nozzle in the upper position. The ethanol solution of the active substance was sprayed onto the fluidized starch at a spraying rate defined in the experimental design (12 or 23 g/min). Next the product in the chamber was allowed to dry until the product temperature reached 30 °C. The HPMC solution was sprayed onto the fluidized product at 15 g/min to achieve a composition according to the experimental design (14 or 24 mg per tablet). Finally the product was allowed to dry until the product temperature reached 40 °C. The product was sieved through a 0.5 mm sieve to obtain a homogeneous granulate. This granulate was mixed with glyceryl behenate, low substituted HPC and silicified microcrystalline cellulose. Round tablets weighting 360 mg with diameter of 10 mm and concavity of 10 (R) were compressed from the final mixture on a rotary tablet press to obtain the tablets of desired tablet hardness (55 or 70 N). According to the experimental design selected tablets were dried in a hot air tray dryer at 35 °C for 2 hours.

2. 3. 2. The Related Substances, Degradation Products Determination

The levels of related substances and degradation products were determined using in-house developed gradient UPLC method. For the evaluation of changes during the manufacturing process and for the evaluation of stability of drug product three different responses were analysed: the total sum of degradation products, the levels of impurity I and the assay of isomer C.

Impurity I is the main degradation product, which is together with other individual impurities included in the total sum of related substances and degradation products. Isomer C is one of the isomeric forms of sirolimus, which is not included in the total sum of related substances and degradation products, but is observed alone considering its individual specification limits.

2. 3. 3. Experimental Design

A full two-level four-factorial design[17] was applied, where sixteen experiments were performed. After the analysis of all sixteen initial samples, a full two-level three-factorial design was applied to the stressed samples, since one of the tested factors did not show significant effects on any of the observed parameters. Therefore, for the second part only eight samples were analyzed. The scheme of experiments is presented in Table 1, where the experiments (y) in bold are the ones included in three-factorial design.

2. 3. 4. Calculation of the Effects of Factors and Their Interactions

The effect of each factor i (D_i) was calculated as the difference between the average of the experiment responses on the upper (+) and the lower (–) level, respectively:

Table 1: Presentation of sixteen experiments, y_1 to $y_{16,}$ for the resolution of two-level four-factorial design[17]. Experiments y_1, y_3, y_5, y_7, y_9, y_{11}, y_{13}, y_{15} where included in three-factorial design.

Experiment	X_1	X_2	X_3	X_4	X_1X_2	X_1X_3	X_1X_4	X_2X_3	X_2X_4	X_3X_4	$X_1X_2X_3$	$X_1X_2X_4$	$X_1X_3X_4$	$X_2X_3X_4$	$X_1X_2X_3X_4$
y_1	**–**	**–**	**–**	**–**	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**–**	**–**	**–**	**+**
y_2	–	–	–	+	+	+	–	+	–	–	–	+	+	+	–
y_3	**–**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**–**	**+**	**+**	**–**
y_4	–	–	+	+	+	–	–	–	–	+	+	+	–	–	+
y_5	**–**	**+**	**–**	**–**	**–**	**+**	**+**	**–**	**–**	**+**	**+**	**+**	**–**	**+**	**–**
y_6	–	+	–	+	–	+	–	–	+	–	+	–	+	–	+
y_7	**–**	**+**	**+**	**–**	**–**	**–**	**+**	**+**	**–**	**–**	**–**	**+**	**+**	**–**	**+**
y_8	–	+	+	+	–	–	–	+	+	+	–	–	–	+	–
y_9	**+**	**–**	**–**	**–**	**–**	**–**	**–**	**+**	**+**	**+**	**+**	**+**	**+**	**–**	**–**
y_{10}	+	–	–	+	–	–	+	+	–	–	+	–	–	+	+
y_{11}	**+**	**–**	**+**	**–**	**–**	**+**	**–**	**–**	**+**	**–**	**–**	**+**	**–**	**+**	**+**
y_{12}	+	–	+	+	–	+	+	–	–	+	–	–	+	–	–
y_{13}	**+**	**+**	**–**	**–**	**+**	**–**	**–**	**–**	**–**	**+**	**–**	**–**	**+**	**+**	**+**
y_{14}	+	+	–	+	+	–	+	–	+	–	–	+	–	–	–
y_{15}	**+**	**+**	**+**	**–**	**+**	**+**	**–**	**+**	**–**	**–**	**+**	**–**	**–**	**–**	**–**
y_{16}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

$$Di = \frac{\sum_{j=1}^{all\ experiments} y_{ij}^{(+)} - \sum_{j=1}^{all\ experiments} y_{ij}^{(-)}}{n} \quad (1)$$

The experimental error can be calculated from repeated measurements, dummy factor effects or interaction effects.[17,18,19] When the full factorial design with more than three factors is used, the most convenient way for the calculation of experimental error is the use of high-order interaction effects. Assuming that there are no interactions between three or more factors, the effects of such interactions would be equal to zero. If they are not zero (what is usually the case), such effects can be regarded as a measure of the lack of experimental precision, any analytical error in measuring the response and all other possible variability that can affect the final results.[20] Therefore, the experimental error, s_E, can be expressed as follows:

$$s_E = \sqrt{\frac{1}{n_d}\sum_{i=1}^{n_d}(D_i)^2} \quad (2)$$

where D_i was the effect of the *i*-th interaction and n_d was the number of high-order interactions.

The critical effect, $|D_i|$, was calculated as:

$$|\mathrm{Di}|_{\mathrm{crit.}} = s_E \cdot t_{df}^{0.05} \quad (3)$$

where t is the tabulated value for the Student distribution at 95% confidence and at (n_d-1) degrees of freedom of two-tailed t-test. For the classification of effects, a parameter R_i was used. R_i is calculated as the ratio of the absolute effect $|D_i|$ of factor *i* and the critical effect $|D_i|_{crit.}$:

$$\mathrm{R_i} = \frac{|D_i|}{|D_i|_{crit.}} \quad (4)$$

When the ration R_i is less than 1, the *i*-th effect was not statistically significant at 95% confidence.[17,18,19]

3. Results and Discussion

3. 1. Results on the Basis of DoE

The factors chosen and the range over which they were examined are presented in Table 2. Three of the selected factors were quantitative, whereas the fourth factor (drying or no drying) was qualitative. Low levels of factors were also the lowest values of the factors (the spraying rate of ethanol solution 12 g/min, the HPMC content 14 mg/tablet, the tablet hardness 50 N).

Water content was controlled with inclusion/exclusion of final tablet drying in technological procedure. Factor on low level presented dried tablets and therefore lower water content, whereas factor on high level presented the samples with higher water content since the tablets were not dried.

With preliminary experiments (results not presented in the article) it was determined that only the levels of HPMC could be varied. All other components were fixed, whereas the HPMC content could be adjusted in order to achieve optimal characteristics of the formulation. In order to keep the tablet mass constant, also the quantity of silicified microcrystalline cellulose was varied. Both silicified microcrystalline cellulose and starch had a role of filler in tablet, presenting together more than 80% of tablet mass. The variation of ± 10 mg of silicified microcrystalline cellulose was therefore negligible in terms of the effect that it might had on the results of presented factorial design.

Table 2: Factors and factor levels investigated in full four-factorial design

Label	Factor	Low level (–)	High level (+)
X_1	spraying rate (g/min)	12	23
X_2	HPMC content (mg/tab.)	14	24
X_3	water content	Drying YES	Drying NO
X_4	tablet hardness (N)	50	70

Preliminary testing also confirmed that the operating parameters are achievable. It was established that the spraying rate of ethanol solution, the final drying of tablets and tablet hardness are critical process parameters which can affect the initial quality as well as the stability of tablets. The levels of spraying rate of ethanol solution were set on the basis of capacity of the fluid bed apparatus. The effect of water content in tablets was evaluated with inclusion of final drying into the technological process. The levels of tablet hardness were determined considering minimal hardness enabling the production of tablets and maximal hardness, limited by the rotary tablet press capacity.

The responses were selected in accordance with preliminary stability testing performed on different tablet samples and based on the preformulation studies. The total sum, the levels of impurity I and the assay of isomer C were chosen as the relevant responses, which indicated the stability of the final dosage form.

The analytical results for all tested samples and all three parameters are presented in Table 3. The levels of degradation products were determined for all initial samples, whereas for the stressed stability testing samples only half of the samples were analyzed as explained later on.

3. 2. Determination of Critical and Significant Effects

All effects presented in Table 4 are calculated on the basis of equation 1. The positive sign of the effect indicates that the factor is synergistic and a negative sign of the effect indicates the antagonistic effect on the response.[21]

The critical effect for each response was determined on the basis of equations 2 and 3 in which the four three-factor interaction effects, determined for each response separately, were used. For example, for determination of critical effect of isomer C the effects of $X_1X_2X_3$ (–0.04), $X_1X_2X_4$ (0.01), $X_1X_3X_4$ (–0.01) and $X_2X_3X_4$ (–0.00) were

Table 3: The content of degradation products and isomer C in initial samples (after manufacturing) and in samples stored at stressed testing conditions

Experiment	Total sum (%)			Impurity I (%)			Isomer C (%)		
	Initial	60 °C, 7 days	Difference	Initial	60 °C, 7 days	Difference	Initial	60 °C, 7 days	Difference
y_1	0.75	1.20	0.45	0.36	0.38	0.02	2.95	3.16	0.21
y_2	0.63	/	/	0.32	/	/	2.97	/	/
y_3	0.47	0.90	0.43	0.29	0.39	0.10	3.09	3.73	0.64
y_4	0.55	/	/	0.32	/	/	3.08	/	/
y_5	0.69	1.05	0.36	0.36	0.42	0.06	3.21	3.54	0.33
y_6	0.58	/	/	0.31	/	/	3.20	/	/
y_7	0.38	0.86	0.48	0.23	0.39	0.16	3.31	4.63	1.32
y_8	0.43	/	/	0.28	/	/	3.27	/	/
y_9	0.62	1.13	0.51	0.34	0.40	0.06	3.23	3.58	0.35
y_{10}	0.54	/	/	0.31	/	/	3.26	/	/
y_{11}	0.42	0.83	0.41	0.28	0.36	0.08	3.42	4.70	1.28
y_{12}	0.44	/	/	0.29	/	/	3.39	/	/
y_{13}	0.48	0.90	0.42	0.28	0.31	0.03	3.33	3.72	0.39
y_{14}	0.42	/	/	0.24	/	/	3.37	/	/
y_{15}	0.44	0.61	0.17	0.35	0.18	– 0.17	3.34	4.91	1.57
y_{16}	0.45	/	/	0.35	/	/	3.32	/	/

Table 4: Statistical evaluation of the effects on responses for initial samples. Critical effects were calculated on the basis of three-factor interaction effects (shaded rows).

Factors and their interactions	Calculated effects		
	Total sum	Impurity I	Isomer C
X_1	–0.08	–0.00	**0.20** → R = 2.9
X_2	–0.07	–0.01	**0.12** → R = 1.7
X_3	**–0.14** → R = 1.6	–0.02	**0.09** → R = 1.3
X_4	–0.03	–0.01	–0.00
$X_1 X_2$	0.01	0.01	**–0.11** → R = 1.6
$X_1 X_3$	0.06	0.04	–0.02
$X_1 X_4$	–0.00	–0.01	0.01
$X_2 X_3$	0.02	0.02	–0.06
$X_2 X_4$	–0.00	–0.00	–0.01
$X_3 X_4$	0.07	0.03	–0.02
$X_1 X_2 X_3$	***0.05***	***0.04***	***–0.04***
$X_1 X_2 X_4$	***0.00***	***–0.00***	***0.01***
$X_1 X_3 X_4$	***–0.02***	***–0.01***	***–0.01***
$X_2 X_3 X_4$	***–0.01***	***0.00***	***–0.00***
$X_1 X_2 X_3 X_4$	0.00	–0.00	0.00
Critical effect	0.09	0.07	0.07

used, giving the critical effect of 0.07. In Table 4 presented R value (calculated according to eq. 4) is the ratio of the absolute effect of each factor or its interaction with other factors and the calculated critical effect.

The effects of factors and their interactions on the total sum of degradation products and isomer C were calculated considering the difference in levels of all degradation products or assay of isomer C determined in initial and in stressed samples (Table 5). In the difference two variables are included each with its own analytical variability. Therefore, the critical effect was multiplied by two. This simplification was possible due to the fact that the same samples were stored at stress testing conditions and

Table 5: Statistical evaluation of the effects on stability of the samples

Factors and their interactions	Calculated effects	
	Total sum	Isomer C
X_1	–0.05	**0.27** → R = 1.9
X_2	–0.09	**0.28** → R = 2.0
X_3	–0.06	**0.88** → R = 6.3
$X_1 X_2$	–0.07	–0.12
$X_1 X_3$	–0.11	**0.17** → R = 1.2
$X_2 X_3$	–0.00	**0.20** → R = 1.4
$X_1 X_2 X_3$	–0.07	–0.08
Critical effect	0.09 × 2 = 0.18	0.07 × 2 = 0.14

the same analytical method was used for the determination of responses for stressed samples. In the first part of experiments, the high-order interaction effects were the measure of experimental precision and analytical error. In the second part the experimental error, in addition to the experimental precision and analytical error, included also variability of stress testing conditions and analytical error of analysis of stressed samples.

3. 3. Evaluation of the Factor Effects on Three Responses

Sixteen experiments were performed for the two-level four-factorial design in order to evaluate the effects of three process parameters and one formulation parameter (HPMC content) on quality of drug product after manufacturing, and eight experiments were performed for the two-level three-factorial design in order to evaluate the same effects on stability of final dosage form (Table 1).

From the obtained results for initial samples it was concluded that three factors, i.e. the spraying rate of ethanol solution (factor X_1), the HPMC content (factor X_2), and the final drying of tablets (factor X_3) had significant effect on isomer C, whereas non of the factors had significant effect on levels of impurity I. Regarding the total sum, only the final water content, i.e. the inclusion of tablet drying in technological procedure had significant effect. The results also indicated that the fourth factor (factor X_4, tablet hardness) had no effect on any of the observed responses.

Considering the effects of the interactions between the two factors, significant effect was observed only for the interaction between spraying rate of ethanol solution and the HPMC content. This interaction significantly affected the initial assay of isomer C.

On the basis of the results gained with the first group of experiments, which were performed to evaluate the initial quality of tablets the following observations were found to be important:

– Tablet hardness is not a critical process parameter, since no significant effect was determined on any of the observed responses. The specification limits can be set with no limitations, except for the visual compliance and the equipment capacity.
– In order to assure lower levels of the total sum of degradation products, all three factors (spraying rate of ethanol solution, HPMC content and final drying of tablets) have to be at their highest levels, but the effects of the first two factors are not significant (Table 4).
– In order to achieve the lowest assay of isomer C in tablets after the manufacturing, all three factors should be on the low level. Spraying rate of ethanol solution showed the most significant effect and should be therefore considered as the most critical.

On the basis of the results of the initial samples, it was decided to include only the first three factors in further experiments for the stressed stability testing samples since tablet hardness did not show any significant effect. Further, for the stressed stability testing samples only the total sum and isomer C were monitored, since no effect on impurity I was determined in the initial samples.

From the results determined for stressed stability testing samples it was evident that practically none of the observed factors had significant effect on the total sum of degradation products. Nevertheless, the results of statistical evaluation of effects indicated that all three factors together with two factor interactions had significant effect on assay of isomer C at stress testing condition (Table 5).

In general, altering the levels for qualitative factor from "+" to "–" (inclusion of final drying of tablets in technological procedure) and for quantitative factors (spraying rate of ethanol solution, HPMC content) from high to low, would decrease the assay of isomer C.

On the basis of detailed evaluation of stability testing results the following could be concluded:

Final drying of tablets has the most significant effect (three times higher effect compared to X_1 and X_2) and should be included in technological procedure in order to prevent increases in isomer C during stability testing. The possibility of drying the tablets with desiccant in final packaging could also be considered and evaluated in the future, since drying can increase the length and costs of technological procedure.

The effect of factor X_1 (spraying rate) and X_2 (HPMC content) on the assay of isomer C is also significant ($R > 1$), while two-factor interactions are less important. The lower assay of isomer C is achieved, if factor X_1 and X_2 are at the low level. Therefore, the spraying rate and HPMC content should also be optimized, since the current limits (12 to 23 g/min or 14 to 24 mg/tab., respectively) are too wide.

4. Conclusion

With the presented experiments effects of four factors were evaluated in terms of the initial quality of drug product, whereas for stability characteristics of drug product the effects of three factors were evaluated.

Using experimental design three different process parameters together with one formulation parameter were evaluated. The effects of the four factors including the interactions between the two factors were evaluated with preparation of only four different granulates, prepared with variations in spraying rate of ethanol solutions and HPMC content. All granulates were further processed on four different ways (with or without final drying and compressed to low or high tablet hardness). Together with the first three factors (spraying rate of ethanol solution, HPMC content and final drying), some of the factor interactions also showed significant effects.

Our study presented an optimization of pharmaceutical development since in one design both formulation and process parameters were examined at the same time. Three important steps were evaluated: formulation itself

(with variation of one of the excipients), manufacturing process (with variation of spraying rate and the tablet hardness) and the final drug product manipulation (evaluation of the effects of drying of tablets). Interactions between factors that effect different steps are not significant, specially three factor interactions. Therefore, for estimation of experimental error and evaluation of significance of main effects and two factor interactions, these high order interactions were used. This way our study was additionally optimized since our experiments were performed in one replicate and the evaluation of results was done using simple calculations.

The comparison has shown that with the use of the one factor at a time experiments, thirty two experiments would be needed in order to evaluate the effects of individual factors and their interactions, as they were evaluated with only sixteen experiments using factorial design.

With the presented study it is also shown that with proactive work and simultaneous evaluation of the results of one part of testing the number of further experiments can be efficiently reduced. With evaluation of results for the initial samples the number of factors and responses for stressed stability testing samples was decreased presenting additional optimization of our work by factor three.

Quality by design became an essential part of the modern approach to pharmaceutical quality. This study demonstrated the usefulness of the quality by design approach, comprising the multi factor data analysis to gain a comprehensive understanding of the preparation and processing of investigated tablets. It was confirmed that experimental design methodology, namely factorial design could efficiently be applied also for characterization of critical process and formulation parameters. There can be no doubt that factorial design is a very economic way for extracting the maximum amount of information in a very short time period and with the fewest number of experiments.

5. References

1. Y. Qiu, Y. Chen, G. G. Z. Zhang: Developing Solid Oral Dosage Forms, Pharmaceutical Theory and Practice, Elsevier, **2009**, pp. 885–898.
2. S. Verma, Y. Lan, R. Gokhale, D.J.Burgess, *Int. J. Pharm.* **2009**, *377*, 185–198.
3. R. A. Lionberger, S. L. Lee, L. Lee, A. Raw, L. X. Yu, *AAPS J.* **2008,** *10*(2)*,* 268–276.
4. L. X. Yu, *Pharm. Res.* **2008**, *25*(4), 781–791.
5. US Food and Drug Administration (November 2009). Guidance for Industry: Q8 (R2) Pharmaceutical Development.
6. T. Martinello, T. M. Kaneko, M. V. Robles Velasco, M. E. Santos Taqueda, V. O. Consiglieri, *Int. J. Pharm.* **2006**, *322*, 87–95.
7. L. Eriksson, E. Johansson, N. Kettaneh-Wold, C. Wikström, S. Wold : Design of Experiments, Principles and Applications, Third revised and enlarged edition, Umetrics Academy, **2008**, pp. 1–6.
8. A. G. Gonzalez, *Anal. Chim. Acta* **1998**, *360*, 227–241.
9. L. C. Chang, S. C. Wu, J. W Tsai, T. J Yu, T. R Tsai, *Int. J. Pharm.* **2009**, *376*, 195–203.
10. J. C. Miller, J. N. Miller, Statistics for analytical chemistry, 3rd Edition, Ellis Horwood PTR Prentice Hall, **1993**, pp. 181.
11. S. Bohanec, T. Rozman-Peterka, P. Blažič, R. Jurečič, J. Grmaš, A. Krivec, J. Zakrajšek, *Acta Chim. Slov.* **2010**, *57*, 895–903.
12. M. Kincl, S. Turk, F. Vrečer, *Anal. Chim. Acta* **2004**, *502*, 107–113.
13. F. G. Vogt, A. S. Kord: Development of Quality-By-Design Analytical Methods, Published online 9 September 2010 in Wiley Online Library (wilexonlinelibrary.com), DOI 10.1002/jps.22325.
14. Y. Vander Heyden, F. Questier, L. Massart, *J. Pharm. Biomed. Anal.* **1998,** *18,* 43–56.
15. B. Dejaegher, Y. Vander Heyden, *J. Chromatogr. A* **2007**, *1158*, 138–157.
16. J. T. Carstensen, C. T. Rhodes: Drug Stability, Principles and Practices, 3rd Edition, (Revised and Expanded), Marcel Dekker, New York, **2000**, pp. 435–436.
17. E. Morgan: Chemometrics: Experimental Design, John Wiley and Sons, London, **1995**, pp. 138–145.
18. M. Moder, S. Bohanec, J. Zupan, *Acta Chim. Slov.* **1997**, *44*(2), 181–196.
19. Y. Vander Hayden, C. Hartmann, D. L. Massart, L. Michel, P. Kiechle, F. Erni, *Anal. Chim. Acta* **1995**, *316*, 15–26.
20. R. A. Stowe, R. P. Mayer, *Ind. Eng. Chem.,* **1966**, *58*(2), 36–40.
21. S. Bolton: Pharmaceutical Statistics: Practical and Clinical Applications, 2nd ed. (Revised and Expanded), Marcel Dekker, New York, **1990**, p. 308.

Povzetek

Z uporabo faktorske analize smo ovrednotili vpliv štirih tehnoloških parametrov (hitrost razprševanja etanolne raztopine, sušenje tablet, trdnost tablet in vsebnost HPMC) na kakovost tablet po izdelavi. Z namenom določitve stabilnosti končnega izdelka, smo tablete izpostavili stresnim pogojem shranjevanja in spremljali tri faktorje s signifikantnim vplivom (hitrost razprševanja etanolne raztopine, sušenje tablet, in vsebnost HPMC). Odgovori, s katerimi smo ovrednotili stabilnost Sirolimusa, so bili vsota razkrojnih produktov, nivoji nečistote I ter vsebnost izomere C. Raziskava je pokazala, da proučevani faktorji ter njihove interakcije najbolj vplivajo na vsebnost izomere C tako v začetnih kot v vzorcih izpostavljenih stresnim pogojem shranjevanja.

Faktorska analiza se je izkazala kot zelo ekonomičen pristop, s katerim pridobimo maksimalno količino informacij v kratkem času. To je posebej pomembno pri študijah, ki vključujejo veliko različnih faktorjev ter njihovih medsebojnih interakcij.

APPENDIX

Three different approaches of statistical analysis are presented. With all the same effects of factors are determined. The only difference is in the determination of critical effects (via experimental error).

1. Comparison of factors' effects and their significance between results presented in the article and the results gained with computer software MODDE 9.0 (Umetrics AB, Umea, Sweden) for initial samples

Comparing the calculated effects of factors and their interactions it can be seen that both with computer software MODDE 9.0 and with the program EFFECTS approximately the same values are gained since they are in both cases calculated according to equation 1. However, it can be seen that there are some differences in determination of significance of the effects.

Table 1: Factor effects for initial samples calculated with program EFFECTS and presented in the article. The significant effects are printed in bold.

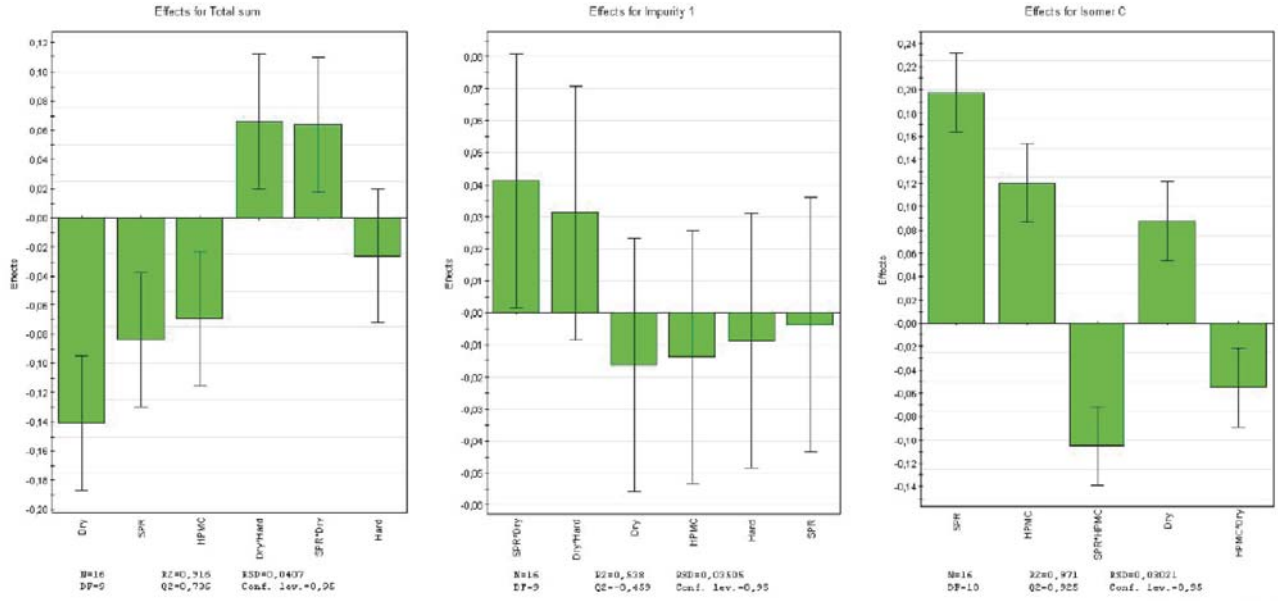
Factors and their interactions	Calculated effects		
	Total sum	Impurity I	Isomer C
X_1	–0.08	–0.00	**0.20**
X_2	–0.07	–0.01	**0.12**
X_3	**–0.14**	–0.02	**0.09**
X_4	–0.03	–0.01	–0.00
$X_1 X_2$	0.01	0.01	**–0.11**
$X_1 X_3$	0.06	0.04	–0.02
$X_1 X_4$	–0.00	–0.01	0.01
$X_2 X_3$	0.02	0.02	–0.06
$X_2 X_4$	–0.00	–0.00	–0.01
$X_3 X_4$	0.07	0.03	–0.02

With computer program EFFECTS the significance of the effects was evaluated on the basis of determination of experimental error using high order interactions (according to equation 2 presented in the article). This way the effects **in bold** presented in Table 1 were determined as significant.

With the calculation with program EFFECTS, the following factors and/or interactions were determined as significant (listed descending according to the size of the effect):

– **Total sum:** factor X_3 (final drying);
– **Impurity I**: none of the factors;
– **Isomer C:** factors X_1 (spraying rate), X_2 (HPMC content), interaction $X_1 X_2$ (interaction between spraying rate and HPMC content), X_3 (final drying).

The results gained with computer software MODDE 9.0 are graphically presented below in Graph 1. The effects of the factors are graphically presented in a bar chart. On each bar, the corresponding 95% confidence interval is superimposed. The confidence interval indicates the uncertainty of each effect and the size depends on the size of noise.



Graph 1: Effect plot for all three responses for initial samples

The results calculated with MODDE indicate that the following effects and interactions are significant (listed descending according to the size of the effect):

– **Total sum:** factors X_3 (final drying), X_1 (spraying rate), X_2 (HPMC content), interaction X_3X_4 (interaction between final drying and tablet hardness), interaction X_1X_3 (interaction between spraying rate and final drying);
– **Impurity I:** interaction X_1X_3 (interaction between spraying rate and final drying);
– **Isomer C:** factors X_1 (spraying rate), X_2 (HPMC content), interaction X_1X_2 (interaction between spraying rate and HPMC content), X_3 (final drying), interaction X_2X_3 (interaction between HPMC content and final drying).

Comparison of the significance of the effects, which was determined in two different ways (program EFFECTS: significance determined on the basis of experimental error, which was estimated based on the high order interactions; program MODDE: significance determined on the basis of experimental error, which was estimated based on the least square analysis, the outcome of which is a regression model consisting of coefficients reflecting the influence of the factors) has shown, that in both cases none of the factors had a significant effect on initial levels of impurity I. Although, one of the interactions (interaction X_1X_3) could be evaluated as significant using MODDE, the model itself has a calculated value Q^2 (the goodness of prediction, meaning it estimates the predictive power of the model) less than 0, which indicates that the model is not good and can therefore not be used.

Considering the initial assay of isomer C, both with MODDE and EFFECTS the first three factors were evaluated as significant (X_1, X_2, X_3). Additionally, both programs determined interaction X_1X_2 (interaction between spraying rate and HPMC content) as significant. With MODDE however one additional interaction was evaluated as significant, which was not detected as such with program EFFECT. That is the X_2X_3 (interaction between HPMC content and final drying). This interaction indicates that in order to achieve lower initial assay of isomer C, this interaction should be positive. To achieve a positive interaction, both X_2 and X_3 factors should be at their high or both at their low level. Since both individual factors X_2 and X_3 had larger effects than their interaction, they were used in final conclusion, i.e. both factors should be at their low level.

The main difference between the results gained with EFFECTS and the ones calculated with MODDE is for total sum of degradation products. Namely, with EFFECTS only one factor was designated as significant (factor X_3, i.e. final drying), while with MODDE three factors (X_1, X_2, and X3) were calculated as significant together with two interactions (X_3X_4, X_1X_3). It has to be pointed out that all these effects were on the borderline of significance also when calculating with EFFECTS. However since the critical value was set higher they were not designated as significant.

Considering the technological procedure and the parameters we were evaluating the conclusions remain the same. Namely, all three factors, which had according to the MODDE significant effect on total sum, had also a significant effect on isomer C (for which the predictive power of the model was the greatest). This was calculated with EFFECTS and with MODDE. Therefore, the technological procedure would be performed in a way that all three factors would be at their low level. Consequently, lowest assay of isomer C would be achieved together with lowest levels of total sum of degradation products.

2. Comparison of factors' effects and their significance between results presented in the article and the results gained with computer software MODDE 9.0 for stressed samples

As described above for the initial samples, the same way the factors' effects and their significance were evaluated with program EFFECTS and MODDE. The results are presented below in Table 2 for EFFECTS and in Graph 2 for MODDE.

Table 2: Factor' effects for stressed samples calculated with program EFFECTS and presented in the article. The significant effects are printed in bold.

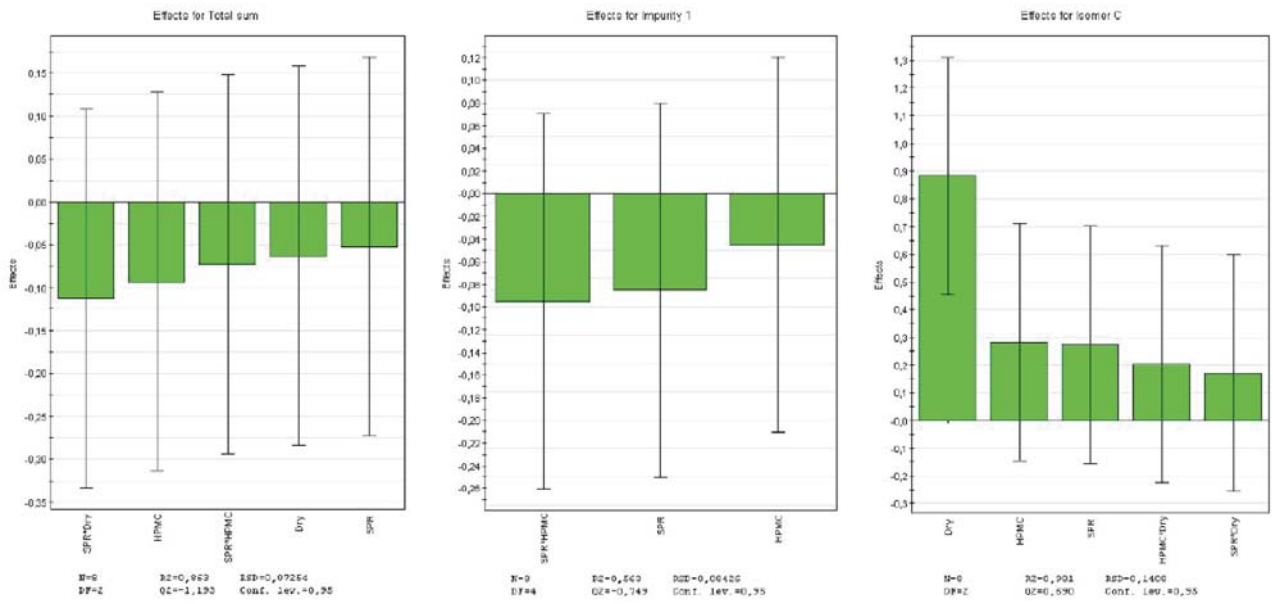
Factors and their interactions	Calculated effects	
	Total sum	Isomer C
X_1	–0.05	**0.27**
X_2	–0.09	**0.28**
X_3	–0.06	**0.88**
X_1X_2	–0.07	–0.12
X_1X_3	–0.11	**0.17**
X_2X_3	–0.00	**0.20**

With the calculation with program EFFECTS, the following factors and/or interactions were determined as significant (listed descending according to the size of the effect):

– **Total sum:** none of the factors;
– **Impurity I**: none of the factors;
– **Isomer C:** factors X_3 (final drying), X_2 (HPMC content), X_1 (spraying rate), interaction X_2X_3 (interaction between HPMC content and final drying), interaction X_1X_3 (interaction between spraying rate and final drying).

The results calculated with MODDE indicate that the following effects and interactions are significant (listed descending according to the size of the effect):

– **Total sum:** none of the factors;
– **Impurity I:** none of the factors;
– **Isomer C:** factor X_3 (final drying).



Graph 2: Effect plot for all three responses for stressed stability samples

The comparison of the results showed that both with EFFECTS and with MODDE none of the investigated factors had a significant effect on total sum or impurity I (after evaluation of initial samples impurity I was already excluded from the study as presented in the article). However, there is a difference between the results for isomer C. While MODDE designated only factor X_3 as significant, the evaluation of results with EFFECTS showed that all three factors are significant. Additionally, also interaction between spraying rate and final drying X_1X_3 is significant.

Our study presented a screening study of technological parameters. This means that we determined those parameters which could have significant effect on the chosen responses and should therefore be further investigated. With designation of more than just one significant effect (for isomer C) we therefore minimized the risk that some significant factor would be overlooked and hence not included in further investigation.

3. Comparison of factors' effects and their significance between results presented in the article and the results obtained by calculation given in ref. no. 12 for initial samples

According to ref no. 12, the experimental error, s_{pooled}, for each response is expressed as:

$$s_{pooled} = \sqrt{\frac{1}{2}\left(s^2_{(+)} + s^2_{(-)}\right)}$$

where $s^2_{(+)}$ and $s^2_{(-)}$ were calculated for each factor and for each group of experiments taking into account the level of factor at certain experiment. Therefore, for analysis of initial responses eight experiments are in group – and eight in group +. For example, expression $s^2_{(+)}$ means variances of responses at level + of the factor.

The effect is significant if fulfilled the condition:

$$|\text{effect}| > t^{0.05/2}_{2n-2} \cdot \sqrt{\frac{2}{n}} \cdot s_{pooled}$$

where t is the tabulated value for the Student's one-sided t-test (with $\alpha = 0.05$) at *(2n–2)* degrees of freedom (n is a number of experiments with factor at the same level; it is eight for four-factor two-level full factorial design and four for three-factor two-level full factorial design).

The results are presented below in Table 3 where critical effects are added to each calculated effect for all responses for initial analysis.

The significant effects obtained with methodology described in our article using program EFFECTS are printed italic while those obtained by methodology given in ref. no. 12 are printed bold. There is no difference in determination of significant effects for total sum and impurity I but a huge difference for isomer C: four significant effects were obtained with EFFECTS and only one with methodology given in ref. no. 12. The reason is in very high critical effects.

4. Comparison of factors' effects and their significance between results presented in the article and the results obtained by calculation given in ref. no. 12 for stressed samples

As described above for initial samples, the same comparison was done also for stressed samples, presented in Table 4.

Table 3: Factor effects for initial samples calculated according to ref no. 12.

Factors and their interactions	Total sum		Impurity I		Isomer C	
	Calculated effects	Critical effects	Calculated effects	Critical effect	Calculated effects	Critical effect
X_1	–0.08	0.13	–0.00	0.05	***0.20***	0.13
X_2	–0.07	0.13	–0.01	0.05	*0.12*	0.17
X_3	***–0.14***	0.11	–0.02	0.05	*0.09*	0.18
X_4	–0.03	0.14	–0.01	0.05	–0.00	0.19
$X_1 X_2$	0.01	0.14	0.01	0.05	*–0.11*	0.17
$X_1 X_3$	0.06	0.13	0.04	0.04	–0.02	0.19
$X_1 X_4$	–0.00	0.14	–0.01	0.05	0.01	0.19
$X_2 X_3$	0.02	0.14	0.02	0.05	–0.06	0.18
$X_2 X_4$	–0.00	0.14	–0.00	0.05	–0.01	0.19
$X_3 X_4$	0.07	0.13	0.03	0.05	–0.02	0.19
$X_1 X_2 X_3$	0.05	0.14	0.04	0.04	–0.04	0.19
$X_1 X_2 X_4$	0.00	0.14	–0.00	0.05	0.01	0.19
$X_1 X_3 X_4$	–0.02	0.14	–0.01	0.05	–0.01	0.19
$X_2 X_3 X_4$	–0.01	0.18	0.00	0.07	–0.00	0.23
$X_1 X_2 X_3 X_4$	0.00	0.14	–0.00	0.05	0.00	0.19

Table 4: Factor effects for stressed samples calculated according to ref no. 12.

Factors and their interactions	Total sum		Isomer C	
	Calculated effects	Critical effect	Calculated effects	Critical effect
X_1	–0.05	0.23	*0.27*	1.18
X_2	–0.09	0.21	*0.28*	1.18
X_3	–0.06	0.23	***0.88***	0.60
$X_1 X_2$	–0.07	0.22	–0.12	1.22
$X_1 X_3$	–0.11	0.19	*0.17*	1.21
$X_2 X_3$	–0.00	0.24	*0.20*	1.20
$X_1 X_2 X_3$	–0.07	0.22	–0.08	1.22

There is no difference in determination of significant effects for total sum but a huge difference for isomer C: five significant effects obtained with EFFECTS and only one with methodology given in ref. no. 12. The reason is in very high critical effects.

5. Overall comparison of factors' effects and critical effects for all three approaches

Different methodologies for critical effects' determinations gave different results. We compared critical effects obtained with (1) high-order interaction effects (program EFFECTS), (2) program MODDE and (3) methodology described in ref. no. 12. All three methodologies differ in the determination of critical effects only.

In Table 5 presented critical effects that are lower than calculated effects are printed bold (Table 5).

Table 5: Critical effects for initial samples obtained on three different ways

Factors and their interactions	Total sum				Impurity II				somer C			
	effect	1	2	3	effect	1	2	3	effect	1	2	3
X_1	–0.08	0.09	**0.05**	0.13	–0.00	0.07	0.04	0.05	0.20	**0.07**	**0.03**	**0.13**
X_2	–0.07	0.09	**0.05**	0.13	–0.01	0.07	0.04	0.05	0.12	**0.07**	**0.03**	0.17
X_3	–0.14	**0.09**	**0.05**	**0.11**	–0.02	0.07	0.04	0.05	0.09	**0.07**	**0.03**	0.18
X_4	–0.03	0.09	0.05	0.14	–0.01	0.07	0.04	0.05	–0.00	0.07	0.03	0.19
$X_1 X_2$	0.01	0.09	0.05	0.14	0.01	0.07	0.04	0.05	–0.11	**0.07**	**0.03**	0.17
$X_1 X_3$	0.06	0.09	**0.05**	0.13	0.04	0.07	0.04	0.04	–0.02	0.07	0.03	0.19
$X_1 X_4$	–0.00	0.09	0.05	0.14	–0.01	0.07	0.04	0.05	0.01	0.07	0.03	0.19
$X_2 X_3$	0.02	0.09	0.05	0.14	0.02	0.07	0.04	0.05	–0.06	0.07	**0.03**	0.18
$X_2 X_4$	–0.00	0.09	0.05	0.14	–0.00	0.07	0.04	0.05	–0.01	0.07	0.03	0.19
$X_3 X_4$	0.07	0.09	**0.05**	0.13	0.03	0.07	0.04	0.05	–0.02	0.07	0.03	0.19
$X_1 X_2 X_3$	0.05	0.09	0.05	0.14	0.04	0.07	0.04	0.04	–0.04	0.07	0.03	0.19
$X_1 X_2 X_4$	0.00	0.09	0.05	0.14	–0.00	0.07	0.04	0.05	0.01	0.07	0.03	0.19
$X_1 X_3 X_4$	–0.02	0.09	0.05	0.14	–0.01	0.07	0.04	0.05	–0.01	0.07	0.03	0.19
$X_2 X_3 X_4$	–0.01	0.09	0.05	0.18	0.00	0.07	0.04	0.07	–0.00	0.07	0.03	0.23
$X_1 X_2 X_3 X_4$	0.00	0.09	0.05	0.14	–0.00	0.07	0.04	0.05	0.00	0.07	0.03	0.19

Below is given the explanation of differences between results obtained with different approaches.

– Total sum:

When considering the directives of regulatory agencies for pharmaceutical industry regarding the reporting thresholds of impurities (about 0.10%), the approach presented in our article fulfils the requirements for the described example. Critical effects determined by MODDE and those determined according to the ref. no. 12 are considering above explanation too low.

– Isomer C:

Although the effects of two-factor interactions on isomer C are significant (being –0.11 and –0.06 for X_1X_2 and X_2X_3, respectively) they are lower compared to the effects of main factors (being 0.20, 0.12 and 0.09 for factors X_1, X_2 and X_3, respectively) that form certain interaction. Because of that only factors are taken into account as significant. With both methods (1 and 2) the same factors are determined as significant.

With program MODDE and methodology in ref. no. 12 the critical effect for stressed samples (Table 6) especially for isomer C was again set to high, that is more than 0.4% and 0.6% in MODDE and ref. no. 12, respectively. Namely, according to the previous knowledge of the investigated system (sirolimus tablets) much lower changes in assay of isomer C are important and give information about the quality of presented drug product.

Table 6: Critical effects for stressed samples obtained on three different ways

Factors and		**Total sum**				**Isomer C**		
their interactions	**effect**	**1**	**2**	**3**	**effect**	**1**	**2**	**3**
X_1	–0.05	0.18	0.22	0.23	0.27	**0.14**	0.43	1.18
X_2	–0.09	0.18	0.22	0.21	0.28	**0.14**	0.43	1.18
X_3	–0.06	0.18	0.22	0.23	0.88	**0.14**	**0.43**	**0.60**
X_1 X_2	–0.07	0.18	0.22	0.22	–0.12	0.14	0.43	1.22
X_1 X_3	–0.11	0.18	0.22	0.19	0.17	**0.14**	0.43	1.21
X_2 X_3	–0.00	0.18	0.22	0.24	0.20	**0.14**	0.43	1.20
X_1 X_2 X_3	–0.07	0.18	0.22	0.22	–0.08	0.14	0.43	1.22