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Antioxidant extraction process for Andean Oca by a Photodiode Array Detector using Response Surface Methodology

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Dedicado à minha Família.

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Abstract

Application of Experimental Design techniques has proven to be essential in various research fields, due to its statistical capability of processing the effect of interactions among independent variables, known as factors, in a system's response. Advantages of this methodology can be summarized in more resource and time efficient experimentations while providing more accurate results.

This research emphasizes the quantification of 4 antioxidants extraction, at two different concentration, prepared according to an experimental procedure and measured by a Photodiode Array Detector.

Experimental planning was made following a Central Composite Design, which is a type of DoE that allows to consider the quadratic component in Response Surfaces, a component that includes pure curvature studies on the model produced.

This work was executed with the intention of analyzing responses, peak areas obtained from chromatograms plotted by the Detector's system, and comprehending if the factors considered – acquired from an extensive literary review – produced the expected effect in response. Completion of this work will allow to take conclusions regarding what factors should be considered for the optimization studies of antioxidants extraction in a Oca (*Oxalis tuberosa*) matrix.

Keywords: Experimental Design, Response Surfaces, antioxidants, Oca

Resumo

A aplicação de técnicas de Desenho de Experiências provou ser essencial nos mais diversos campos de investigação, devido à sua capacidade estatística de processar o efeito de interações entre variáveis independentes, também conhecidas por fatores, na resposta produzida pelo sistema. As vantagens desta metodologia traduzem-se num aumento da eficiência de recursos e tempo experimental, produzindo ainda resultados com maior precisão.

Esta investigação foca-se na quantificação da extração de 4 antioxidantes, para duas concentrações diferentes, preparado de acordo com um procedimento experimental e medido por um Detetor de Fotodíodos.

O planeamento experimental foi concebido com base num Desenho do Compósito Central, um tipo de DoE que permite considerar a componente quadrática nas Superfícies de Resposta, componente essa que estuda a curvatura pura nos modelos produzidos.

Este trabalho foi realizado com a intenção de analisar as respostas, áreas de picos obtidas a partir de cromatogramas produzidos pelo Detetor, e compreender se os fatores considerados – adquiridos através de uma revisão literária extensiva – produziram o efeito esperado na resposta. O culminar deste estudo irá permitir tirar conclusões acerca dos fatores que devem ser considerados para os estudos de otimização da extração de antioxidantes numa matriz de Oca (*Oxalis tuberosa*).

Palavras-chave: Desenho de Experiências, Superfícies de Resposta, antioxidantes, Oca

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List of Abbreviations

ANOVA	Analysis of Variance
CCC	Circumscribed Central Composite
CCD	Central Composite Design
DoE	Design of Experiments
OVAT	One Variable At a Time
RSM	Response Surface Methodology
TAA	Total Antioxidant Activity
TPC	Total Phenolic Content

α	Distance from center point to axial point
β	Regression coefficient
2^k	Two-level full factorial form
3^k	Three-level full factorial form
d.f.	Degrees of freedom
e_{ijk}	Residual for a particular experiment
E	Ethanol concentration
k	Factors
l	Levels
l^k	Full factorial form
SS	Sum of Squares
MS	Mean Square
N	Total number of experiments
n	Number of replicas
n_C	Number of center points
R^2	Coefficient of determination
x_c	Coded level
x_{min}	Minimum real level
$x_{máx}$	Maximum real level
x	Real level

1. Introduction

1.1. Contextualization

Throughout times the importance of statistical and mathematical models in life and decision making has taken a tremendous importance. Statistics is the mathematical science involving the collection, analysis and interpretation of data. While before this science used to be only applied to what could be called directly related fields, nowadays its usage is spread to various fields, because organizations and single individuals felt the need to stay on track with economical, technological and market demand. Its application can be found in diversified fields, such as Chemometrics, Business Analytics, Demography, Quality Control, Reliability Engineering, Quantitative Psychology, etc.

Chemometrics is the science of extracting information from chemical systems by data-driven means. It is a highly interfacial discipline, using methods frequently employed in core data-analytic disciplines such as multivariate statistics, applied mathematics, and computer science, in order to address problems in chemistry, biochemistry, medicine, biology and chemical engineering.

Chromatography is a chemical methodology used for separating compounds of a mixture and posterior analysis of concentrations. It was invented by Mikhail Tsvet on 1903, who used it to study the separation of plant pigments. Considered a powerful technology nowadays, it possesses a wide variety of techniques available for experimenters, allowing them to choose the most suitable processes for fulfilling the intended objective. Therefore, Chromatography plays an important role in many laboratorial researches and industrial applications (Ali et al. n.d.). An important activity in the Food certification process consists in quantifying all compounds concentrations present in the product, which will then be displayed in its label. Many companies hire the services of chemistry laboratories to separate and analyze these compounds, which is frequently made through chromatographic separation methods.

In a Quality context, an experiment is a test that measures changes in system's results, caused by intentional alterations in process's input. As a result of the procedure one or multiple responses, depending on the type of system, are obtained from each experiment. Design Of Experiments (DOE) is used with the purpose of studying the conformity of a characteristic(s), measured by response(s), to its expected value(s). The first registered application of this methodology was conducted by Sir Ronald A. Fisher in the 20s, who developed this statistical technique for agricultural purposes (Bhote 1999). It was only in the 80's that eastern companies started to take advantage of DOE, due to improvements made to the original method by Genichi Taguchi. Taguchi's methods had only been put in practice by Japanese companies, up until his

achievements became known internationally and started being applied in a global scale (Pereira & Requejo 2012).

Design Of Experiments filled in an important gap in the chemical field, by introducing a way that allows experimenters to study several parameters at the same time, demanding a smaller number of experiments, so consequently time and material were also reduced.

1.2. Motivation

This study intends to quantify the extraction process, by a Photodiode Array Detector, of four antioxidants using standards: Ellagic Acid, Ferulic Acid, Rutin and Cinnamic Acid. The success of this study is expected to lead to the quantification of this compounds for an Andean Oca (*Oxalis tuberosa*).

Experimental Design will allow to study the influence of Column Temperature, Flux and solvent concentration (% of Acetonitrile) in the extraction process. Supported by Response Surface Methodology (RSM) experimenters will be able to take conclusions regarding system's response to the interaction of factors at different levels. Responses are quantified by the chromatographic peak areas obtained from the system being studied, the Photodiode Array Detector. An Analysis Of Variance will also inform if the alterations being made, to the independent variables that are considered to be important for this system, are responsible for response alterations.

1.3. Objectives

For the successful accomplishment of this thesis, the following objectives were determined:

- Optimization of extraction process of antioxidants for the Oca matrix
- Identification of the best significant factors combinations, and its respective levels, through the DOE methodology

1.4. Thesis structure

The present dissertation is organized in five chapters. The initial chapter contains a brief introduction to the thematic approached, stating this study's objectives, the methodology used and this document's structure.

On the second chapter are described the fundamentals of Experimental Design, covering different techniques for several applications as the Response Surface Methodology (RSM) and considering various DoE typologies including the Central Composite Design (CCD). Following through, also the principals of Chromatography are covered presenting the Photodiode Array Detector system.

Next chapter focuses on the Methodology used in this study, presenting the experimental plan and justifying the choices of factors, levels and Design. Furthermore, it includes the Analysis of Results for each of the four antioxidants studied: Ellagic Acid, Ferulic Acid, Rutin and Cinnamic Acid. In this part are examined all parameters considered to have importance for comprehending this study's success.

Finally, the last chapter contains the final conclusions of this work, comments and suggestions based on the crucial parts it, expecting to open a path for new studies to come.

2. Literature review

2.1. Design Of Experiments

Design of Experiments is a technique for discovering about new processes, acquire a better comprehension on existing products and optimizing these products/processes.

Design of Experiments (DoE) is a tool used for optimization of products or services, when correctly applied it can save time and money, along with other useful resources. Optimizing consists in improving the performance of a system, or process, in order to obtain the maximum benefit from it. It can be applied for products, services.

The DoE methodology was created by Sir Ronald A. Fisher in the 20s, who developed this statistical technique for agricultural purposes (Bhote 1999). Its scope only included enterprises after the improvements made by the Japanese Genichi Taguchi, who applied it first on his country, and later exported those ideas for eastern companies, turning it into a global scale methodology (Pereira & Requejo 2012).

In a Quality context, an experiment is a test that measures changes in system's results, caused by overseen alterations in process's input. Input variables, known as factors, are independent and may assume different values, the levels, these data may be of a quantitative or qualitative kind. Quantitative levels always need to be scalable (i.e. temperatures), while in the second case levels represent the variation of a condition, whether is to use or not an equipment, or trying out different materials in a process, etc. As a result of the procedure one or multiple responses, depending on the type of system, are obtained from each experiment. System's performance is measured through comparison of changes in response values, as a reaction to factors' manipulation. Hence, responses must be quantitative, in order to have well accurate and measurable indicators of optimization (Pereira & Requejo 2012).

Experimental design (DOE) is used with the purpose of studying the conformity of a characteristic(s), measured by response(s), to its expected value(s). Traditionally approaches on experimental studies were only about adjusting the average response. Although, this approach was limited and there was a need to know more about response's behavior. Improvements were made so that variability in response, caused by factors, could be measured in order to take conclusions about which factors influenced response significantly, and the levels yielding better responses (Pereira & Requejo 2012).

Optimization process can be divided in two types of experiments, screening and optimization experiments. Screening experiments are performed in an early stage, period in which researchers have few knowledge about system's behavior regarding all possible variables. In this phase experimenter must consider all factors that might possibly influence response, in order to gauge the factors that have significant influence in it. An optimization plan is then delineated with only

the factors that revealed to have significant influence in response's variability, to discover the levels yielding optimal response (Montgomery 2009).

Many enterprises, nowadays, utilize experimental designs in conception and development of new products, achieving major benefits in costs and time reductions by doing well at first. Also in Chemistry, it's regularly applied, due to being an improvement in relation to traditional methods. Before the demystification of this statistical technique, optimization was carried out by monitoring the influence of One-Variable-At-a-Time (OVAT) on response, keeping the other variables at a fixed value. This approach depends on intuition, experience and luck for its success and even though is frequently unreliable and inefficient (Antony 2003). Comparing to this method, factorial designs have the benefit to investigate more than a factor per experiment, therefore obtaining in each response an estimation of several factors influence. It's a more efficient option, demands less experiments to perform a complete study, and this efficiency level proportionally increases with the number of factors (Montgomery 2009).

2.1.1. Full Factorial Design with two levels – 2^k

Full Factorial designs are one type of DoE represented by an l^k exponentiation form. In comparison with OVAT, it requires an inferior number of experiments, and allows to study when a factor's effect on response is altered by a different level of another factor, known as interaction. This exponentiation indicates the total number of experiments that need to be performed to study *k* factors (exponent) at *l* different levels (base). Replication of experiments is the act of repeating the original experimental conditions for several sets of samples *n*. It's used to estimate experimental error and also allows a more accurate estimation of factors/interactions effects. The total number of experiments is expressed by $N = n \times l^k$. All factors are included in each experiment and studied for the same number of levels (Pereira & Requejo 2012; Montgomery 2009; Antony 2003). During this work only the 2^k basis structure will be approached, although full factorials can also be created for three levels (3^k designs).

For an easier display, it was established that factors would be represented by capital letters, according to the alphabetic order. Experimental levels are assigned with codified levels of x_c=+1 (or "+") and x_c=-1 (or "-"), known as high and low levels. The relation between coded (x_c) and experimental levels (x) is given by Equation 2.1. Previous to the experiments, researcher needs to assign these codes to experimental values (Pereira & Requejo 2012).

$$x_c = \frac{x - \frac{(x_{max} + x_{min})}{2}}{\frac{(x_{max} - x_{min})}{2}} \tag{2.1}$$

Experimental Plan

Experiments are planned according to a specific non-randomized order, called "standard order" (or Yates order). This procedure can be accomplished by following sequential instructions described:

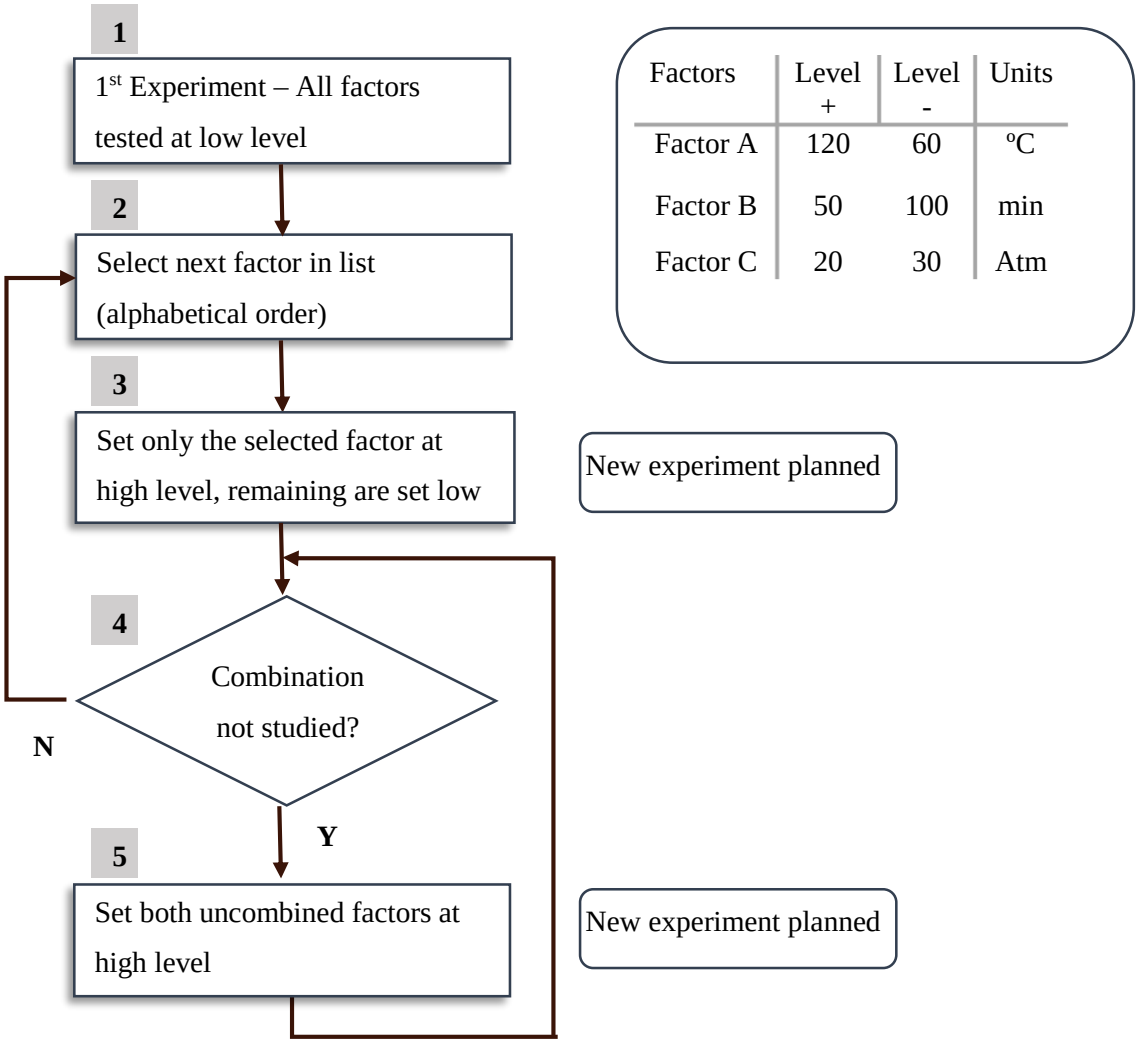


Figure 2. 1 - Experimental procedure for a 2^k Design

A Design Matrix (Table 3) is a visual representation of the experimental plan. Initial experiment (step 1) tests all factors at a low level, and is represented by "(1)" in the matrix. Next, a factor chosen according to priority criteria (Factor A) – alphabetic order – is set at high level, all others are set to low level, and experiment "a" is introduced in the plan (steps 2 and 3). Researcher then proceeds to a verification (step 4), if there are other experiments with high level factor(s) already planned, although yet to be combined with the one just planned, if so those combinations are next in plan (step 5). Otherwise, next factor in list enters the plan. This procedure is repeated until all factors and combinations are inserted in the plan. Coefficients "+" and "–" in the Matrix represent the levels, high or low, that factors are set. Design matrix displayed is a 2^3

full factorial with all interactions AB, AC, BC and ABC. This experimental region can also be geometrically represented by a cube (Fig. 2).

Number of replications n indicates how many times the whole set of experiments is ran under equivalent experimental conditions. Replica is the name given to an individual response that is replicated. It's possible to observe there are two sets of responses (n=2) in Table 1, y_{x1} and y_{x2}.

Table 2. 1- Design Matrix for a 2^3 full factorial

Standard Order	Factors A	B	C	Response
(1)	–	–	–	y_{11}
a	+	–	–	y_{12}
b	–	+	–	⋯
ab	+	+	–	
c	–	–	+	
ac	+	–	+	
bc	–	+	+	
abc	+	+	+	y_{1N}

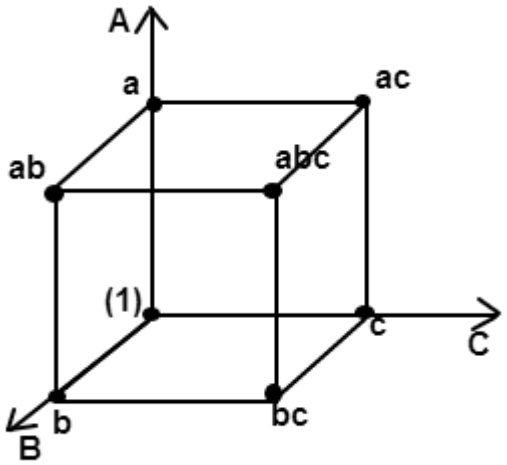


Figure 2. 2 - Geometrical representation of a 2^k Design

By fulfilling these obligatory requirements, a matrix is automatically orthogonal. This characteristic verifies if (Pereira & Requejo 2012):

- In each column the number of low levels (-) is the same as the number of high levels (+);
- The previous condition implies that each factor is experimented the same amount of times at both levels;

- The columns are orthogonal given that the sum of any coefficients' product (product of any two columns) will always equals zero;
- A column multiplied by itself results in the identity column, which is composed only by + signs in the matrix;
- The product of any two columns results always in other column of the matrix.

Regression Model

From a factorial design it's possible to produce a regression model predictive of system's response. All factors and interactions are initially considered to be part of this model, and for that reason it's called a full model (Eq. 2.2). For a 2^3 design, average of total responses is measured by β_0 – also called the intercept –, regression coefficients, β_1 to β_{123}, measure the effect on response y given by a unit alteration in factor/interaction's level. Variables x_1 to x_3 represent the codified levels, although, they can also be replaced by experimental values by means of Eq. 2.1. Including more factors increases the number of regression coefficients and codified variables. With the results achieved from the multi-way ANOVA, a model can be built with only the significant factors and/or interactions, eliminating from the equation all other components.

$$\hat{y} = \hat{\beta}_0 + \hat{\beta}_1 x_1 + \hat{\beta}_2 x_2 + \hat{\beta}_3 x_3 + \hat{\beta}_{12} x_1 x_2 + \hat{\beta}_{13} x_1 x_3 + \hat{\beta}_{23} x_2 x_3 + \hat{\beta}_{123} x_1 x_2 x_3 + \varepsilon \qquad (2.2)$$

In this model there are main effects, used to estimate linear responses, and interaction effects, used to estimate small curvature responses. A 2^k regression model can only go this far, not being able to yield good estimates of highly curved responses. In the next subchapter will be presented a method more fit for this type of response surfaces.

Calculus of Effects

A contrast is the total sum of responses obtained by a factor, or interaction of factors. Responses (y_1 to y_N) are multiplied by the respective coded levels (x_1 to x_N) and summed (Eq. 2.3). This information can easily be extracted from Design Matrix's columns.

$$Contrast_X = x_1 y_1 + x_2 y_2 + \cdots + x_N y_N \qquad (2.3)$$

Experimenter's intention is to prove that variation in response derives from factors' manipulation, and therefore must be measured. So the influence in response attained from changing a factor's level is called an effect. There are main and interactive effects, first refers to changes in a single factor's level while the second is due to changes in interaction's levels(Antony 2003; Montgomery 2009; Pereira & Requejo 2012). A generalized equation is used to represent effects:

$$\text{Effect}_X = \frac{Constrast\ X}{2^{k-1} \times n} \tag{2.4}$$

Model Validation

A regression model can only be tested for variance if certain assumptions are fulfilled. These assumptions are that responses must be normally distributed, factors must be independent variables and system's variance must be constant. Several tests can be applied to verify these assumptions, although through an analysis of residuals is possible to confirm all of them.

A residual (Eq. 2.5), calculates the difference between one experiment's response and the average of responses.

$$e_{ijk} = y_{ijk} - \bar{y}_{ijk} \tag{2.5}$$

A normal probability plot of residuals can be used to verify the normality assumption, although an examination of standardized residuals (Eq. 2.6) is required to check for outliers. Approximately 68% of these values should fall within the limits ±1, about 95% of them in ±2, and all of them in ±3. If standard residuals are approximately according to these percentages it means that there aren't outliers (Montgomery 2009).

$$d_{ijk} = \frac{e_{ijk}}{\sqrt{MS_{Resid}}} \tag{2.6}$$

In order to verify the independence of variables and constant variance assumption, residuals are plotted vs. the run order of time and vs. the fitted value. If a particular tendency can be observed in these graphics then it's likely for these assumptions to be broken. Summing up, no obvious patterns or tendencies can be found in residuals plots for assumptions to be verified (Montgomery 2009).

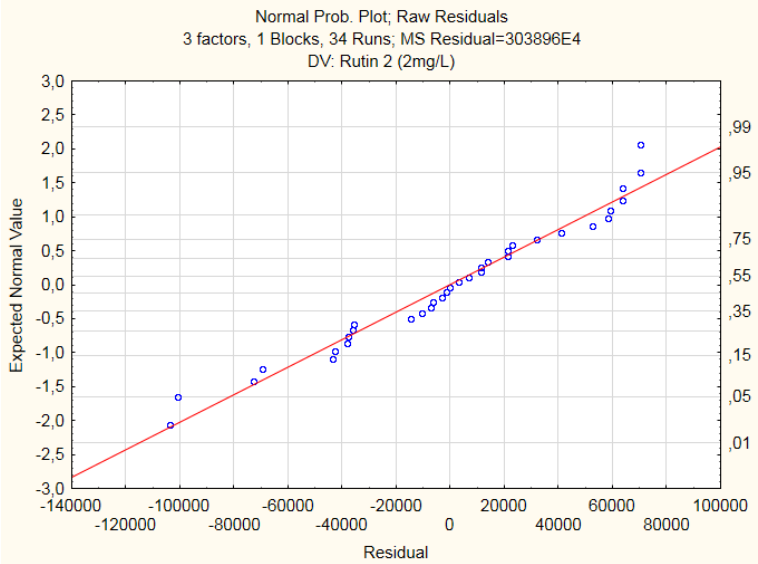


Figure 2. 3 - Normal Probability Plot of Raw Residuals

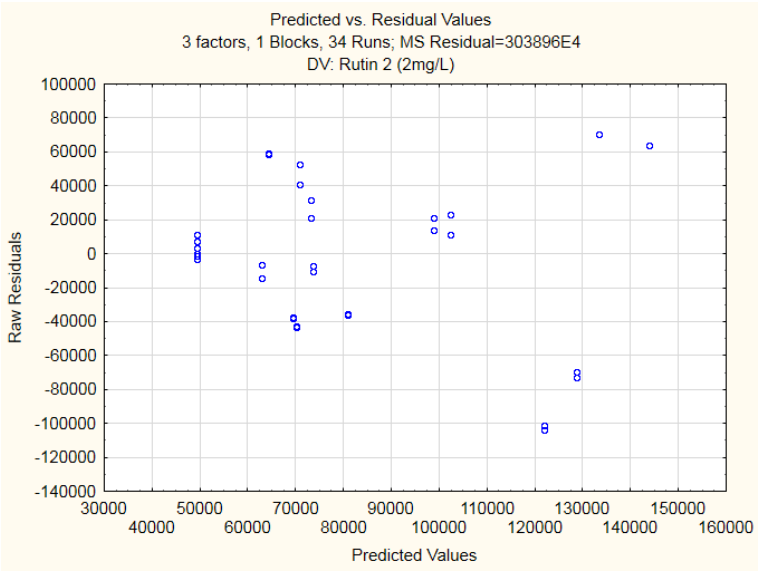


Figure 2. 4 - Predicted Values vs. Residual Values Graphic

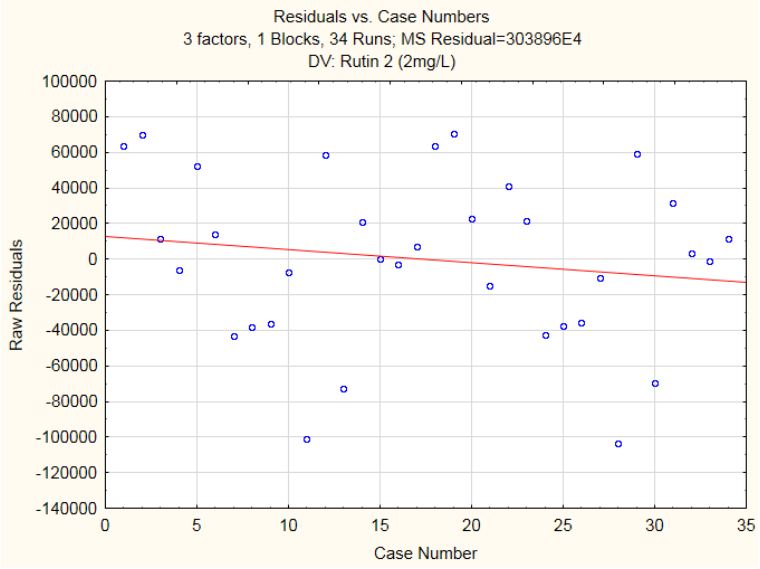


Figure 2. 5 - Residuals vs. Case Numbers Plot

Analysis of Variance

Variation in response caused by a factor or interaction is measured by the Sum of Squares, SS_X (Eq. 2.7). Total system's variation SS_{Total} (2.8) is the sum of variation explained by factors and its interactions SS_{Model}, while the variation caused by other variables not accounted in the system is SS_{Resid}. The total variation can also be expressed by the sum of each response's square value minus the square of all responses dividing by the total number of experiments.

$$SS_X = \frac{(Constrast\ X)^2}{2^k \times n} \tag{2.7}$$

$$SS_{Total} = SS_{Model} + SS_{Residual} = \sum_{i=1}^{2}\sum_{j=1}^{2}\sum_{k=1}^{n} y_{ijk}^2 - \frac{\left(\sum_{i=1}^{2}\sum_{j=1}^{2}\sum_{k=1}^{n} y_{ijk}\right)^2}{2^k n} \tag{2.8}$$

Mean Square, MS_X, is an estimative of system's variance that measures variability explained by a factor or interaction. Variability that isn't explained by the model is given by MS_{Resid}.

$$MS_X = \frac{SS_X}{1} \tag{2.9}$$

$$MS_{Resid} = \frac{SS_{Resid}}{2^k \times (n-1)} \tag{2.10}$$

A multi-way ANOVA is a statistical tool used to test the significance of model's dependent variables through a Fischer exact test. A Fischer test compares a statistic F_0 to a critical value, $F_{\alpha;\text{d.f. Model; d.f. Resid}}$ obtained from a Fischer distribution. Two possible conclusions can be taken:

- Factor/interaction is significant if $F_0 > F_{\alpha;\text{d.f. Model; d.f. Resid}}$
- Factor/interaction is insignificant if $F_0 < F_{\alpha;\text{d.f. Model; d.f. Resid}}$

$$F_0 = \frac{MS_X}{MS_{Resid}} \tag{2.11}$$

An ANOVA table is used to summarize information regarding model's significance. Factors that don't have significant influence should be removed and put on the variation that isn't explained by the model, SS_{Resid}. From this changes made, a reduced ANOVA containing only significant factors can be built (Table 2). In case significant factors are altered by this reduction, measures should be taken to address this problem. Model's reduction causes a division of residual variance in two components, $SS_{\text{Pure Error}}$, resultant from factorial experiments, and $SS_{\text{Lack of Fit}}$, resultant from non-significant variables excluded from the model.

Table 2. 2 - Reduced Multi-Way ANOVA

Source of variation	**SS**	**d.f.**	**MS**	**F_0**
A	SS_A	1	$\frac{SS}{1}$	$\frac{MS_A}{MS_{Resid}}$
AC	SS_{AC}	1	$\frac{SS}{1}$	$\frac{MS_{AC}}{MS_{Resid}}$
ABC	SS_{ABC}	1	$\frac{SS}{1}$	$\frac{MS_{ABC}}{MS_{Resid}}$
Residual	SS_{Resid}	$2^k(n-1)+\text{d.f.}_{\text{insig.}}$	$\frac{SS}{d.f._{\cdot Resid}}$	

After discovering the significant factors, an assessment of effects must be done to take conclusions regarding which experimental values yield better responses. Effect's magnitude tells which factor influence more response, while direction gives information on which levels to choose. Summarizing, it's the combination of ANOVA and effects that allow to learn more about the experimental values that are representative of the system.

For scenarios in which replication occurs ($n \geq 2$), as the one demonstrated, it's possible to create an estimation of the residual variation. Although, it's not always possible to conduct replication experiments. Limited resources, budget and time may appear as obstacles to it.

To verify if responses estimated by the model are in accordance with system's production, measures as R^2 and R^2_{adj} are used. Both parameters measure the total percentage of response

estimated by the model, although the R^2_{adj} is considered more reliable due to having a better adjustment to non-significant factors.

$$R^2 = \frac{SS_{Model}}{SS_{Total}} \tag{2.12}$$

$$R^2{}_{adj.} = 1 - \frac{\frac{SS_{Resid}}{d.f_{\cdot Resid}}}{\frac{SS_{Total}}{d.f_{\cdot Total}}} \tag{2.13}$$

2.1.2. Central Composite Design

A Central Composite Design is used to study a region of interest larger than the one confined between two factorial levels. It's highly used in optimization experiments when standard factorial designs can't reach optimal solutions. Designs are built from a factorial structure, to which central and axial experiments are added (Hibbert 2012; Ferreira et al. 2007). The Composite experiments for a two-level full factorial can be described by:

$$N = 2^k + 2 \times k + n_c \tag{2.14}$$

As expressed in the equation, experiments can be divided in three sequenced block. The first one is a full factorial design (2^k), in which factors are tested at two levels, which are selected by researchers from previous screening experiments, or alternatively from performing a literary review. A second block (n_c) is added, in which a new level centered between the two factorial levels is generated, it's codified as *x=0*. For this block to be created all factors need to be quantitative (Ferreira et al. 2007). Several experiments are performed in this point, where all factors possess the same codified level, called the centerpoint of the design region. The number of centerpoint replications, n_C, is established by the researcher according to criteria that will be further explained with more detail.

Axial experiments ($2 \times k$), represented by the 3rd block are dependent on an evaluation of system's curvature. If the test proves that curvature is significant, should be added to the design. An analysis of variance is applied for that purpose:

$$SS_{Curvature} = \frac{n_F n_{CP} (\bar{Y}_F - \bar{Y}_{CP})^2}{n_F + n_{CP}} \tag{2.15}$$

$$MS_{Curvature} = \frac{SSCurvature}{1} \tag{2.16}$$

$$MS_{Error} = \frac{\sum_1^{n_{cp}} \left(y_j - \bar{Y}_{cp}\right)^2}{n_{cp} - 1} \quad (2.17)$$

Where $\bar{Y}_F$ and $\bar{Y}_{CP}$ represent the average response for factorial and centerpoint experiments, while n_F and n_{CP} stand for the number of experiments made for each block. Using the Fischer exact test (Eq. xx) it's possible to verify if curvature is significant. If significance is verified, axial experiments are performed creating two new levels, codified as $x=\alpha$ and $x=-\alpha$. The parameter α represents the distance between axial level and centerpoint level.

It's possible that sometimes experimenters don't use this test, and instead take a conservative approach considering that curvature might exist and therefore performing a complete CCD, with the three blocks.

Design Features

According to the requirements of the system investigated, researcher needs to define two main features to characterize a design, the distance from axial level to the center of design (α) and the number of replications (n_C) at the centerpoint (Montgomery 2009). For a cubical region of interest a center faced design (CCF) can be applied, this design features are α=1 and a small number of replications at the center, usually one or two (Ferreira et al. 2007).

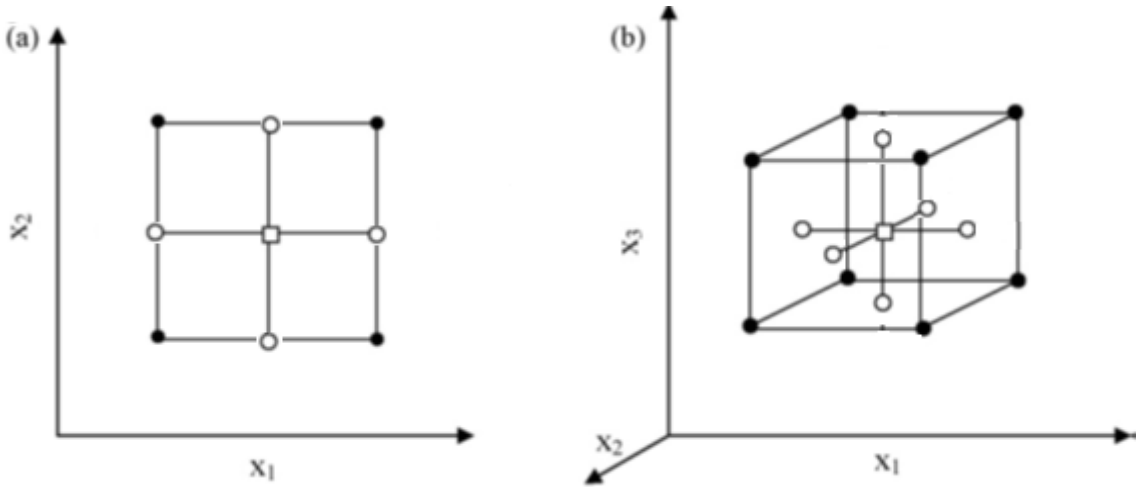


Figure 2. 6 - CCF Design representation for (a) 2 and (b) 3 factors

In case the system produces an experimental surface that might surpass the factorial region, then it's recommended to use a circumscribed design (CCC) in which $\alpha>1$ and a higher n_C is required. A significant curvature requires a design with special features, Box and Hunter developed a solution to attend these requirements. According to them, a design is rotatable if all

estimates of variance depend exclusively on their distance to the design centerpoint, making the precision of predicted response be the same for all points located in the hypersphere around the design center. For this reason, the distance from axial points and factorial points to the center of the design has to be equal, generating a hypersphere region (Montgomery 2009; Ferreira et al. 2007). This rotatability is verified for:

$$\alpha = 2^{k/4} \tag{2.18}$$

Creating a circumscribed design for the biggest axial distance ($\alpha=\sqrt{k}$) demands a consequently higher number of centerpoint replications, because the growth of experimental error increases with the experimental region.

(b)

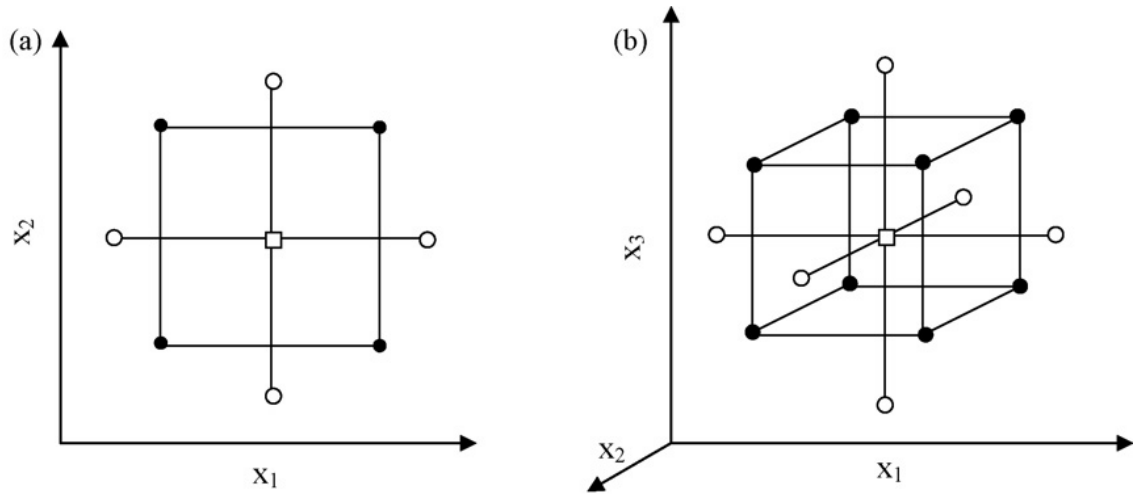


Figure 2. 7 - CCC Design representation for (a) 2 and (b) 3 factors

Selecting α depends mainly on the type of response system is expected to generate, while the number of centerpoint replications is chosen according to α and the accuracy of experimental error intended, it produces an independent estimative of this error without changing estimative of effects (Pereira & Requejo 2012).

Experimental Plan

Experimental planning is displayed by means of a Design Matrix divided in three parts. Factorial component is the first subset of experiments that appears on the design, it's composed by the first 8 experiments, followed by centerpoint replications in which all factors are set at the same level "0". Axial experiments are the last component added, two experiments are performed

for each factor studied, one at the high level "α", and the other at the low level "-α". Remaining factors, not set for axial levels, are all fixed at level "0"(Montgomery 2009).

Defining the levels with clarity is crucial to attain a successful optimization (Baş & Boyacı 2007). The proximity of experimental values can compromise response's variation, not causing enough variation to have a clear distinction between different levels responses (Hibbert 2012).

Table 2. 3- Design Matrix for a Central Composite Design

Standard Order	Factors A	B	C	
1	–1	–1	–1	
2	+1	–1	–1	
3	–1	+1	–1	
4	+1	+1	–1	
5	–1	–1	+1	
6	+1	–1	+1	
7	–1	+1	+1	
8	+1	+1	+1	
9	0	0	0	
10	0	0	0	
11	0	0	0	
12	1,682	0	0	
13	-1,682	0	0	
14	0	1,682	0	
15	0	-1,682	0	
16	0	0	1,682	
17	0	0	-1,682	

Factorial Component

Central Component

Axial Component

$$\begin{bmatrix} \alpha & 0 & 0 & \cdots & \cdots & \cdots & 0 \\ -\alpha & 0 & 0 & \cdots & \cdots & \cdots & 0 \\ 0 & \alpha & 0 & \cdots & \cdots & \cdots & 0 \\ 0 & -\alpha & 0 & \cdots & \cdots & \cdots & 0 \\ \vdots & & & & & & \vdots \\ 0 & 0 & 0 & 0 & \cdots & \cdots & \alpha \\ 0 & 0 & 0 & 0 & \cdots & \cdots & -\alpha \end{bmatrix}.$$

Calculus of Effects and Analysis of Variance

The main difference from the Central Composite Design to the Full Factorial Design is the ability to include pure curvature in its model. Therefore, in its ANOVA are included the square factors such as it's demonstrated under.

Table 2. 4- Example of an ANOVA for two factors that includes quadratic levels

Source of variation	**SS**	**d.f.**	**MS**	**F_0**
A	SS_A	1	$\frac{SS}{1}$	$\frac{MS_A}{MS_{Resid}}$
B	SS_B	1	$\frac{SS}{1}$	$\frac{MS_B}{MS_{Resid}}$
AB	SS_{AB}	1	$\frac{SS}{1}$	$\frac{MS_{AB}}{MS_{Resid}}$
A^2	$SS_A{}^2$	1	$\frac{SS}{1}$	$\frac{MS_{A^2}}{MS_{Resid}}$
B^2	$SS_B{}^2$	1	$\frac{SS}{1}$	$\frac{MS_{B^2}}{MS_{Resid}}$
Residual	SS_{Resid}	n_{cp} -1	$\frac{SS}{d.f._{Resid}}$	

Regression Model

A regression model to study pure curvature applies for cases in which there's indication of significant curvature. In that case, the model is given by:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j>i}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \varepsilon \qquad (2.19)$$

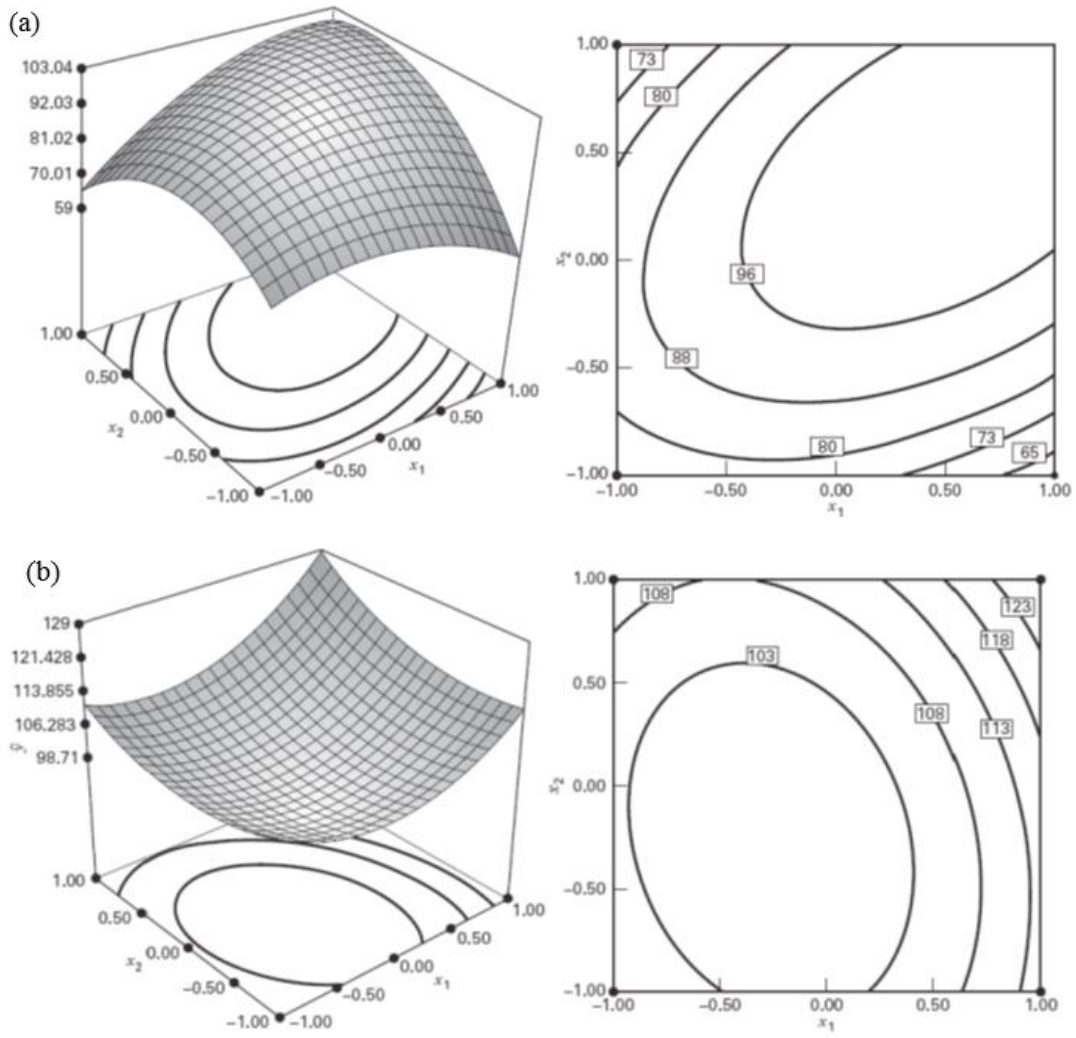
This model can be reduced to only the significant components after running the multi-way ANOVA.

Response Surface Methodology

A Response Surface Methodology is a set of statistical techniques used to develop a surface that reflects the response system is expected to return, produced by a regression model, to changes in factors' levels. This tool is used by statistical computer softwares that allow to process a bigger amount of information faster. According to Montgomery "the eventual objective of RSM is to determine the optimum operating conditions for the system or to determine a region of the factor space in which operating requirements are satisfied" (Montgomery 2009).

It starts by using techniques to improve response produced by a first-order model, by increasing or decreasing it until the maximum/minimum response that this model can produce. These responses are attained through the method of the steepest ascent for increasing response, and method of the steepest descent to decrease it. A second-order model is then used to find the optimum, this point can be a maximum, minimum or saddle point. This point is known as stationary point and it's located where partial derivatives $\partial\hat{y}/\partial x_1 = \partial\hat{y}/\partial x_2 = \partial\hat{y}/\partial x_k = 0$.

A two-dimensional display of the surface plot, named Contour Plot, can be developed by this tool. It helps to understand the shape a response surface might have. Lines of constant response are drawn in the plane of independent variables reproducing response's behavior. It's a valid option as a guideline for researchers to help in response surface visualization. The final three-dimensional plot of the predictive model, including the optimum, is given by a response surface plot (Baş & Boyacı 2007).



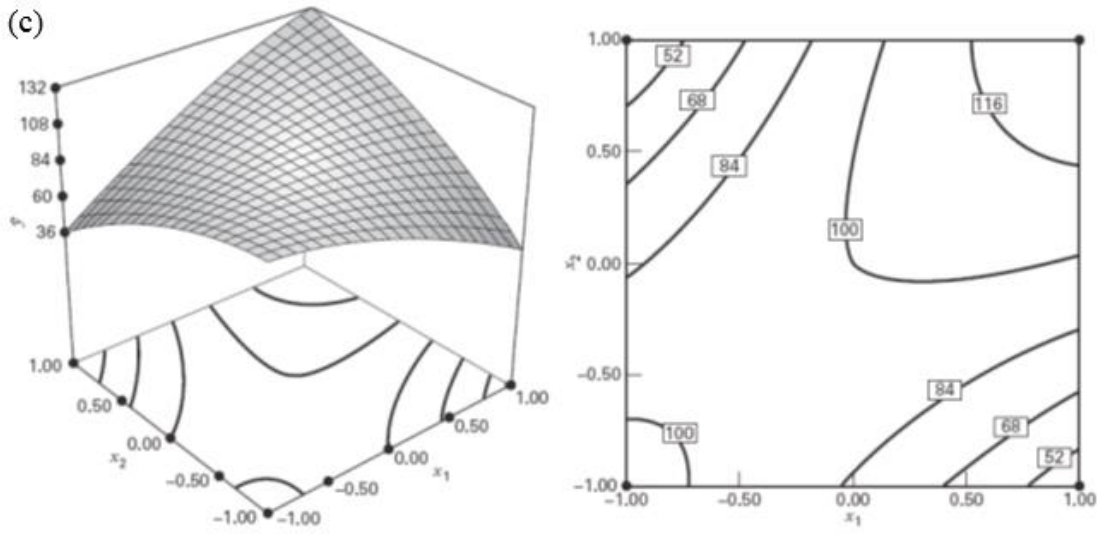


Figure 2. 8 - Response Surface Plot for (a) maximum (b) minimum and (c) saddle point

2.2. Chromatography

Chromatography is a chemical method used for separating compounds that are mixed in a solution. Usually, the procedure is separated in three chronological stages, sample preparation, analysis of compounds and analyte extraction. All chromatographic processes have at least two points in common: a solvent designated as mobile phase that passes through the mechanism, and a stationary phase that retains compounds temporarily. The interaction of these two phases can be used to classify the different methods used. In Column Chromatography, the stationary phase is fixed in a tube and the mobile phase is forced to pass through this tube, while in Plain Chromatography the stationary phase is supported by a plaque or by a paper, and the mobile phase passes through it by capillarity or with the help of gravity. The other way to classify it is according the type of mobile phases used: gas chromatography, liquid chromatography and supercritical fluid chromatography (Gonçalves 2001).

2.2.1. Separation of compounds

Compounds in a mixture can be separated through diverse techniques, such as normal phase chromatography, liquid-liquid partition chromatography, reversed phase chromatography, ion exchange chromatography, etc.

In reversed phase chromatography, the sample enters the system, gets dragged by the mobile phase – a polar eluent – through a column containing the stationary phase – a non-polar solid compound. It's this interaction of both phases that creates conditions for separation, in which the components of the mixture (sample) take different times to get dragged through the column, being the more polar components attracted to the mobile phase, therefore dragged faster, while the less

polar components are attracted to the stationary phase. Polarity is the characteristic used by the separation mechanism by this method (Meyer 1988).

2.2.2. Chromatograms: obtaining and analyzing results

After the solid-mobile phase interaction, separated compounds are sent to the mass detector which registers an electrical sign, this sign is sent to the computer and displayed graphically as a Chromatogram. A chromatogram is a graphic containing Gaussian curves, also known as peaks, which supply qualitative and quantitative information about the mixture. Retention time is a qualitative information represented by the time separating injection moment, from the moment when signal maximum is reached, and it's only obtained under identical chromatographic conditions: column dimensions, type of stationary phase, mobile phase composition and flow velocity. When these variables are repeatedly experimented with the same values, it's possible to identify a peak through retention times' comparison.

Compounds main data that can be retrieved from a chromatogram is the peak width, w_1, at the baseline, and the dead time, t_0, or time required by the mobile phase to pass through the column. Quantitative information such as areas and concentrations of compounds can be calculated from graphic parameters, this data is used to establish comparisons between compounds and take conclusions regarding separation's efficiency.

2.3. Response Surface Methodology applied to antioxidant extraction

The usage of RSM as a support to investigate the statistical importance of various variables in a response as revealed itself fundamental. From its advantages, the one that must be underlined is its capacity of studying curvature in models by plotting the response surface, a three dimensional representation of the effect caused by two independent variables in a dependent variable.

An appropriate selection of factors has a crucial role in experimental success, and there are two paths that can be followed to arrive to conclusions:

- Screening experiments – A set of experiments is performed according to an experimental design, in which all factors that might be important of factors should be considered. The experimental goal is to reduce the number of factors to only the significant ones.
- State-of-the-Art Review – Through a review of similar studies, it's possible to verify which factors are being considered for the process intended.

Ideally both tasks should be materialized in order to achieve a successful optimization, even if sometimes it's not possible due to being time and resource constraining.

State-of-the-Art Review

In order to optimize the Total Phenolic Compounds extraction and antioxidant activity on fresh dark figs, an experimental design was done. The factors considered were temperature (25-65ºC), time (60-120 min) and acetone concentration (40-80%). Box-Behnken was the design selected for these experiments, due to a need of studying factors at 3 levels each.

Experimenters concluded that all factors considered influenced significantly TPC and antioxidant activity of fig extracts. The optimal conditions to obtain the highest extraction of phenolics from fresh dark fig, as well as maximum antioxidant activity, were acetone concentration of 63.48%, temperature of 48.66 ºC, and extraction time of 115.14 min. Under optimal conditions, the experimental values for TPC and antioxidant activity were 536.43 ± 5.53 and 68.77 ± 1.43 mg GAE/100 g DM, respectively. These experimental results were in agreement with the predicted values which corresponding to 540.10 mg and 71.86 mg GAE/100 g DM (Bachir Bey et al. 2014).

A solid-liquid extraction process was optimized using a Response Surface Methodology for three factors: Temperature, pH and Ethanol concentration. The design applied for this optimization was a Central Composite, in the form: 2^3+2×3+6. This optimization was conducted for *Pyracantha fortuneana* fruits with a focus on total phenolic content (TPC) and total antioxidant activity (TAA). Based on the ANOVA, experimenters gauged that EtOH concentration, extraction temperature, and solution pH had significant positive linear effects on antioxidant activity, whereas their quadratic interaction had significant negative effects on TAA. Extraction conditions for optimized responses were EtOH = 71%, T = 51 ºC, and pH = 3.2. The maximum TAA predicted by contour plots was 1755 U/g dried PFF sample weight, whereas the value in our results under the optimum conditions was 1728 ± 14 U/g dried sample weight(Zhao et al. 2013).

Optimal conditions for antioxidant extraction from potato peel waste in a HPLC system were studied using a Response Surface Methodology. The extraction of antioxidants as a function of Temperature (T), Ethanol concentration (E) and Time (t) was studied using a rotatable second order design (CCD) with six replicates– 2^3 + 2x3 + 6 – in the centre of the experimental domain, and therefore α = 1,681. The conditions of the independent variables studied ranged from 25-90ºC for T, 20-100% for E and t between 5-150 min. The main phenolic compounds identified in the extracts were chlorogenic (Cl) and ferulic (Fer) acids. (Amado et al. 2014).

With the purpose of testing a new method for extracting natural antioxidants of espresso spent coffee grounds (SCG), a waste material abundant in restaurants and cafeterias, Marija Ranic et al. used response surface methodology as a statistical support. This method is a Microwave-assisted

extraction (MAE) and the independent variables considered were extraction time (ET), liquid-to-solid ratio (LSR), and microwave power (MWP). A central composite design was used and a total of 30 experiments was performed (Ranic et al. 2014). The investigated factors, Extraction Time and Ethanol percentage, ranged from 40s to 360s and 20% to 80% (v/v) respectively. Deriving from these factors combination, the total extract yield ranged from 7.694 to 31.216 mg/g d.w SCG. The maximum yield was recorded on the 24th run, under following experimental conditions: 180s, 12 ml/g, and 550W MWP. Concerning the Total Phenolic Content (TPC) expressed in percentage (%, w/w) of dry SCGE, the yield ranged from 18.83 to 79.83%. The maximum yield was recorded in sample run 16, for the following levels: 40s, 240 W MWP and 6 fold solvent to SCG ratio(Ranic et al. 2014).

Extraction conditions for Mangifera Pajang peels were optimized through a Response Surface Methodology. System's performance was measured in terms of total phenolic content and antioxidant capacity, and the variables studied were solvent concentration (ethanol), extraction temperature and liquid-to-solid ratio. A total of 20 experiments, divided in 8 factorial plus 6 star points and 6 center points, were performed following a central composite design. Three optimal conditions were developed for the responses, which were ethanol concentration 68%, 55 ºC and 32.7 mL/g, for TPC generating a response of 14.6 mg GAE/g, while for AC it was 68%, 56 ºC and 31.8 mL/g with a response of 0.2065 nm.(Prasad et al. 2011).

The Subcritical water extraction method was optimized for seeds of Coriandrum sativum. The parameters measured were the total phenolics and total flavonoids, which were conditioned by temperature (100-200º C), pressure (30-90 bar), and extraction time (10-30 mins). The experimental design applied for this case was a Box-Behnken with 3 factors and 3 levels (Zeković et al. 2014).

Extraction of polyphenols and antioxidants from the rice bran with resource to ultrasonic technology, was optimized via an RSM. The variables solvent percentage (ethanol), temperature and time were taken into account for this study. Experiments were planned and run following a center faced central composite design, combining a total of 16 experiments divided in: 8 factorial experiments, 6 face centered experiments and 2 center points. The indepentent variables considered for this stage were: ethanol percentage (50-90%), extraction temperature (40-60ºC), and extraction time (15-45 min). Response variables were TPC (mg GAE/g of rice bran), FRAP (lmol Fe2+/g of rice bran), DPPH free radical scavenging (%) and yield of extraction (%). The optimum extraction conditions were: temperature of 51-54ºC, extraction time in the range of 40-45 min and concentrations 65-67% of ethanol, resulting in optimal total phenols (6.05 mg GA/g dw), antioxidant activity (54.14 lmol Fe2+/g dw) and antiradical activity (52.83% inhibition) from rice bran (Tabaraki & Nateghi 2011).

2.4. Oca Tuber

The Oca is an amylase tuber that grows in the Andean regions, especially in Peru and Bolivia, which is very similar to a potato and can be visually distinguished by two characteristics: shape and color. The second most widely cultivated native tuber in the Andes is Oxalis tuberosa, which belongs to the Oxiladaceae family. It's actually harvested as a substitute product of the common potato, given its similar nutritional value and the fact that they are more resistant to plagues and diseases. It's a culture that easily grows in poor soils and adverse climacteric conditions(Alcalde-eon et al. 2004).

The phytopharmaceuticals that are applied in the harvesting have a beneficial effect for the end consumer, due to the antioxidants compounds present that prevent several human deceases such as cardiovascular deceases, cancer and oxidant stress. Therefore, it's important to quantify the presence of these compounds in food. These type of experiments, that provide information regarding aliment's characteristics, assume a major importance for health organizations that can than approach nutritional problems of the local cultures with more knowledge. It allows them to develop nutritional plans and campaigns based on reliable information(Chirinos et al. 2009).

2.5. Photodiode Array Detector

These detectors can detect any light absorption from the ultraviolet region (190 nm) until the visible region (720 nm). Its biggest advantage is to allow experimenters to monitor a wide range of wavelengths at once. Consequently, it provides benefits such as reduction of run time and solvent expenditure (Meyer 2010).

In its mechanism, a light from a broad emission source such as a deuterium lamp is collimated by an achromatic lens system so that the total light passes through the detector cell onto a holographic grating. In this way the sample is subjected to light of all wavelengths generated by the lamp. The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc. At the end of the run, the output from any diode can be selected and a chromatogram produced employing the UV wavelength that was falling on that particular diode. Most instruments will permit the monitoring of a least one diode in real time so that the chromatogram can be followed as the separation develops. This system is ideal in that by noting the time of a particular peak, a spectrum of the solute can be obtained by recalling from memory the output of all the diodes at that particular time. This gives directly the spectrum of the solute, i.e., a curve relating adsorption against wavelength (Scott n.d.).

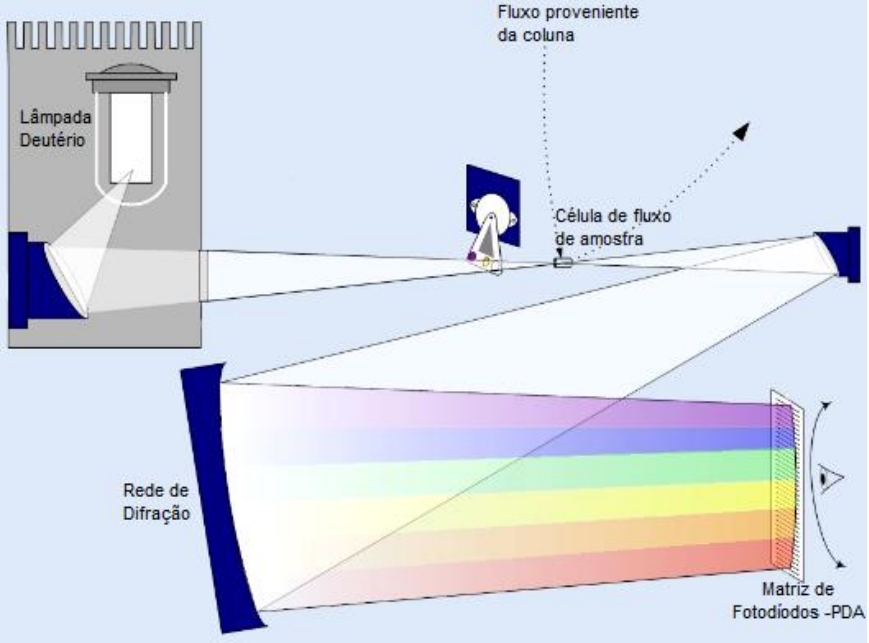


Figure 2. 9 - Representative scheme of a Photodiode Array Detector

3. Methodology and Results

This study was designed with the objective of identifying the factors influencing the extraction process, using a Photodiode Array Detector system, for four antioxidants: Ellagic Acid, Ferulic Acid, Rutin and Cinnamic Acid. In order to characterize the antioxidants were used the standards of each compound. Except for Ellagic Acid, standards applied had two concentrations: 0,2mg/L and 2mg/L. The reason for using different standards for the first antioxidant – 0,4mg/L and 4mg/L – is that in previous experiments made, the researchers observed that the standards previously defined weren't producing responses.

Experimental Plan

The experimental plan was made according to DoE methodology, more specifically a Central Composite Design (CCD) was the option selected due to giving the possibility of including pure curvature studies in its predictive model. The factors considered for this study were: the Column Temperature in ºC, the Solvent Concentration in % of Acetonitrile, and the Flux in ml/min. These were the independent variables considered by the researchers to have an important influence over the dependent variable extraction, which is measured in Peak Area (system's response).

A total of n= 17 experiments were performed with two replicas for each standard solution as shown:

Table 3. 1 - Experimental Factors and respective levels

Factors	Level -1,682	Level -1	Level 0	Level +1	Level +1,682	Units
Column Temperature	30	34	40	46	50	ºC
Dissolution Solvent	1	21	50	79	99	% Acetonitrile
Flux	0,20	0,25	0,33	0,40	0,45	ml/min

Table 3. 2 - General Experimental plan

	Factors		
Standard Order	A	B	C
1	34	21	0,25
2	46	21	0,25
3	34	79	0,25
4	46	79	0,25
5	34	21	0,40
6	46	21	0,40
7	34	79	0,40
8	46	79	0,40
9	40	50	0,33
10	40	50	0,33
11	40	50	0,33
12	50	50	0,33
13	30	50	0,33
14	40	99	0,33
15	40	1	0,33
16	40	50	0,45
17	40	50	0,20

The number of experiments isn't sorted randomly, it results from a quantitative and a qualitative analysis made by the experimenters relaying mainly on how accurately we need to comprehend the system. If the system requires a light comprehension due to the requirements of its practical application being low, not demanding precision in response then a standard experimental design can be enough, such as a two level full factorial (2^k) or fractional factorial (2^{k-p}). The three level experiments (3^k or 3^{k-p}) aren't commonly considered because they demand a high number of experiments to study few factors, achieving easily an unbearable number of experiments giving the exponential characteristic of factorial experiments. Therefore when laboratorial resources are strictly limited, or when the time is crucial, this option is not valid.

For this experiment, the more suitable option was a Central Composite Design with 3 centerpoint experiments – $N= 2^3+ 2x3 + 3= 17$ experiments. This experimental plan allows to retrieve information regarding the curvature of the system, allowing to produce a predictive model that includes linear components, interaction components and quadratic components.

The data resulting from these experiments was then inserted in Statistica 10, in order to make a proper statistical study of the experimental design.

3.1. Ellagic Acid

3.1.1. Experimental results

A set of 17 solutions were prepared for two different concentrations of Ellagic Acid standard – therefore achieving a total of 34 experiments – subject to variations in Column Temperature, Flux and Acetonitrile Concentration. The first experimental set was prepared using a standard concentration of 0,4mg/L, while the second was made using a 4mg/L concentration. The solutions were then inserted into the Photodiode Array Detector and responses were measured in peak areas, as shown in Table 3.3. The highest extractions for both Ellagic Acid standards were achieved on the 3rd experiment, in which peaks of 52939 and 53424 were observed for the two low standard replicas, while 402019 and 427993 were the peaks for the high standard replicas. Consequently, it's possible to observe that the real experimental conditions yielding best results are: 34ºC, 79%, and 0,25 ml/min.

Table 3. 3 - Experimental plan and respective responses for Ellagic Acid solution

				Responses			
	Factors			0,4 mg/L		4 mg/L	
Std. Order	A	B	C	1st Injection	2nd Injection	1st Injection	2nd Injection
1	34	21	0,25	33428	31926	323043	327177
2	46	21	0,25	32031	30902	298781	307117
3	**34**	**79**	**0,25**	52939	53424	402019	427993
4	46	79	0,25	28712	30550	217184	240422
5	34	21	0,40	11800	12520	118842	124667
6	46	21	0,40	10327	10330	122451	127954
7	34	79	0,40	18175	18722	64399	62685
8	46	79	0,40	14081	15276	50704	50497
9	40	50	0,33	21572	20240	223849	234490
10	40	50	0,33	27384	25872	129708	214036
11	40	50	0,33	10895	11294	33186	34154
12	50	50	0,33	5274	5335	41592	44819
13	30	50	0,33	9121	9902	53093	91611
14	40	99	0,33	20681	23761	215023	236036
15	40	1	0,33	18699	19523	186780	192124
16	40	50	0,45	17339	17849	176084	179993
17	40	50	0,20	15066	15924	106701	108163

3.1.2. Analysis of Variance

Ellagic Acid (0,4 mg/L)

The Analysis of Variance obtained for an Ellagic Acid low standard (0,4mg/L) is represented in Table 3.4. Significant factors are identified with the bold effect, therefore it's possible to observe that these factors are: Column Temperature (Linear), Column Temperature (Quadratic), Acetonitrile (Linear) and Flux (Linear). The last factor is the one that has higher influence (p=0,000002) in response for this low standard compound. It's imperative to perform an ANOVA Reduction to verify if by fitting other non-significant factors in the Error, more factors will become significant.

Table 3. 4 - ANOVA for 0,4 mg/L Ellagic Acid

	Var.:Ellagic Acid 1 (0,4 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	**2,341469E+08**	**1**	**2,341469E+08**	**4,92115**	**0,036243**
Column Temp. (ºC) (Q)	**4,271174E+08**	**1**	**4,271174E+08**	**8,97687**	**0,006263**
Acetonitrile (%) (L)	**2,232602E+08**	**1**	**2,232602E+08**	**4,69234**	**0,040452**
Acetonitrile (%) (Q)	2,989626E+07	1	2,989626E+07	0,62834	0,435731
Flux (ml/min) (L)	**1,860561E+09**	**1**	**1,860561E+09**	**39,10404**	**0,000002**
Flux (ml/min) (Q)	5,480615E+07	1	5,480615E+07	1,15188	0,293829
1L by 2L	1,473614E+08	1	1,473614E+08	3,09714	0,091172
1L by 3L	9,177161E+07	1	9,177161E+07	1,92880	0,177645
2L by 3L	1,612223E+07	1	1,612223E+07	0,33885	0,565924
Error	1,141914E+09	24	4,757976E+07		
Total SS	4,306910E+09	33			

Displayed in Table 3.5, the Reduced ANOVA shows that this procedure didn't acquire any new significant factors. As a result, it's possible to state that 4 factors had significant influence in 0,4mg/L Ellagic Acid standard.

Table 3. 5 - Reduced ANOVA for 0,4mg/L Ellagic Acid

	Var.: Ellagic Acid (4 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	**2,341469E+08**	**1**	**2,341469E+08**	**4,47855**	**0,043027**
Column Temp. (ºC) (Q)	**4,727691E+08**	**1**	**4,727691E+08**	**9,04270**	**0,005402**
Acetonitrile (%) (Q)	**2,232602E+08**	**1**	**2,232602E+08**	**4,27032**	**0,047816**
Flux (ml/min) (L)	**1,860561E+09**	**1**	**1,860561E+09**	**35,58713**	**0,000002**
Error	1,516173E+09	29	5,228184E+07		
Total SS	4,306910E+09	33			

Through an analysis of the coefficient of determination, R^2= 0,64797, we can verify that for Ellagic Acid (0,4mg/L) the regression model approximates the real data.

Ellagic Acid (4 mg/L)

The Reduced ANOVA (Table 3.6) made for the Ellagic Acid 4mg/L standard demonstrates that Column Temperature (Q), Acetonitrile (Q) and Flux (L) are the factors influencing significantly response. Highest influence in response for this high concentration standard is caused by the Linear component of Flux. The ANOVA produced for this standard with all factors can be found in the Appendixes.

Table 3. 6 - Reduced ANOVA for 4mg/L Ellagic Acid

	Var.: Ellagic Acid (4 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (Q)	1,920336E+10	1	1,920336E+10	4,31934	0,046334
Acetonitrile (%) (Q)	2,138579E+10	1	2,138579E+10	4,81023	0,036180
Flux (ml/min) (L)	1,999161E+11	1	1,999161E+11	44,96639	0,000000
Error	1,333770E+11	30	4,445901E+09		
Total SS	3,858589E+11	33			

Through an analysis of the coefficient of determination, R^2= 0,65434, we can verify that for Ellagic Acid (4mg/L) the regression model approximates the real data.

3.1.3. RSM and Contour plots

Ellagic Acid (0,4 mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and are above displayed from Figure 3.1 to 3.6.

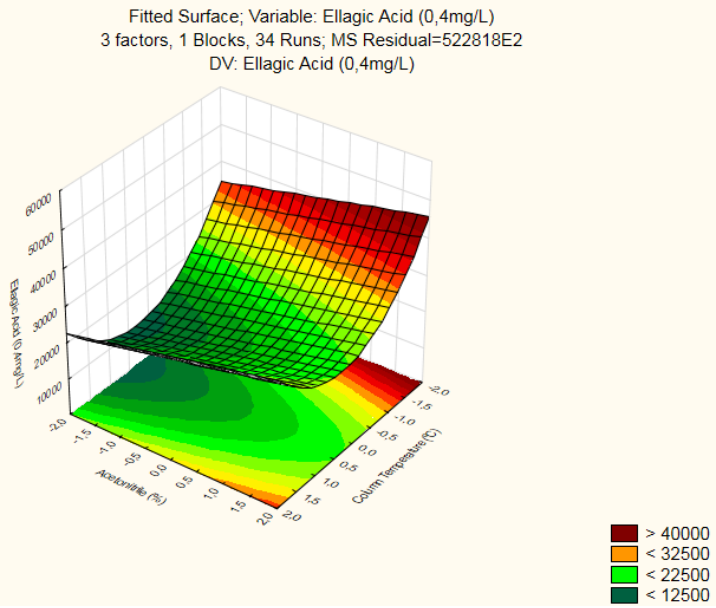


Figure 3. 1 - RSM for Ellagic Acid (0,4mg/L): Acetonitrile x Column Temperature

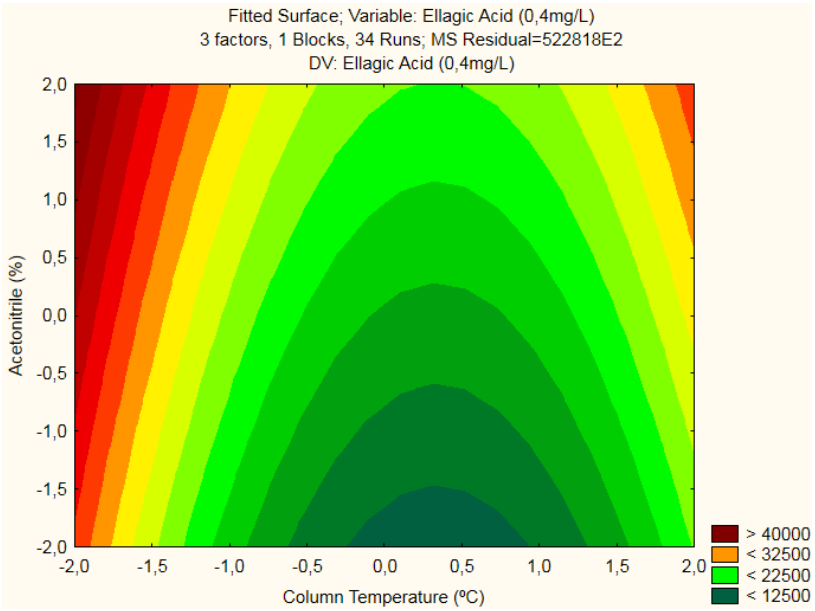


Figure 3. 2 - Contour Plot for Ellagic Acid (0,4mg/L): Acetonitrile x Column Temperature

For the interaction Acetonitrile x Column Temperature, it's possible to observe that the highest peak area (>40000) is obtained for the high level of Acetonitrile and low level of Column Temperature. Figure 3.2 shows a Contour with an eliptical shape centered at the central level (0 or 34ºC) of Column Temperature and low level of Acetonitrile.

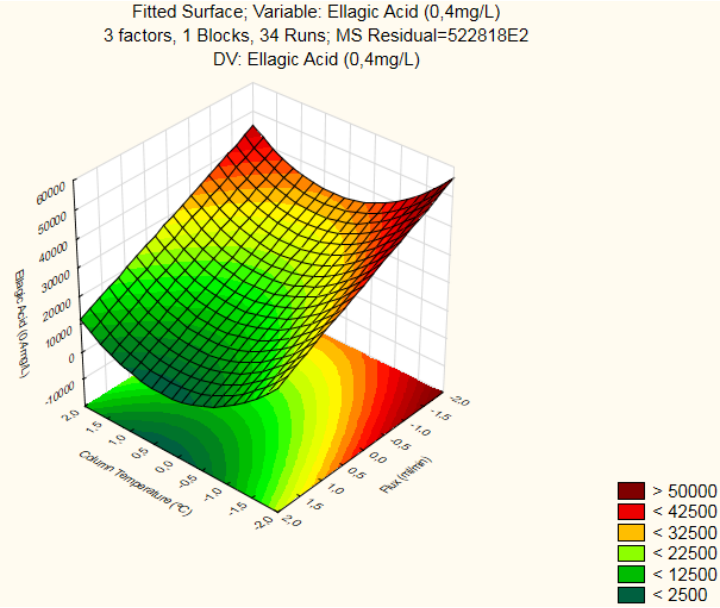


Figure 3. 3 - RSM for Ellagic Acid (0,4mg/L): Flux x Column Temperature

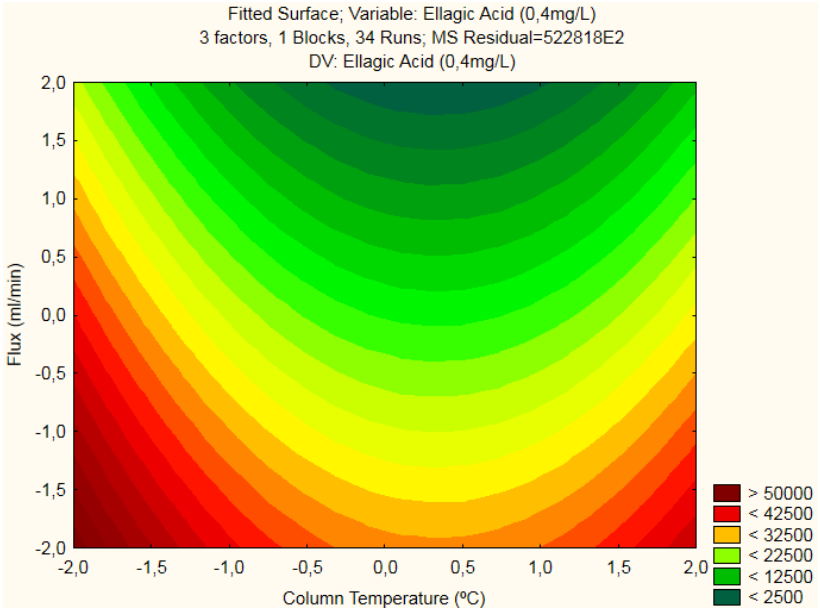


Figure 3. 4 - Contour Plot for Ellagic Acid (0,4mg/L): Flux x Column Temperature

Graphics plotted demonstrate that the highest peak (>50000) for the interaction between Flux and Column Temperature was attained with both factors set at low level. This Contour Plot (Figure 3.4) presents a similar shape to the interaction above, and again the central region of Column Temperature yield the lowest peak areas (<2500).

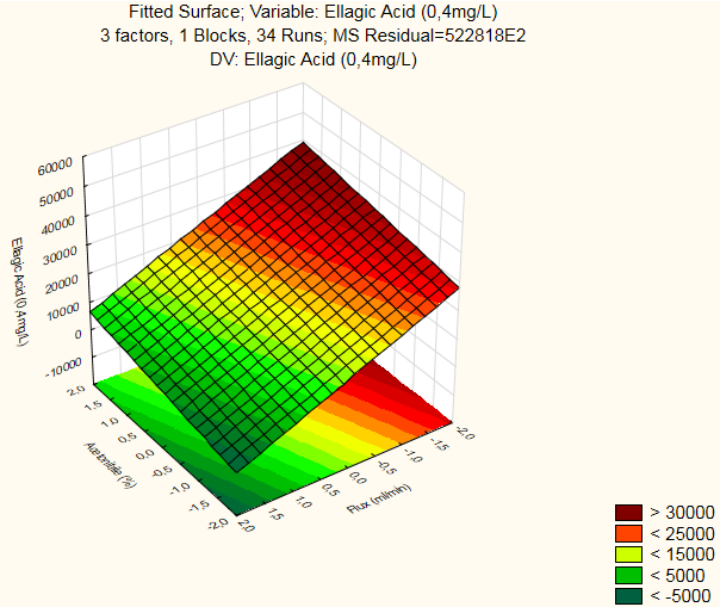


Figure 3. 5 - RSM for Ellagic Acid (0,4mg/L): Flux x Acetonitrile

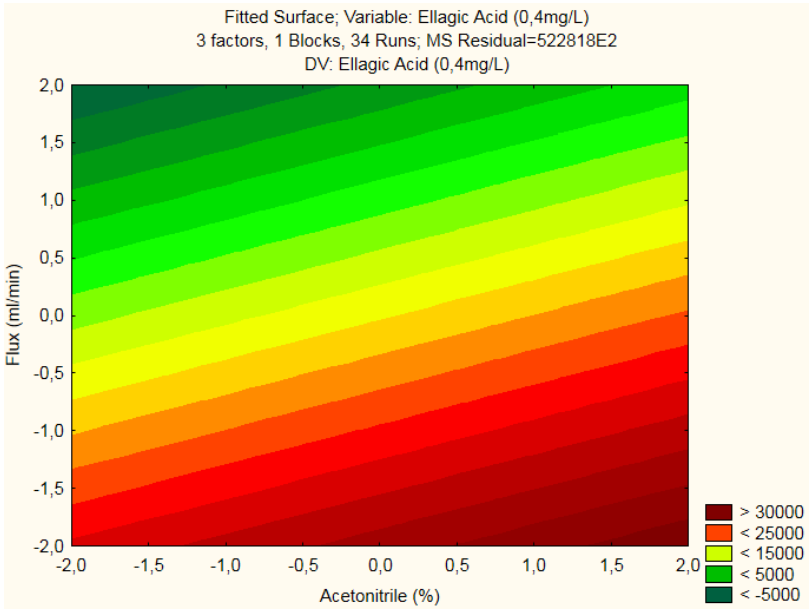


Figure 3. 6 - Contour Plot for Ellagic Acid (0,4mg/L): Flux x Acetonitrile

It's this interaction that yields lower peak areas for Ellagic Acid (0,4mg/L), with its high peak on the >30000 range. This result is obtained when Acetonitrile is set at high level and Flux at low level. The Contour Plot (Figure 3.6) presented in this case doesn't show curvature in opposition to the two interactions shown above.

Summarizing, it's the interaction Flux x Column Temperature that attains the highest peak area (>50000) for both factors low levels, presenting a Response Surface with curvature. This interaction is aligned with the significant factors found in the Reduced ANOVA (Table 3.5).

Ellagic Acid (4 mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

From the three Response Surfaces produced, it was the interaction between Flux and Column Temperature that yielded the highest peak (>400000), which was obtained for a low Flux . This surface also has a strong curvature component as it was expected given that its Reduced ANOVA (Table 3.13) found the two Quadratic components to be significant.

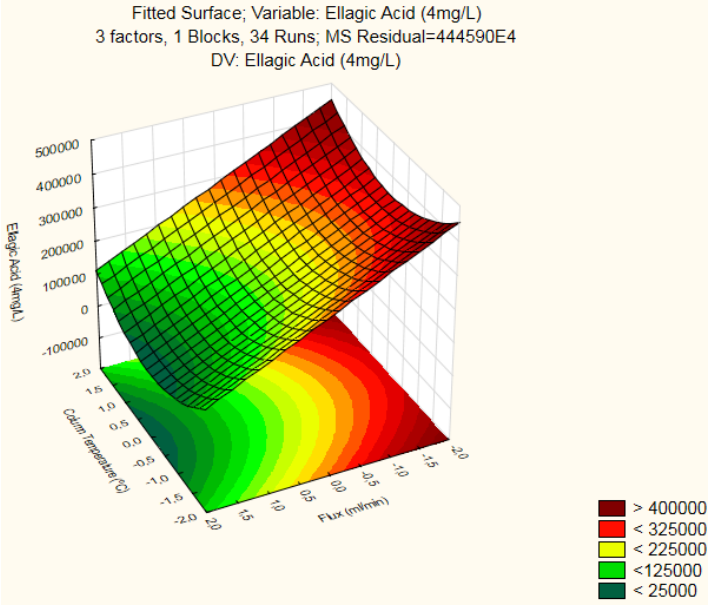


Figure 3. 7 - RSM for Ellagic Acid (4mg/L): Flux × Column Temperature

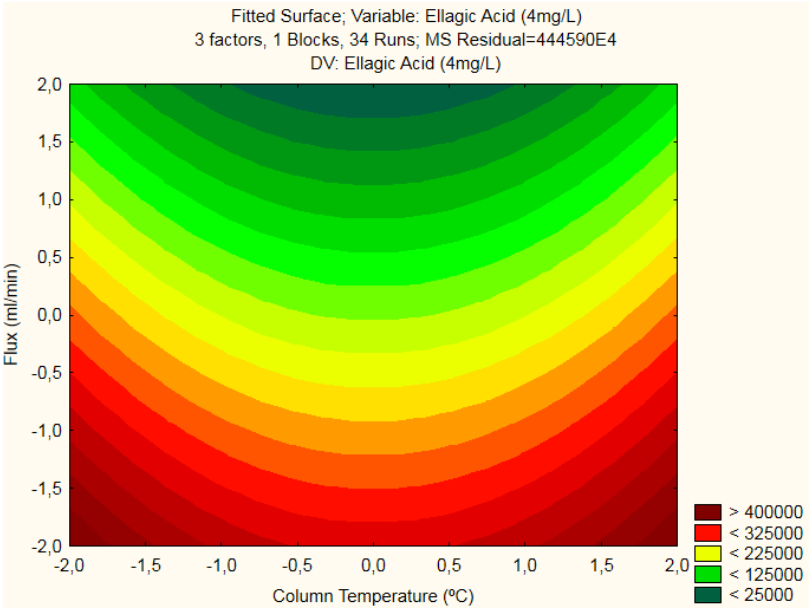


Figure 3. 8 - Contour Plot for Ellagic Acid (4mg/L): Flux × Column Temperature

3.1.4. Regression Model

Ellagic Acid (0,4mg/L)

For building the Regression Model's equation, are needed the coefficients which can be extracted from Table 3.7.

Table 3. 7 – Regression Coefficients for 0,4mg/L Ellagic Acid

	Regress. Coeff.	Std.Err.	t(29)	p	-95,%	+95,%
Mean/Interc.	17192,13	1683,015	10,21508	0,000000	13750,0	20634,28
Column Temp. (ºC)(L)	-2927,88	1383,516	-2,11626	0,043027	-5757,5	-98,27
Column Temp. (ºC)(Q)	4259,45	1416,460	3,00711	0,005402	1362,5	7156,43
Acetonitrile (%)(L)	2859,00	1383,516	2,06648	0,047816	29,4	5688,61
Flux (ml/min)(L)	-8253,36	1383,516	-5,96550	0,000002	-11083,0	-5423,75

The generic form of the Regression Model is presented by the following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j + \varepsilon$$

By replacing β_x for the respective coefficients that can be extracted from this table's second row, it's possible to obtain the regression equation:

$$Y = 2459273 - 87213x_1 + 10715x_2 - 3908594x_3 + 895{x_1}^2 - 31{x_2}^2 + 2213963{x_3}^2 - 130x_{12} + 55750x_{13} - 8300x_{23}$$

Assumptions that are assumed for the mathematical model and respective Analysis of Variance need to be validated, which is done by verifying if residuals are independent, normally distributed, with a null average and constant variance. Residuals are obtained from the difference between observed values and predicted/estimated values.

Normality verification is made by plotting a Normal probability distribution graphic, and if the results are disposed approximately according to a straight line it's possible to conclude that the Normality assumption is satisfied. So, through an observation of residuals exposed in Figure 3.9 it's possible to confirm that the assumption is verified.

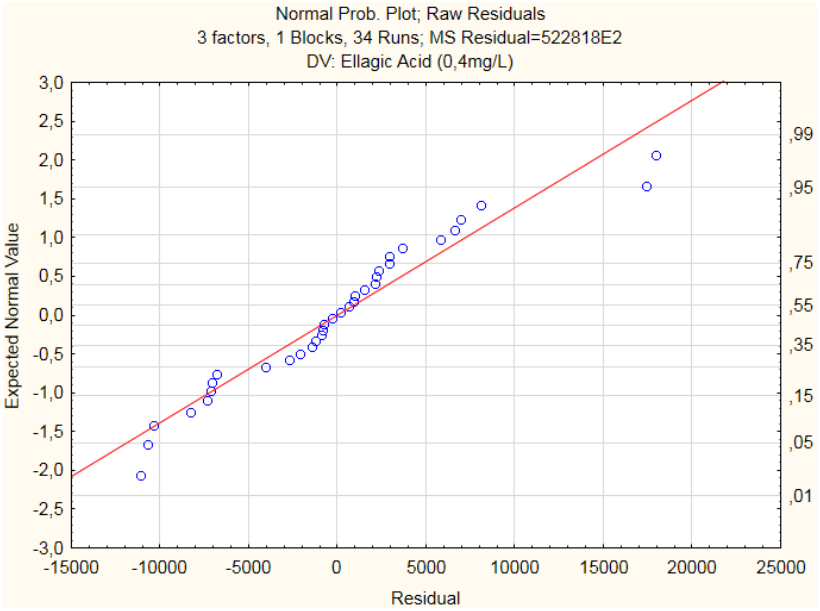


Figure 3. 9 - Normal Probability Plot of Residuals for Ellagic Acid (0,4mg/L)

The simplest method used to verify if variance is constant consists in plotting a graphic of residuals in function of predicted values. Graphic shouldn't present any special structure in order for the constant variance assumption to be verified. Therefore, Figure 3.10 shows that this sample fulfills the assumption.

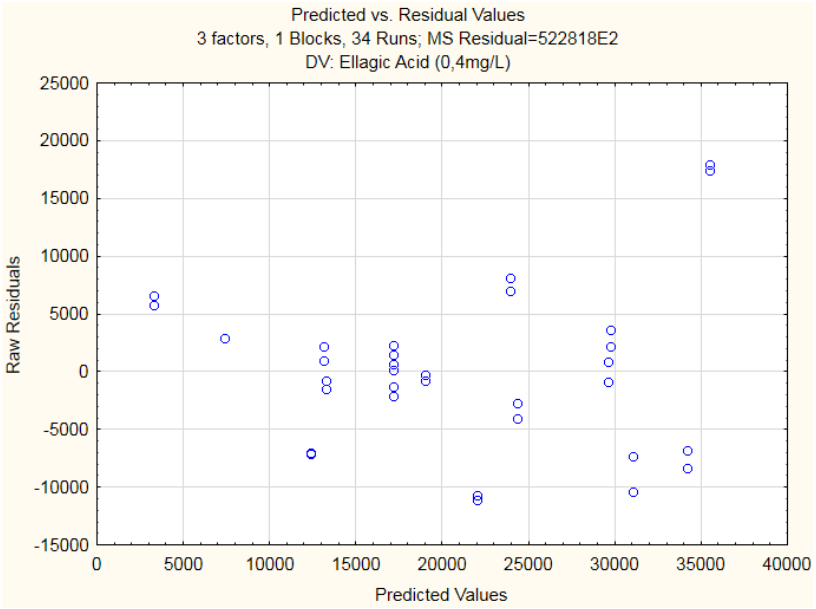


Figure 3. 10 - Predicted vs. Residual Values Plot for Ellagic Acid (0,4mg/L)

By plotting the Residuals vs. Case Numbers it's possible to evaluate the independence of residuals. Its independence is proven if they're randomly distributed in the graphic, such as they're in Figure 3.11.

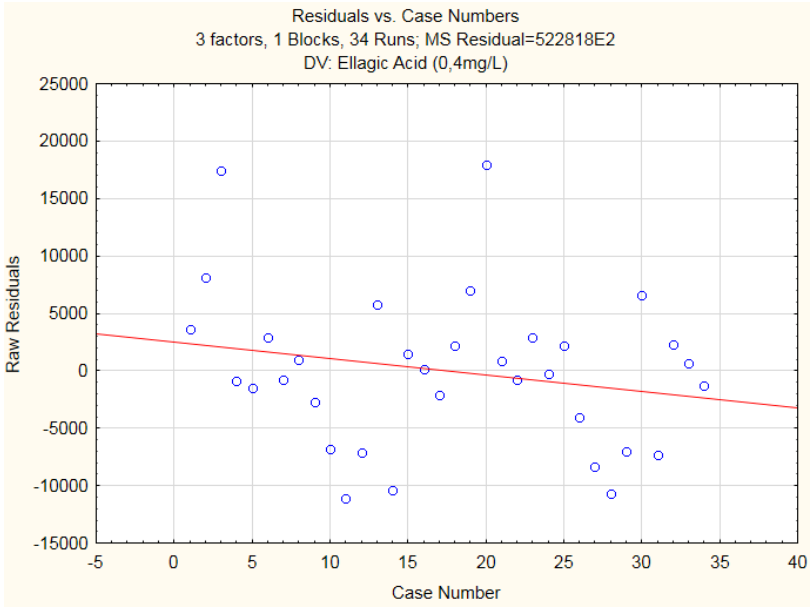


Figure 3. 11 - Residuals vs. Case Numbers for Ellagic Acid (0,4mg/L)

Ellagic Acid (4mg/L)

From the Regression Coefficients Table that can be found in the Appendixes was built the following Regression Model for Ellagic Acid (4mg/L):

$$Y = 170867{,}3 - 85552{,}5x_3 + 27881{,}4{x_1}^2 - 29423{,}1{x_2}^2$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

3.2. Ferulic Acid

3.2.1. Experimental plan

The responses in Table 3.8 show the values resultant from applying the variation of conditions planned to Ferulic Acid samples, for 0,2mg/L and 2mg/L standards. By observing results, it's possible to see that the 1st experiment achieved the highest response (45016 and 44441) for the low concentration standard, while for the high concentration standard (240727 and 241335) it was the 3rd experiment. For this compound, it's possible to verify that low levels of Temperature and Flux are yielding the best results, while the Solvent Concentration (% of Acetonitrile) varied between low and high levels.

Table 3. 8 - Experimental plan and respective responses for Ferulic Acid solution

				Responses			
	Factors			0,2 mg/L		2 mg/L	
Std. Order	A	B	C	1st Injection	2nd Injection	1st Injection	2nd Injection
1	**34**	**21**	**0,25**	25417	25072	240727	241335
2	46	21	0,25	26785	27430	240457	240791
3	**34**	**79**	**0,25**	45016	44441	203279	185410
4	46	79	0,25	30256	30284	109443	112743
5	34	21	0,40	15348	17301	145846	147574
6	46	21	0,40	15300	15681	145898	145751
7	34	79	0,40	18123	17157	56828	42677
8	46	79	0,40	18033	17826	55990	60441
9	40	50	0,33	23606	23198	128176	112903
10	40	50	0,33	21602	21478	122661	122255
11	40	50	0,33	16920	16860	38539	48236
12	50	50	0,33	19839	21178	182354	180875
13	30	50	0,33	14832	14584	89853	90206
14	40	99	0,33	33996	32993	192302	192700
15	40	1	0,33	20924	19985	113945	119009
16	40	50	0,45	21711	21868	121234	116627
17	40	50	0,20	19611	20458	114370	115973

3.2.2. Analysis of Variance

Ferulic Acid (0,2mg/L)

The ANOVA performed for this compound can be found in the Appendixes. By inserting the variance of non-significant factors into the error's variance, a Reduced ANOVA was achieved (Table 3.9). Regarding these results, it's possible to observe that there are 5 significant components: Acetonitrile (L), Flux (L), Flux (Q), Column Temperature x Acetonitrile, Acetonitrile x Flux. Although, it's the Flux's linear component that presents a lower p-value and consequently has more influence in system's response.

Table 3. 9 - Reduced ANOVA for 0,2mg/L Ferulic Acid

	Var.: Ferulic Acid (0,2 mg/L)				
	SS	df	MS	F	p
Acetonitrile (%) (L)	**6,044061E+07**	**1**	**6,044061E+07**	**4,53134**	**0,042553**
Flux (ml/min) (L)	**1,227722E+09**	**1**	**1,227722E+09**	**92,04462**	**0,000000**
Flux (ml/min) (Q)	**5,976066E+07**	**1**	**5,976066E+07**	**4,48037**	**0,043647**
1L by 2L	**5,774480E+07**	**1**	**5,774480E+07**	**4,32924**	**0,047079**
1L by 3L	3,630665E+07	1	3,630665E+07	2,72198	0,110563
2L by 3L	**8,922692E+07**	**1**	**8,922692E+07**	**6,68951**	**0,015411**
Error	3,601351E+08	27	1,333834E+07		
Total SS	1,891337E+09	33			

Through an analysis of the coefficient of determination, R^2= 0,80959, we can verify that for Ferulic Acid (0,2mg/L) the regression model approximates the real data.

Ferulic Acid (2mg/L)

Reduced ANOVA displayed at Table 3.10 shows that four factors had significant influence in Ferulic Acid (2mg/L) standard. Causing significant variation in system's response there were linear components of Acetonitrile and Flux, a quadratic component of Flux and the interaction between Column Temperature and Flux. From these components, it's the first one that presented higher influence in response. For consulting the original ANOVA built for this compound address to the Appendixes.

Table 3. 10 - Reduced ANOVA for 2mg/L Ferulic Acid

	Var.: Ferulic Acid (2 mg/L)				
	SS	df	MS	F	p
Acetonitrile (%) (L)	5,154178E+10	1	5,154178E+10	124,0132	0,000000
Flux (ml/min) (L)	4,574960E+10	1	4,574960E+10	110,0768	0,000000
Flux (ml/min) (Q)	3,366644E+09	1	3,366644E+09	8,1004	0,008042
1L by 3L	2,081002E+09	1	2,081002E+09	5,0070	0,033092
Error	1,205284E+10	29	4,156153E+08		
Total SS	1,147919E+11	33			

Through an analysis of the coefficient of determination, R^2= 0,895, we can verify that for Ferulic Acid (2mg/L) the regression model approximates the real data.

3.2.3. RSM and Contour plot

Ferulic Acid (0,2 mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

Flux x Acetonitrile was the Surface that yielded highest peak area for the Ferulic Acid low standard (0,2mg/L), and it presents a light curvature as can be observed in Figure 3.12. This peak area (>50000) was generated for Acetonitrile's high level and Flux's low level.

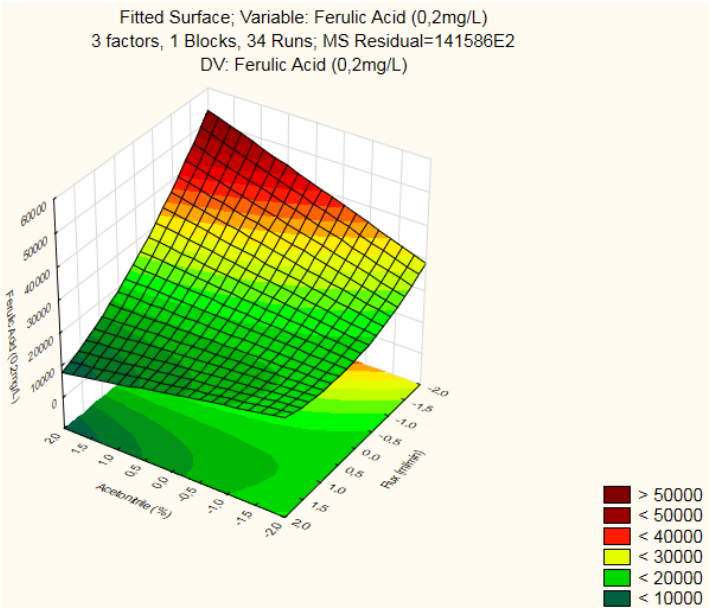


Figure 3. 12 - RSM for Ferulic Acid (0,2mg/L): Flux × Acetonitrile

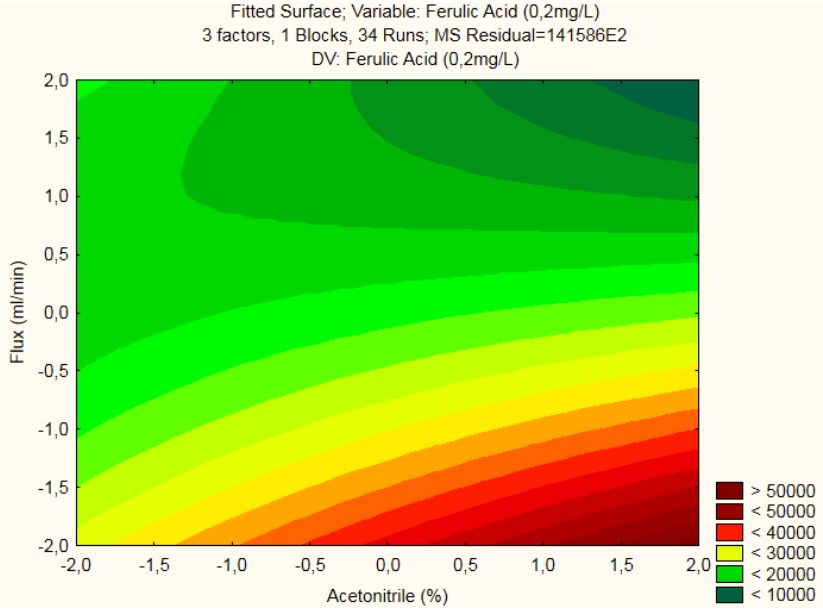


Figure 3. 13 – Contour Plot for Ferulic Acid (0,2mg/L): Flux × Column Temperature

Ferulic Acid (2mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

For this compound's high standard, it was the interaction Flux x Acetonitrile producing the highest extraction value (>300000). The extraction conditions defined to attain this peak were both factors low levels. Surface's curvature can be observed in Figure 3.14, as also in the Contour Plot lines (Fig. 3.15) that exhibit a circular shape.

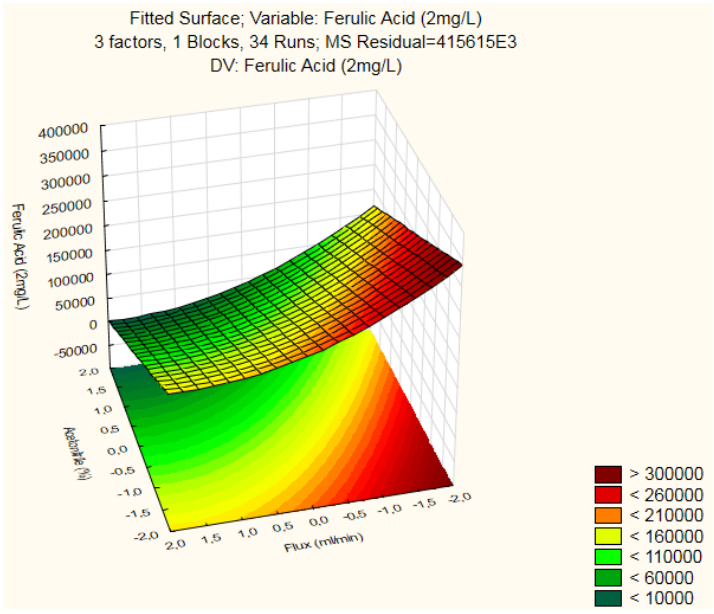


Figure 3. 14 - RSM for Ferulic Acid (2mg/L): Flux × Acetonitrile

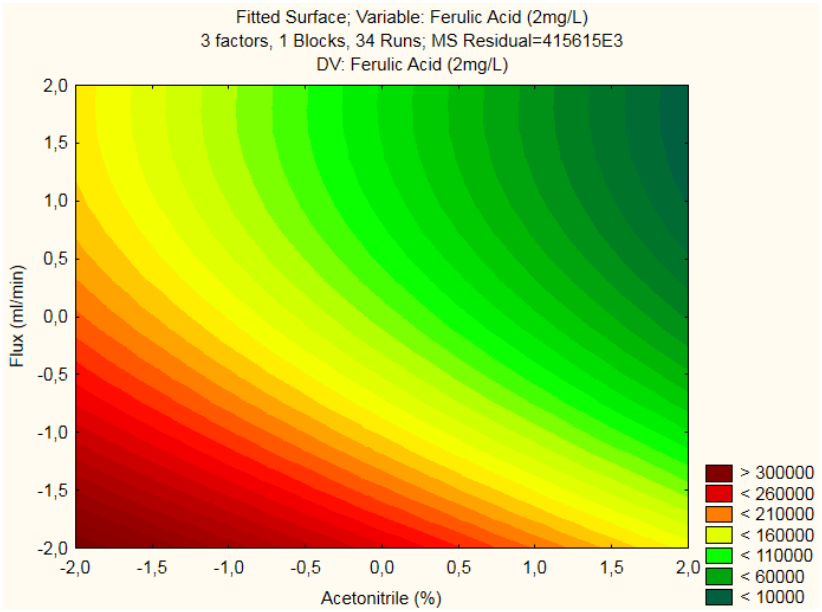


Figure 3. 15 – Contour Plot for Ferulic Acid (2mg/L): Flux × Acetonitrile

3.2.4. Regression Model

Ferulic Acid (0,2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Ferulic Acid (0,2mg/L):

$$Y = 21580{,}86 + 1487{,}56x_2 - 6704{,}39x_3 + 1514{,}39{x_3}^2 - 1899{,}75x_{12} - 2361{,}5x_{23}$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

Ferulic Acid (2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Ferulic Acid (2mg/L):

$$Y = 125498{,}4 - 43439{,}9x_2 - 40926{,}4x_3 + 11366{,}5{x_3}^2 + 11404{,}5x_{13}$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

3.3. Rutin

3.3.1. Experimental plan

The responses in Table 3.11 show the values resultant from applying the variation of conditions planned on Rutin samples, for 0,2mg/L and 2mg/L standards. By observing results, it's possible to see that the 1st experiment achieved the highest response (40571 and 43753) for the low concentration standard, while for the high concentration standard (207700 and 207581) it was the 3rd experiment. For this compound, it's possible to verify that low levels of Temperature and Flux are yielding the best results, while the Solvent Concentration (% of Acetonitrile) varied between low and high levels.

Table 3. 11 - Experimental plan and respective responses for Rutin solution

				Responses			
	Factors			0,2 mg/L		2 mg/L	
Std. Order	A	B	C	1st Injection	2nd Injection	1st Injection	2nd Injection
1	**34**	**21**	**0,25**	21561	21645	207700	207581
2	46	21	0,25	20441	19893	203506	203852
3	**34**	**79**	**0,25**	40571	43753	113890	125313
4	46	79	0,25	26320	23939	56754	48337
5	34	21	0,40	10472	10275	123189	111888
6	46	21	0,40	10040	9842	112805	120213
7	34	79	0,40	17185	19372	26809	27419
8	46	79	0,40	10919	12804	31505	31812
9	40	50	0,33	19637	20053	44779	45250
10	40	50	0,33	16496	18174	66538	63176
11	40	50	0,33	9025	9416	21166	18319
12	50	50	0,33	10123	12584	122874	123453
13	30	50	0,33	10366	11105	56020	59170
14	40	99	0,33	25407	26095	94231	105016
15	40	1	0,33	16817	17113	49273	52711
16	40	50	0,45	15693	15238	46407	48129
17	40	50	0,20	15096	16148	56434	60803

3.3.2. Analysis of Variance

Rutin (0,2mg/L)

Reduced ANOVA (Table 3.12) presents five significant components, showing that linearity, pure cuvature and a two-factor interaction are part of this list. From all significant factors, Flux's linear component proved to have the highest influence in response. The ANOVA generated for this standard can be visualized in the Appendixes.

Table 3. 12 - Reduced ANOVA for 0,2mg/L Rutin solution

	Var.: Rutin (0,2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (Q)	1,166512E+08	1	1,166512E+08	4,86860	0,035720
Acetonitrile (%) (L)	1,477172E+08	1	1,477172E+08	6,16519	0,019289
Flux (ml/min) (L)	1,029884E+09	1	1,029884E+09	42,98368	0,000000
Flux (ml/min) (Q)	1,038606E+08	1	1,038606E+08	4,33477	0,046598
1L by 2L	1,164349E+08	1	1,164349E+08	4,85958	0,035879
Error	6,708766E+08	28	2,395988E+07		
Total SS	2,144565E+09	33			

Through an analysis of the coefficient of determination, R^2= 0,68717, we can verify that for Rutin (0,2mg/L) the regression model approximates the real data.

Rutin (2mg/L)

From this high-concentration standard's ANOVA – can be found in the Appendixes – a Reduced ANOVA (Table 3.13) was generated by fitting non-significant factors' variance in the Residual variance. Quadratic and linear components of the factors Acetonitrile and Flux showed to be significant for this standard's extraction. Linear Flux was the variable showing highest influence in response.

Table 3. 13 – Reduced ANOVA for 2mg/L Rutin solution

	Var.: Rutin (2 mg/L)				
	SS	df	MS	F	p
Acetonitrile (%) (L)	5,069848E+10	1	5,069848E+10	57,47370	0,000000
Acetonitrile (%) (Q)	4,041506E+09	1	4,041506E+09	4,58160	0,040856
Flux (ml/min) (L)	1,911991E+10	1	1,911991E+10	21,67505	0,000066
Flux (ml/min) (Q)	5,798506E+09	1	5,798506E+09	6,57340	0,015798
Error	2,558137E+10	29	8,821162E+08		
Total SS	1,034505E+11	33			

Through an analysis of the coefficient of determination, R^2= 0,75272, we can verify that for Rutin (2mg/L) the regression model approximates the real data.

3.3.3. RSM and Contour Plot

Rutin (0,2mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

The high curvature surface produced for the interaction Flux x Column Temperature was the one yielding highest result. The highest peak yielded for this standard was obtained for Flux's low level, and for both limits of Column Temperature's level range. Contour Plot lines (Fig. 3.17) and Response Surface (Fig. 3.16) for this interaction show a high curvature surface.

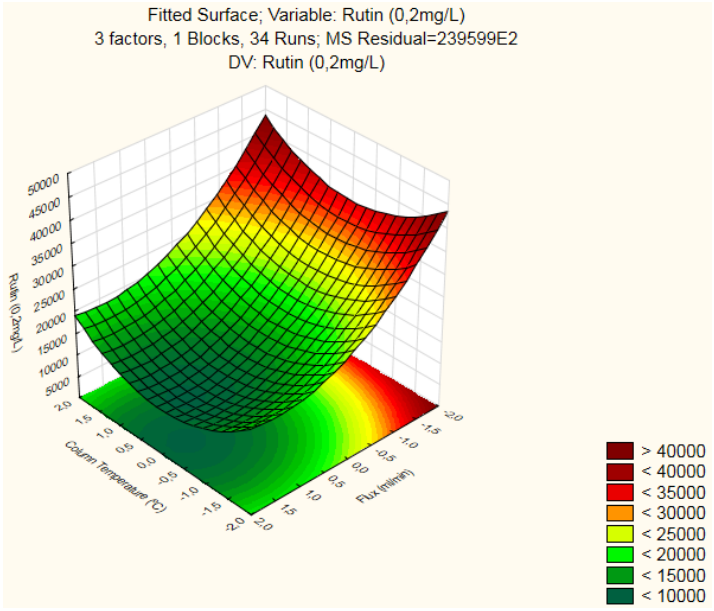


Figure 3. 16 - RSM for Rutin (0,2mg/L): Flux × Column Temperature

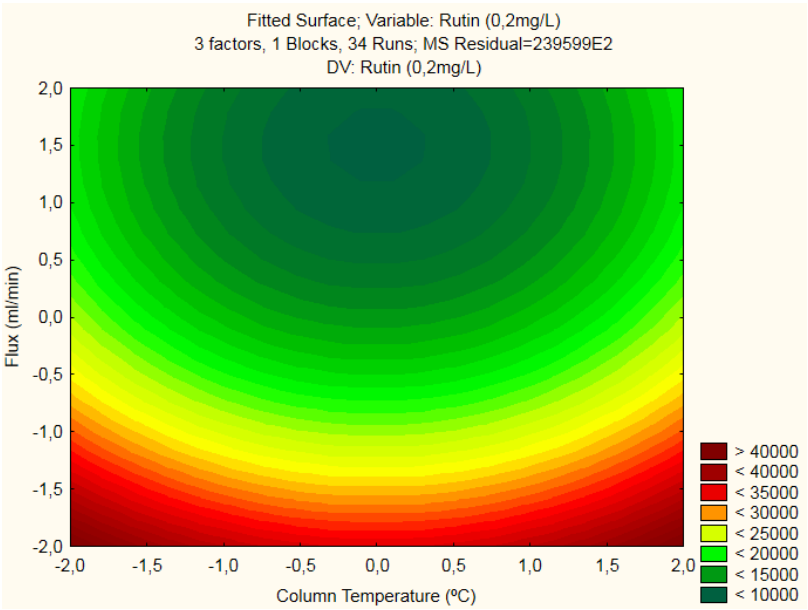


Figure 3. 17 – Contour Plot for Rutin (0,2mg/L): Flux × Column Temperature

Rutin (2mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

From the three Response Surfaces produced, it was the interaction between Flux and Acetonitrile that yielded the highest peak (>300000), which was obtained when both levels were set low. This surface also has a strong curvature component as it was expected given that its Reduced ANOVA (Table 3.13) found the two Quadratic components to be significant.

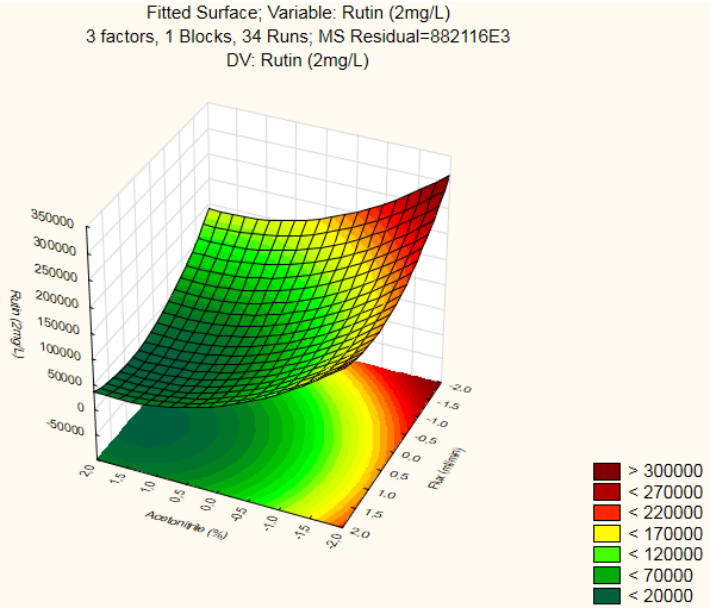


Figure 3. 18 - RSM for Rutin (2mg/L): Flux × Acetonitrile

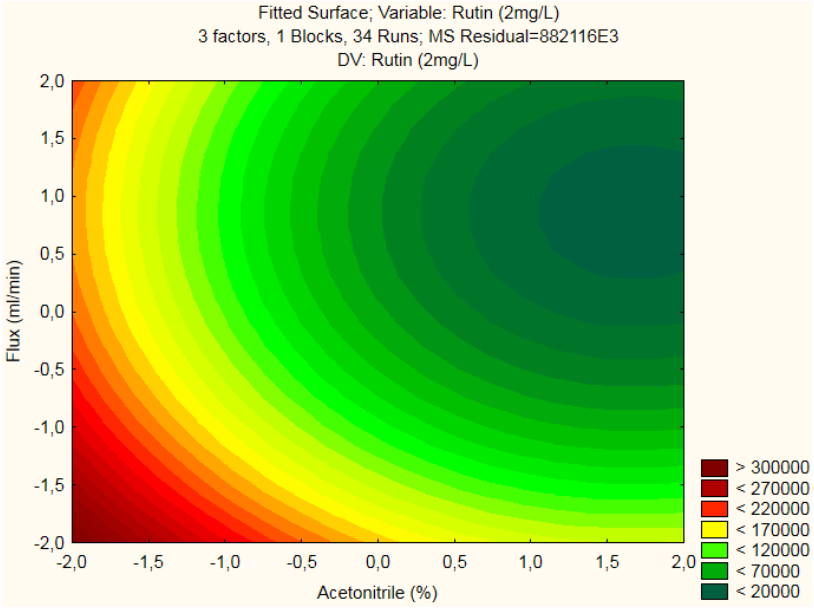


Figure 3. 19 – Contour Plot for Rutin (2mg/L): Flux × Acetonitrile

3.3.4. Regression Model

Rutin (0,2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Rutin (0,2mg/L):

$$Y = 14360{,}52 + 2325{,}54x_2 - 6140{,}49x_3 + 2173{x_1}^2 + 2050{,}46{x_3}^2 - 2697{,}63x_{12}$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

Rutin (2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Rutin (2mg/L):

$$Y = 62308{,}4 - 43083x_1 - 26457{,}7x_2 + 12790{,}8{x_1}^2 + 15320{,}9{x_2}^2$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

3.4. Cinnamic Acid

3.4.1. Experimental plan

In Table 3.14 are represented the extraction results for both Cinnamic Acid standards: 0,2mg/L and 2mg/L. The low-concentration standard yielded extraction levels of 45275 and 48924 for the 3rd experiment, while the high concentration standard yielded results of 304987 and 315913 for the 1st experiment.

Table 3. 14 - Experimental plan and respective responses for Cinnamic Acid solution

				Responses			
	Factors			0,2 mg/L		2 mg/L	
Std. Order	A	B	C	1st Injection	2nd Injection	1st Injection	2nd Injection
1	**34**	**21**	**0,25**	26654	25100	304987	315913
2	46	21	0,25	32738	31078	321666	315602
3	**34**	**79**	**0,25**	45275	48924	227926	199114
4	46	79	0,25	38100	38215	98826	99588
5	34	21	0,40	15966	18757	186172	190236
6	46	21	0,40	16126	18875	196749	184955
7	34	79	0,40	15577	17874	63027	60947
8	46	79	0,40	34706	34273	70639	66488
9	40	50	0,33	24998	25434	146793	143728
10	40	50	0,33	23173	22068	238028	234029
11	40	50	0,33	23390	25219	60053	56237
12	50	50	0,33	17721	21208	245453	244426
13	30	50	0,33	22364	13722	183124	200922
14	40	99	0,33	32465	33517	227619	338795
15	40	1	0,33	31017	28886	150983	154947
16	40	50	0,45	30992	30229	164055	156922
17	40	50	0,20	35735	22988	151738	158689

3.4.2. Analysis of Variance

Cinnamic Acid (0,2mg/L)

From this low-concentration standard's ANOVA – can be found in the Appendixes – a Reduced ANOVA (Table 3.13) was generated by fitting non-significant factors' variance in the Residual variance. Quadratic and linear components of the Flux showed to be the only significant factors for this standard's extraction.

Table 3. 15 – Reduced ANOVA for 0,2mg/L Cinnamic Acid solution

	Var.: Cinnamic Acid 1 (0,2 mg/L)				
	SS	Df	MS	F	p
Flux (ml/min) (L)	1,651076E+10	1	1,651076E+10	8,061492	0,007912
Flux (ml/min) (Q)	1,456418E+10	1	1,456418E+10	7,111059	0,012064
Error	6,349117E+10	31	2,048102E+09		
Total SS	9,456611E+10	33			

Through an analysis of the coefficient of determination, R^2= 0,32861, it was possible to verify that for Cinnamic Acid (0,2mg/L) the regression model doesn't approximate the real data.

Cinnamic Acid (2mg/L)

From this low-concentration standard's ANOVA – can be found in the Appendixes – a Reduced ANOVA (Table 3.13) was generated by fitting non-significant factors' variance in the Residual variance. Quadratic and linear components of the Flux showed to be the only significant factors for this standard's extraction.

Table 3. 16 - Reduced ANOVA for 2mg/L Cinnamic Acid solution

	Var.: Cinnamic Acid (2 mg/L)				
	SS	Df	MS	F	p
Column Temp. (ºC) (L)	9,120554E+09	1	9,120554E+09	8,4502	0,006927
Acetonitrile (%) (L)	1,131537E+11	1	1,131537E+11	104,8373	0,000000
Flux (ml/min) (L)	5,021313E+10	1	5,021313E+10	46,5226	0,000000
Flux (ml/min) (Q)	1,586878E+10	1	1,586878E+10	14,7025	0,000626
Error	3,130047E+10	29	1,079327E+09		
Total SS	2,196567E+11	33			

Through an analysis of the coefficient of determination, R^2= 0,8575, we can verify that for Cinnamic Acid (2mg/L) the regression model approximates the real data.

3.4.3. RSM and Contour plots

Cinnamic Acid (0,2mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

From the three Response Surfaces produced, it was the interaction between Flux and Column Temperature that yielded the highest peak (>150000). The Surfaces produced for this compound showed that something irregular occurred with this experimental set, given that it's the only standard that produced results inconsistent. The causes for this scenario will be discussed ahead.

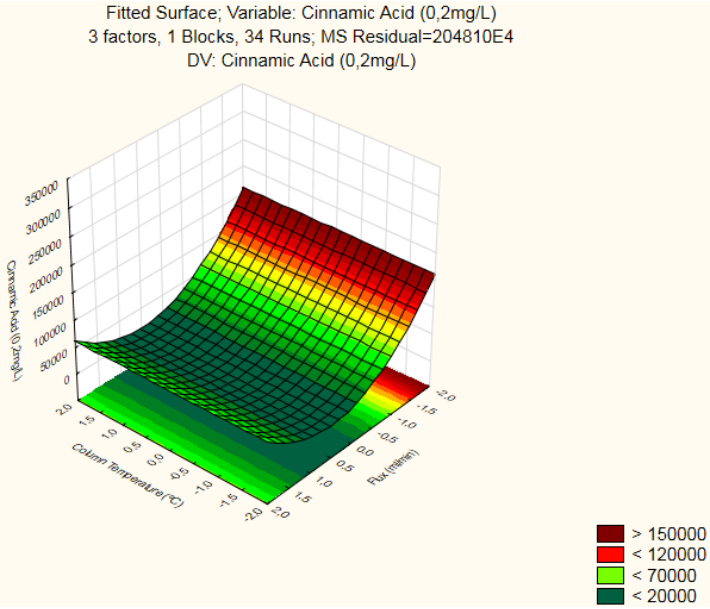


Figure 3. 20 - RSM for Cinnamic Acid (0,2mg/L): Flux × Column Temperature

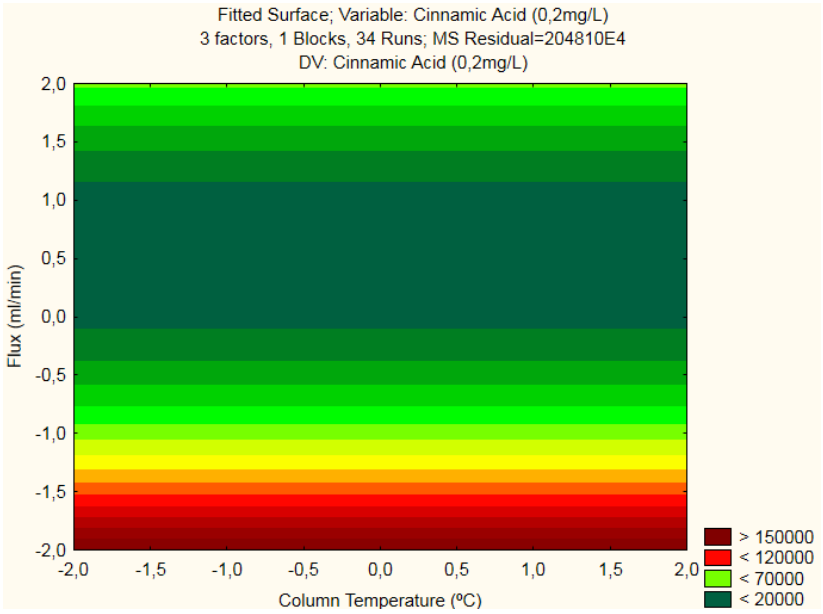


Figure 3. 21 – Contour Plot for Cinnamic Acid (0,2mg/L): Flux × Column Temperature

Cinnamic Acid (2mg/L)

For studying the curvature produced by this standard, RSM and Contour Plots were produced for the 3 factor interactions and can be found in the Appendixes.

For Cinnamic Acid (2mg/L) it's the interaction Flux x Acetonitrile that produced the highest peak area (>400000), in accordance to the significance expressed in Table 3.16.

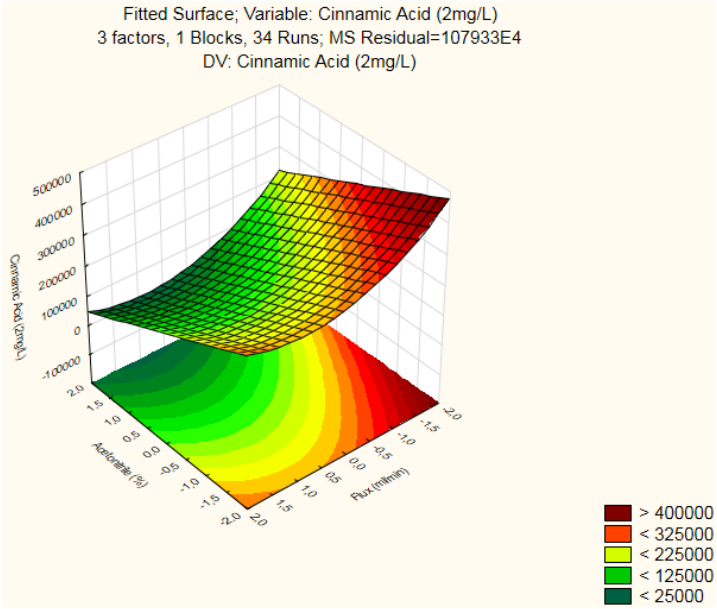


Figure 3. 22 - RSM for Cinnamic Acid (2mg/L): Flux × Acetonitrile

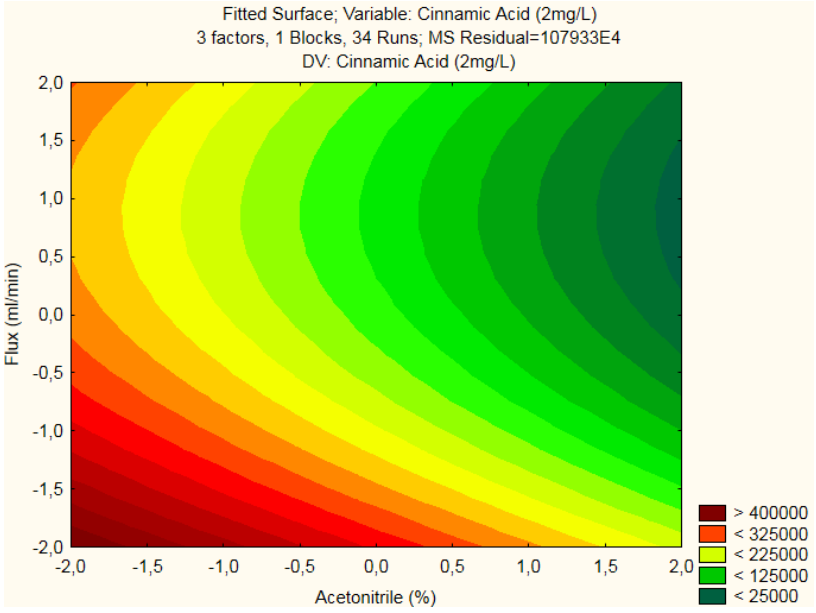


Figure 3. 23 - RSM for Cinnamic Acid (2mg/L): Flux × Acetonitrile

3.4.4. Regression Model

Cinnamic Acid (0,2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Cinnamic Acid (0,2mg/L):

$$Y = 17038 - 24586{,}3x_3 + 23641{,}3{x_3}^2$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

Cinnamic Acid (2mg/L)

From the Regression Coefficients table that can be found in the Appendixes was built the following Regression Model for Cinnamic Acid (2mg/L):

$$Y = 161333{,}5 - 18273{,}4x_1 - 64364x_2 - 42876{,}3x_3 + 24677{,}5{x_3}^2$$

Analysis of Residuals for this compound proving that the data is normal, independent and its variance is constant can be found in the Appendixes.

3.5. Analysis of results

Experiments conducted for the four standards representative of antioxidants present on Andean Oca lead to highly satisfactory results.

The compound that achieved highest peaks, for both low concentration and high concentration standard, was the Ellagic Acid (Figures 3.24 and 3.25). Response Surfaces generating the highest peak areas for these two standards – 0,4 mg/L and 4 mg/L – presented curvature and belong to the same interaction: Column Temperature x Flux.

The factor that proved to have more significance in response was the Flux (L), due to being the unique factor influencing significantly all the eight standards studied. Furthermore, it was the factor with higher significance in five out of eight standards. Consequently, the two interactions that yielded best Response Surfaces were the ones containing this factor: Flux x Column Temperature and Flux x Acetonitrile. The levels yielding these Surfaces were low, excepted for Column Temperature that obtained the best results for both extremes of the level range.

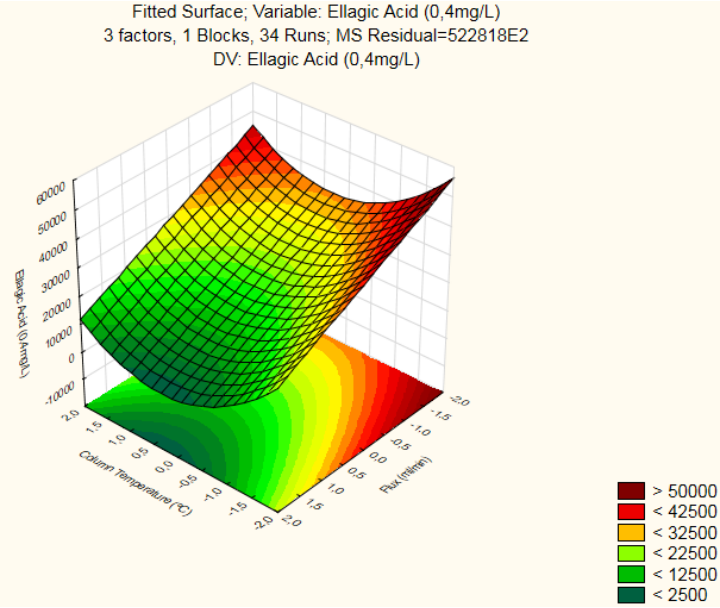


Figure 3. 24 – Highest RSM for low concentration standard: Ellagic Acid (0,4mg/L)

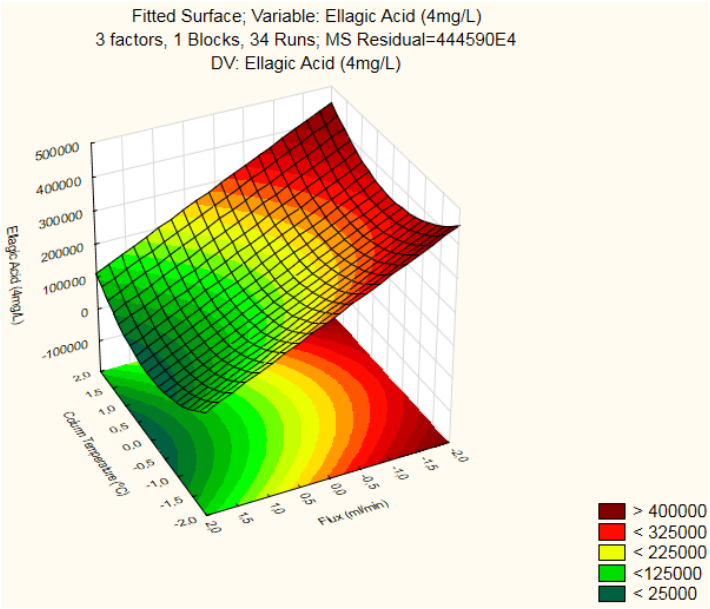


Figure 3. 25 - Highest RSM for high concentration standard: Ellagic Acid (4mg/L)

A Summary Table is displayed under with the most important data relative to the results and analysis made for these experiments:

Table 3. 17 - Summary of relevant parameters obtained for all compounds

Compounds	Results	Levels	RSM	Sign. Factors	R^2
Ellagic Acid (0,4 mg/L)	52939 53424	34ºC 79% 0,25ml/min	Flux x Column Temp.	4	0,64797
Ellagic Acid (4mg/L)	402019 427993	34ºC 79% 0,25ml/min	Flux x Column Temp.	3	0,65434
Ferulic Acid (0,2mg/L)	45016 44441	34ºC 79% 0,25ml/min	Flux x Acetonitrile	5	0,80959
Ferulic Acid (2mg/L)	240727 241335	34ºC 79% 0,25ml/min	Flux x Acetonitrile	4	0,895
Rutin (0,2mg/L)	40571 43753	34ºC 79% 0,25ml/min	Flux x Column Temp.	5	0,68717
Rutin (2mg/L)	207700 207581	34ºC 21% 0,25ml/min	Flux x Acetonitrile	5	0,75272
Cinnamic Acid (0,2mg/L)	45275 48924	34ºC 79% 0,25ml/min	Flux x Column Temperature	2	0,32861
Cinnamic Acid (2mg/L)	304987 315913	34ºC 21% 0,25ml/min	Flux x Acetonitrile	4	0,8575

4. Discussion/Conclusions

This study intended to identify the factors influencing the extraction process, using a Photodiode Array Detector system, for four antioxidants: Ellagic Acid, Ferulic Acid, Rutin and Cinnamic Acid. For that matter, experiments were executed under the form of a Central Composite Design, enabling to include RSM on the study.

Experimental results showed that factors considered for this study were highly influent in system's response, meaning that response varied as a consequence of manipulating these factors. The factor that had presented highest significance in response was the Flux, more specifically its Linear component. These was the only component significant for all eight experimental sets, and was also the most significant one in five of them.

Response Surface Methodology showed that Ellagic Acid was the antioxidant yielding highest peak areas for both low concentration standard and high concentration standard. It was also possible to verify that the CCD was appropriately chosen for this study, given that all Reduced ANOVAs showed significant Quadratic components. Coefficients of Determination, R^2, produced for this study ranged from 0,65 to 0,89 which proved that there was a high correlation between predicted values and the real data. The only exception to this case was the low standard Cinnamic Acid, which lead to incoherent results. Its R^2 was inferior to 0,5 which shows that there wasn't a good fit between the regression model estimated and the real data. Also the RSM yielded low peak areas in comparison to the remaining ones. Several causes can be pointed out for this situation, although it is hard to really comprehend what was the real cause. For that matter new experiments must be executed, in which some alterations must be done to the configuration of this experimental set. The reduced concentration of a low standard, a small alteration on experimental conditions might be possible causes for generating this unexpected results.

As a conclusion it's important to state that objective of this study was successfully achieved. It was possible to identify the factors influencing system's response for the four antioxidants studied. There was a good fit of the regression model produced to the real data, which is the same as saying that there was a high correlation in results. Following this study, a new experimental study should be made in order to validate the results obtained, replacing the standards for samples of Andean Oca. It's also important to conclude that screening experiments should always be made for this type of studies, and when executed should be planned according to a DoE methodology. This way it's possible to perform a continuous work and keep statistical track of all studies made.

Limited resources is other issue that can jeopardize experimental success results. Researchers often see their work limited by resources, and consequently need to shorten experiments in order to try to meet the final objectives with less materials, in less time and with less workers than expected. Especially for public institutes it's important to count with government and European funding to continue having conditions for executing an appropriate research work.

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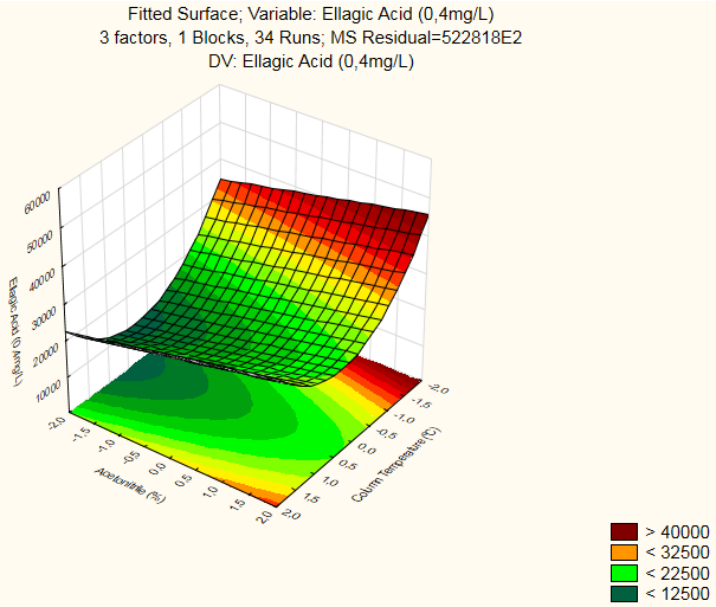
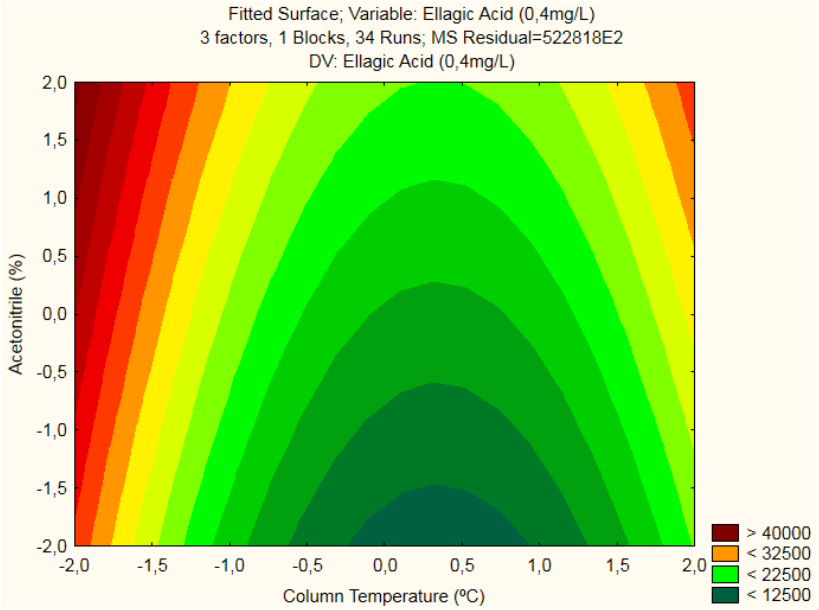
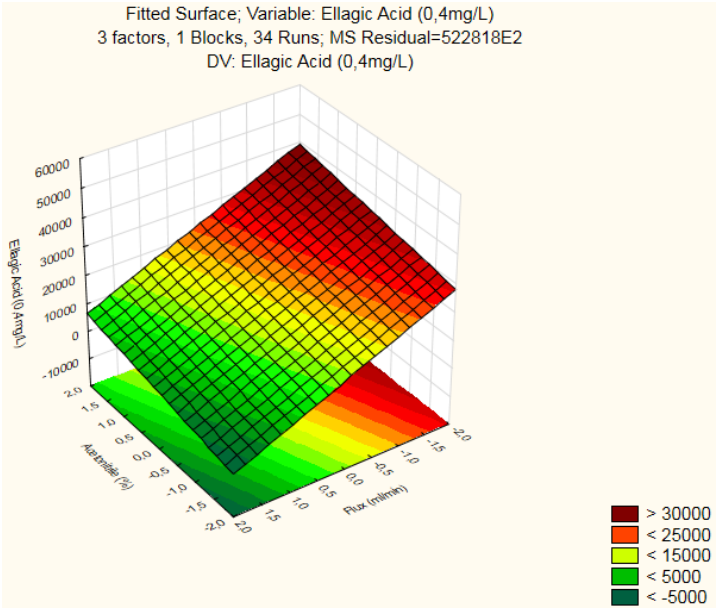
Appendixes

Ellagic Acid (0,4mg/L)



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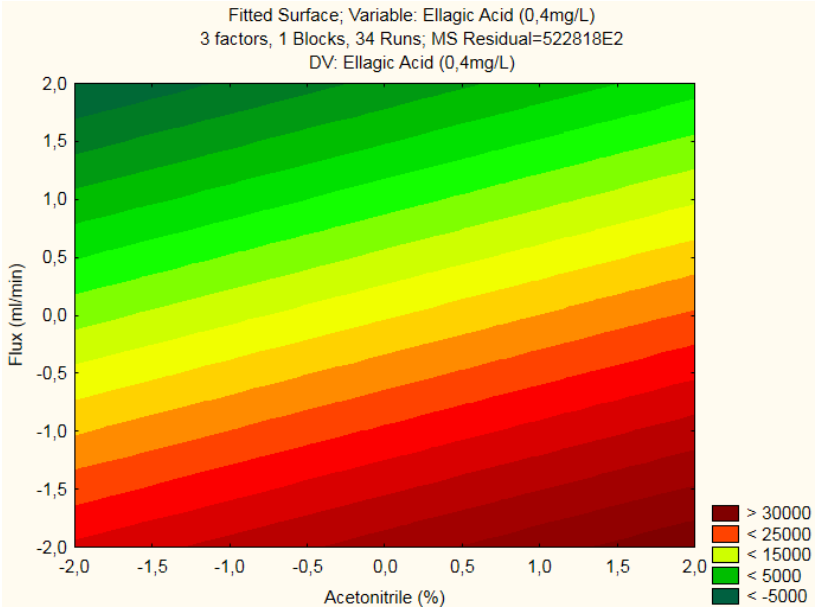
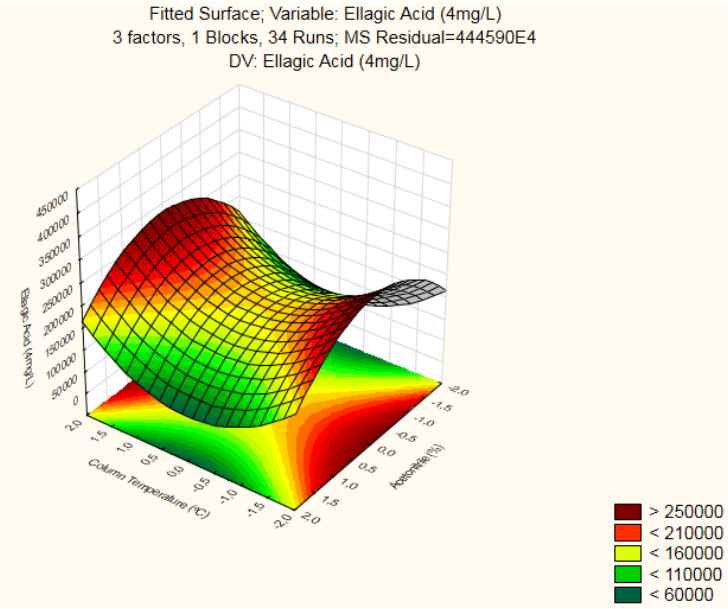
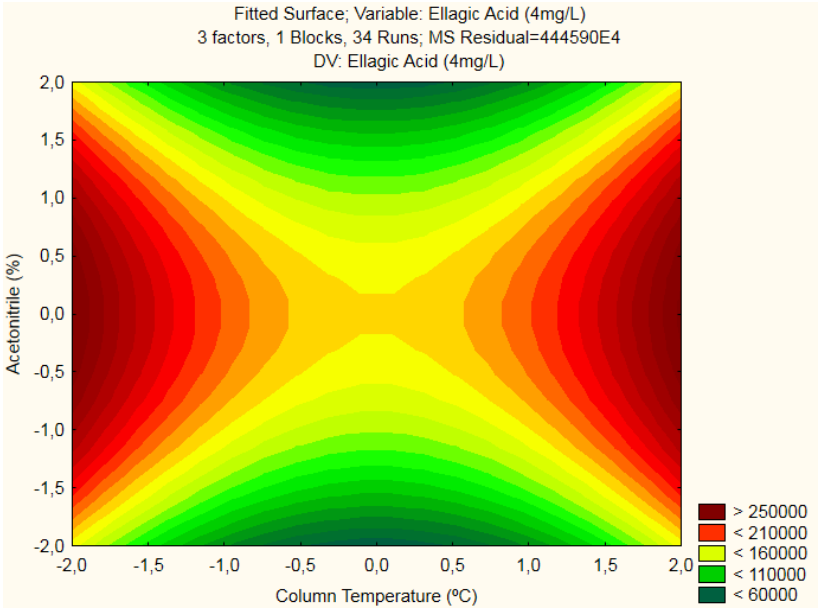
3 factors, 1 Blocks, 34 Runs; MS Residual=522818E2
DV: Ellagic Acid (0,4mg/L)
Ellagic Acid (0,4mg/L)
60000
50000
40000
30000
20000
10000
0
-10000
Acetonitrile (%)
Flux (ml/min)
> 30000
< 25000
< 15000
< 5000
< -5000


Fitted Surface; Variable: Ellagic Acid (0,4mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=522818E2
DV: Ellagic Acid (0,4mg/L)
Flux (ml/min)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
Acetonitrile (%)
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
2,0
> 30000
< 25000
< 15000
< 5000
< -5000

Ellagic Acid (4mg/L)

	Var.: Ellagic Acid (4 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	2,161680E+09	1	2,161680E+09	0,51442	0,480154
Column Temp. (ºC) (Q)	**2,298073E+10**	**1**	**2,298073E+10**	**5,46881**	**0,028019**
Acetonitrile (%) (L)	2,594433E+09	1	2,594433E+09	0,61741	0,439702
Acetonitrile (%) (Q)	1,452251E+10	1	1,452251E+10	3,45597	0,075328
Flux (ml/min) (L)	**1,999161E+11**	**1**	**1,999161E+11**	**47,57474**	**0,000000**
Flux (ml/min) (Q)	4,225256E+09	1	4,225256E+09	1,00550	0,325987
1L by 2L	8,138882E+09	1	8,138882E+09	1,93684	0,176778
1L by 3L	9,887369E+09	1	9,887369E+09	2,35293	0,138126
2L by 3L	5,517853E+09	1	5,517853E+09	1,31310	0,263131
Error	1,008516E+11	24	4,202149E+09		
Total SS	3,858589E+11	33			

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DV: Ellagic Acid (4mg/L)
Acetonitrile (%)
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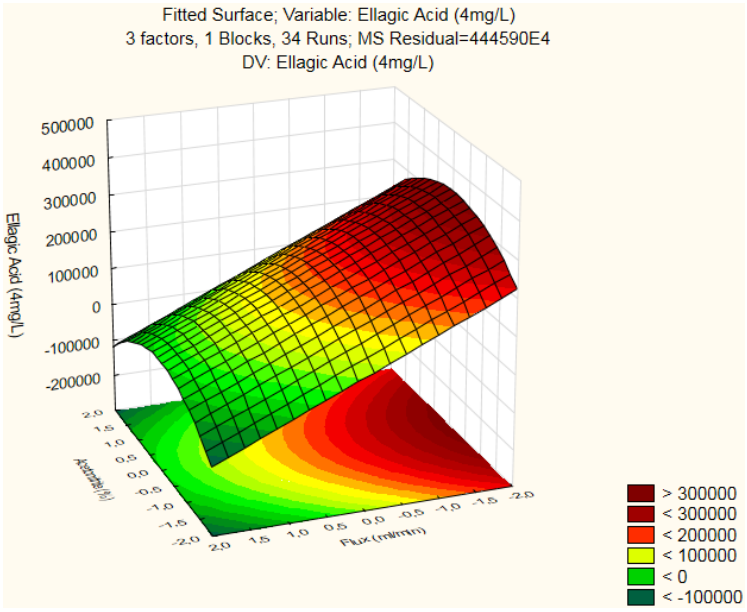
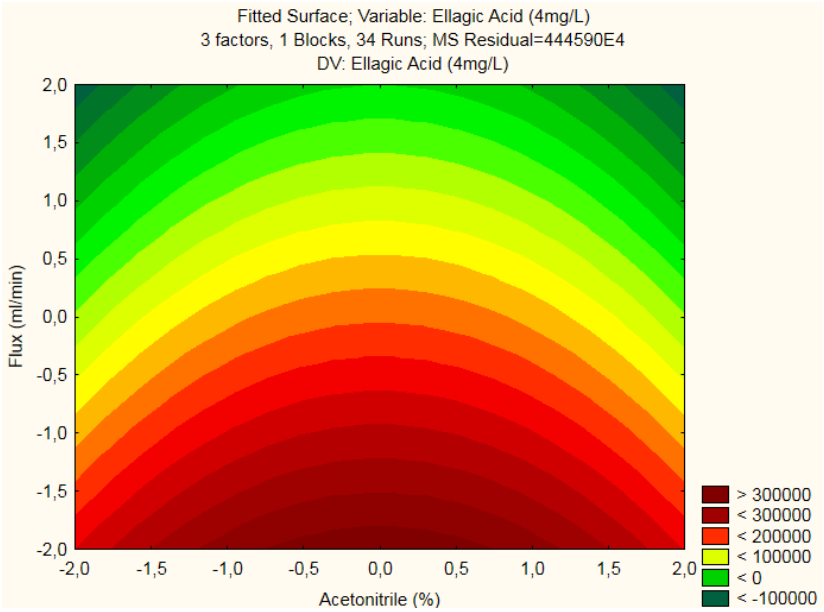
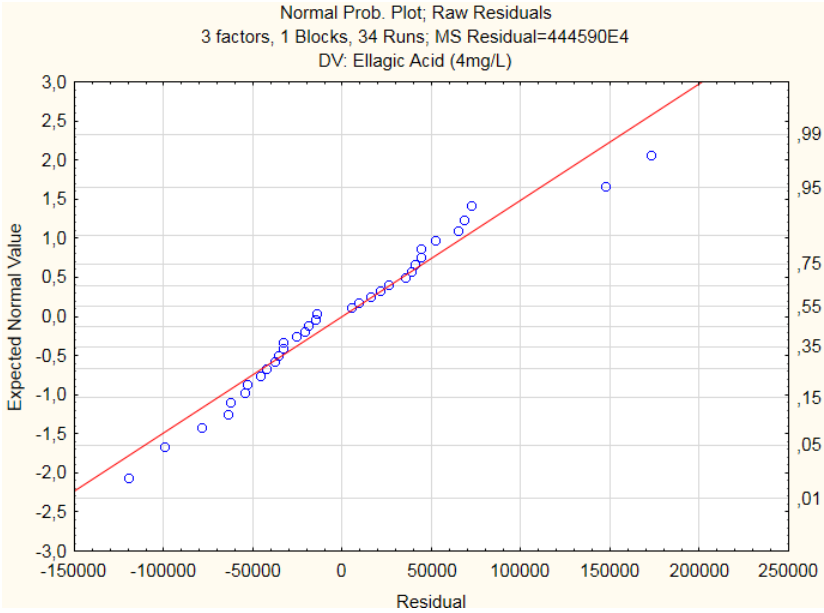
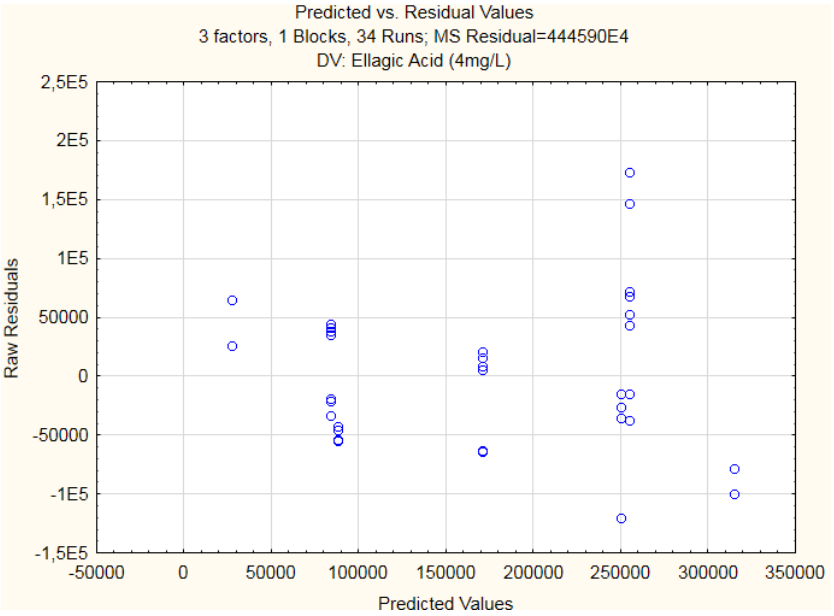
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0
Column Temperature (ºC)
> 250000
< 210000
< 160000
< 110000
< 60000

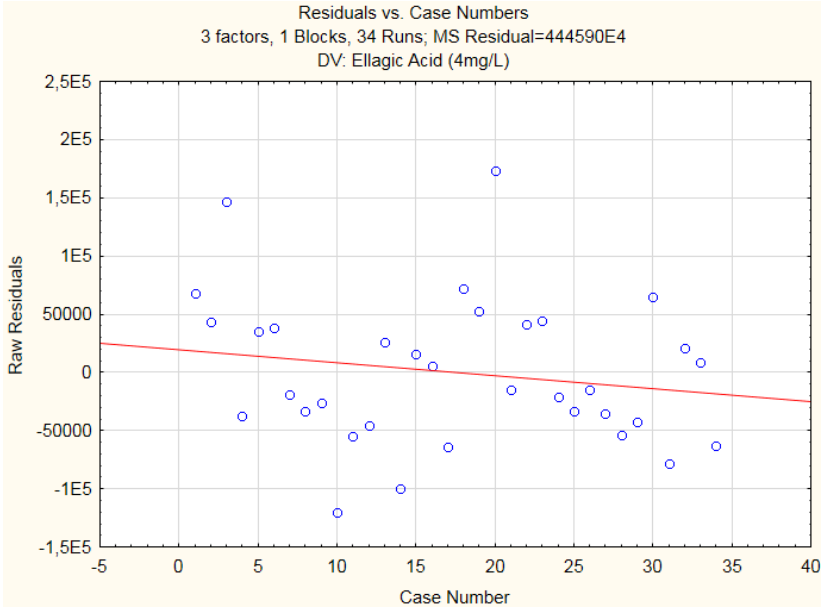

Fitted Surface; Variable: Ellagic Acid (4mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=444590E4
DV: Ellagic Acid (4mg/L)
Ellagic Acid (4mg/L)
500000
400000
300000
200000
100000
0
-100000
-200000
Flux (ml/min)
> 300000
< 300000
< 200000
< 100000
< 0
< -100000



	Regress. Coeff.	Std.Err.	t(30)	p	-95,%	+95,%
Mean/Interc.	170867,3	20396,97	8,37709	0,000000	129211	212523,4
Column Temp. (ºC)(Q)	27881,4	13415,47	2,07830	0,046334	483	55279,4
% Acetonitrile(Q)	-29423,1	13415,47	-2,19322	0,036180	-56821	-2025,1
Flux (ml/min)(L)	-85552,5	12758,18	-6,70570	0,000000	-111608	-59496,8

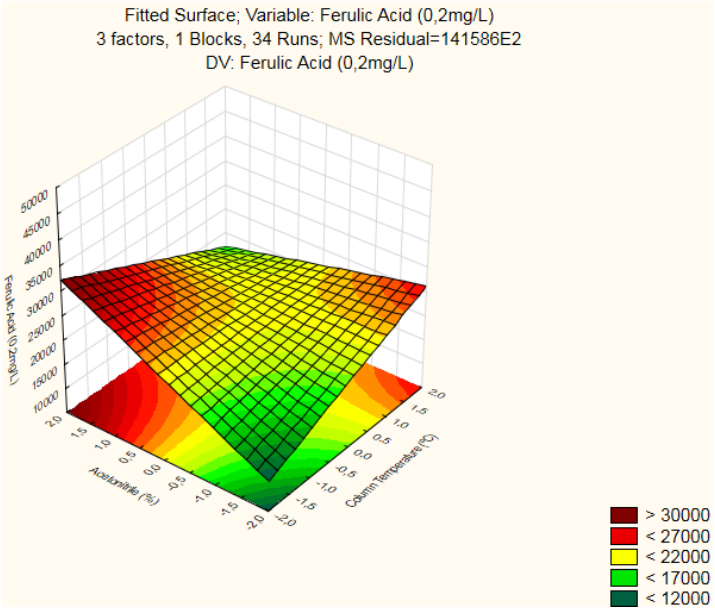


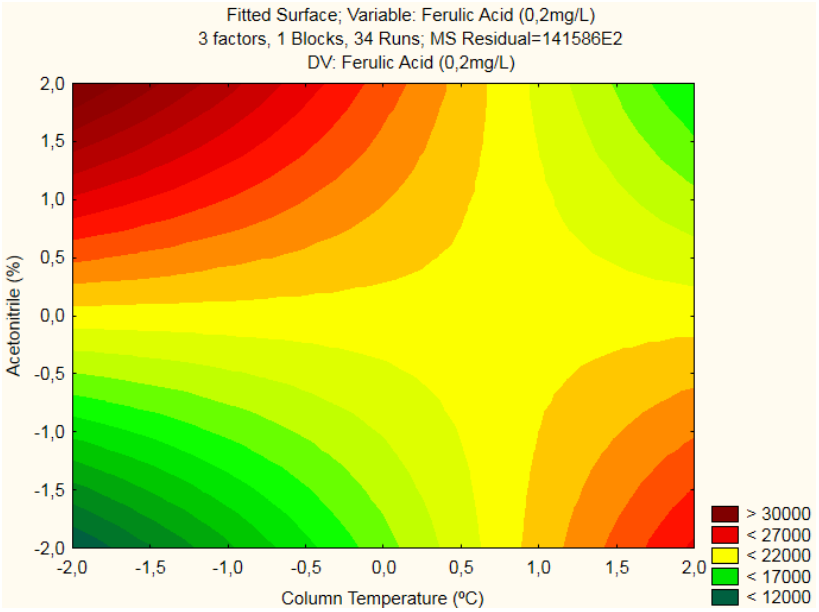

Predicted vs. Residual Values
3 factors, 1 Blocks, 34 Runs; MS Residual=444590E4
DV: Ellagic Acid (4mg/L)
Raw Residuals
2,5E5
2E5
1,5E5
1E5
50000
0
-50000
-1E5
-1,5E5
-50000
0
50000
100000
150000
200000
250000
300000
350000
Predicted Values

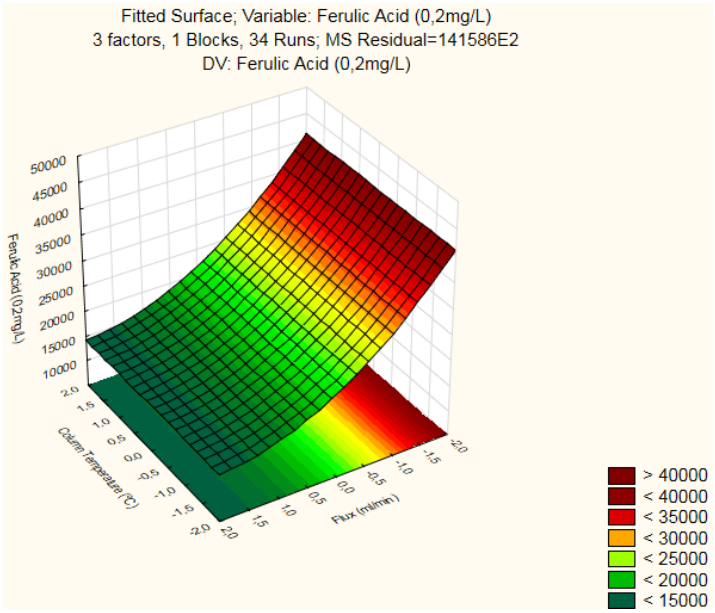

Residuals vs. Case Numbers
3 factors, 1 Blocks, 34 Runs; MS Residual=444590E4
DV: Ellagic Acid (4mg/L)
Raw Residuals
2,5E5
2E5
1,5E5
1E5
50000
0
-50000
-1E5
-1,5E5
-5
0
5
10
15
20
25
30
35
40
Case Number

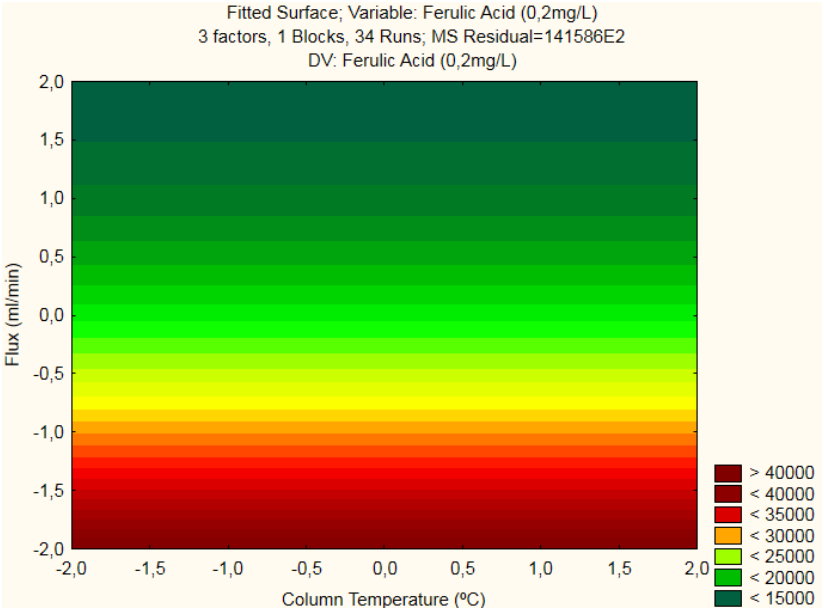
Ferulic Acid (0,2mg/L)

	Var.: Ferulic Acid (0,2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	1,466948E+07	1	1,466948E+07	1,14176	0,295911
Column Temp. (ºC) (Q)	3,062642E+07	1	3,062642E+07	2,38372	0,135689
Acetonitrile (%) (L)	**6,044061E+07**	**1**	**6,044061E+07**	**4,70423**	**0,040220**
Acetonitrile (%) (Q)	6,365858E+05	1	6,365858E+05	0,04955	0,825737
Flux (ml/min) (L)	**1,227722E+09**	**1**	**1,227722E+09**	**95,55647**	**0,000000**
Flux (ml/min) (Q)	**6,840881E+07**	**1**	**6,840881E+07**	**5,32442**	**0,029964**
1L by 2L	**5,774480E+07**	**1**	**5,774480E+07**	**4,49441**	**0,044539**
1L by 3L	3,630665E+07	1	3,630665E+07	2,82583	0,105729
2L by 3L	**8,922692E+07**	**1**	**8,922692E+07**	**6,94474**	**0,014497**
Error	3,083552E+08	24	1,284813E+07		
Total SS	1,891337E+09	33			

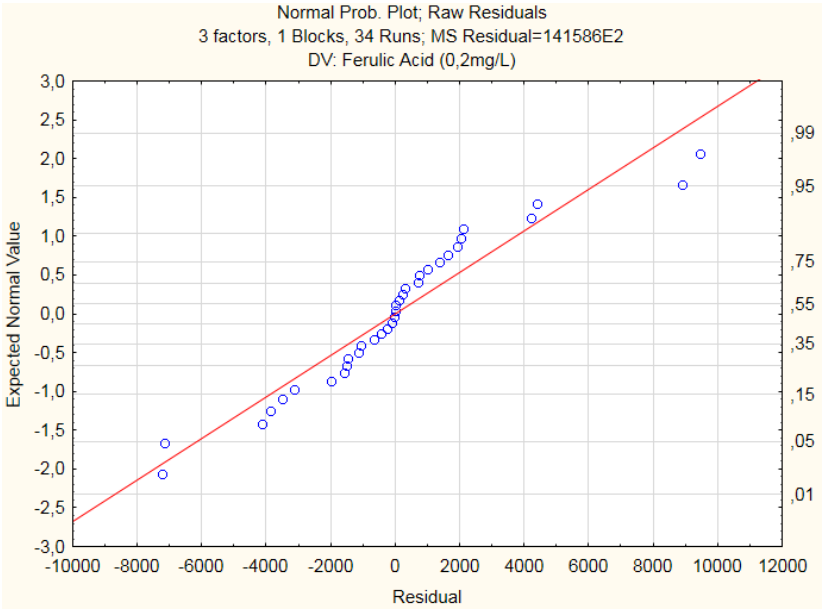


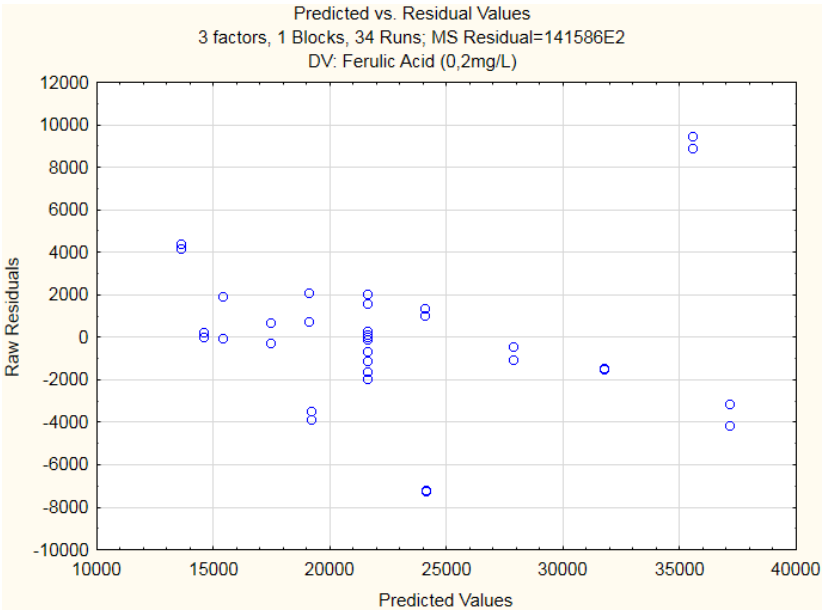

Fitted Surface; Variable: Ferulic Acid (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=141586E2
DV: Ferulic Acid (0,2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0
Column Temperature (ºC)
> 30000
< 27000
< 22000
< 17000
< 12000

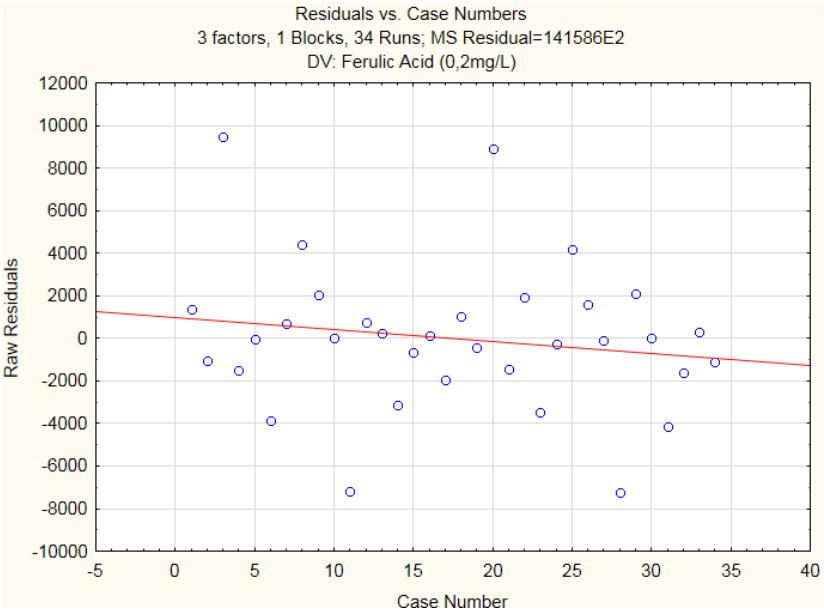

Fitted Surface; Variable: Ferulic Acid (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=141586E2
DV: Ferulic Acid (0,2mg/L)
Ferulic Acid (0,2mg/L)
50000
45000
40000
35000
30000
25000
20000
15000
10000
Column Temperature (ºC)
Flux (ml/min)
> 40000
< 40000
< 35000
< 30000
< 25000
< 20000
< 15000



	Regress. Coeff.	Std.Err.	t(28)	p	-95,%	+95,%
Mean/Interc.	21580,86	875,8367	24,64028	0,000000	19786,79	23374,93
% Acetonitrile(L)	1487,56	719,9786	2,06611	0,048181	12,75	2962,37
Flux (ml/min)(L)	-6704,39	719,9786	-9,31192	0,000000	-8179,19	-5229,58
Flux (ml/min)(Q)	1514,39	737,1222	2,05446	0,049372	4,46	3024,31
1L by 2L	-1899,75	940,6990	-2,01951	0,053099	-3826,68	27,18
2L by 3L	-2361,50	940,6990	-2,51037	0,018114	-4288,43	-434,57

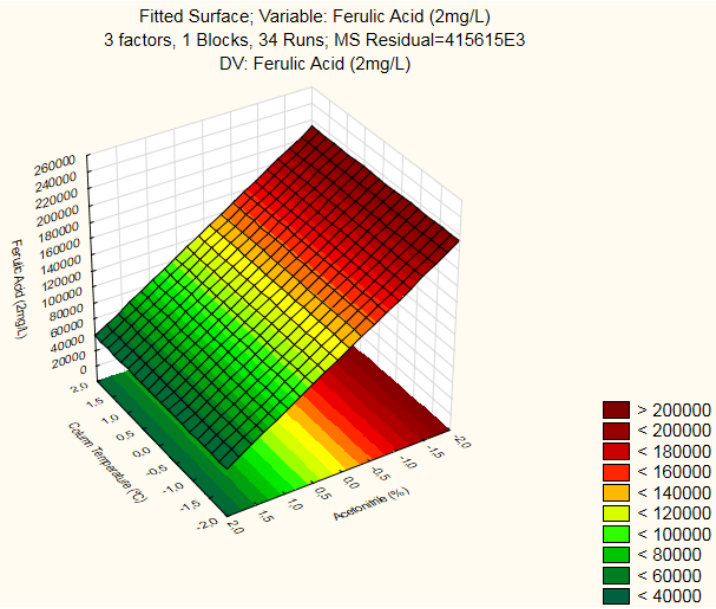


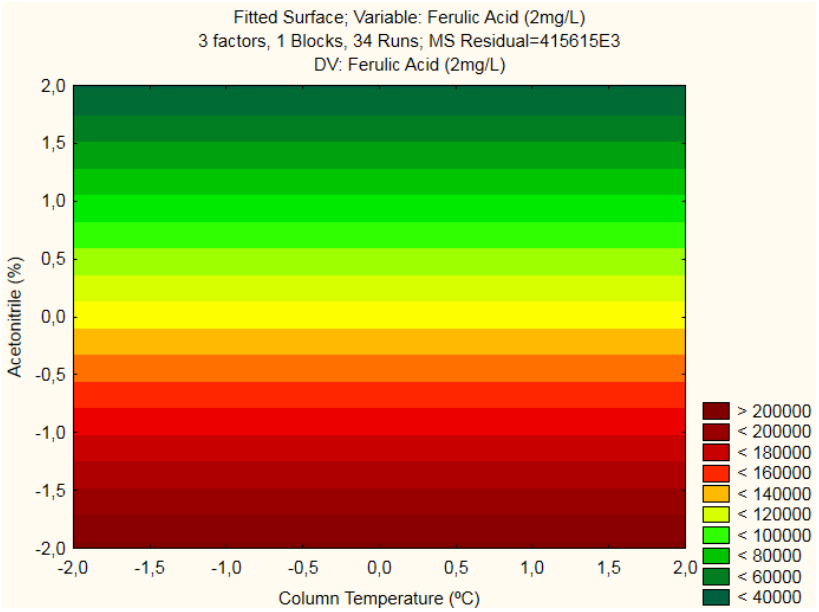

Predicted vs. Residual Values
3 factors, 1 Blocks, 34 Runs; MS Residual=141586E2
DV: Ferulic Acid (0,2mg/L)
Raw Residuals
12000
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
10000
15000
20000
25000
30000
35000
40000
Predicted Values

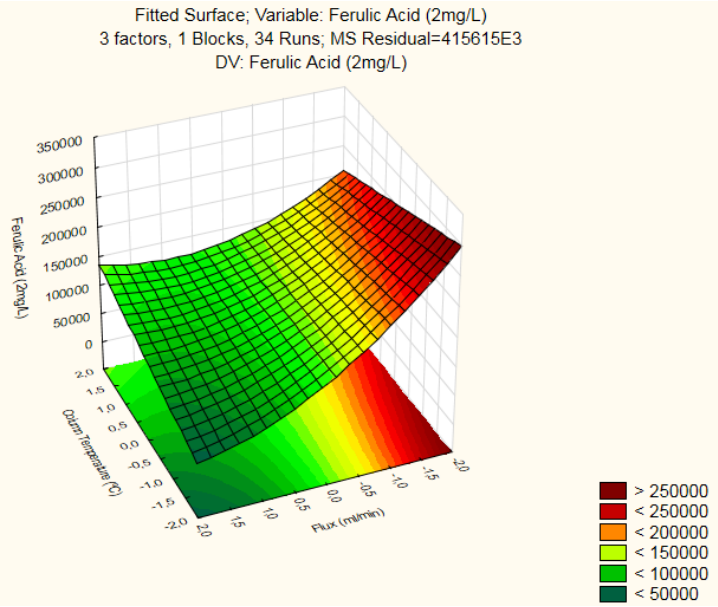

Residuals vs. Case Numbers
3 factors, 1 Blocks, 34 Runs; MS Residual=141586E2
DV: Ferulic Acid (0,2mg/L)
Raw Residuals
12000
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
-5
0
5
10
15
20
25
30
35
40
Case Number

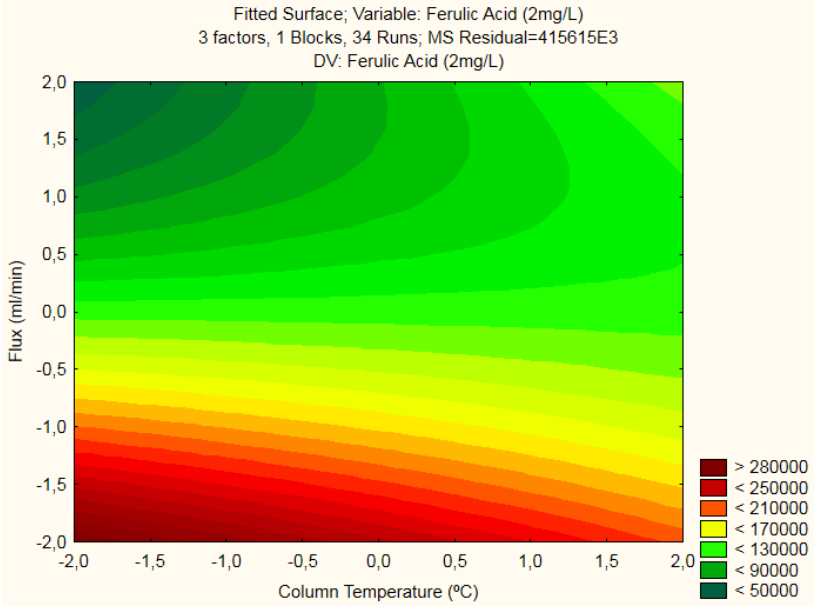
Ferulic Acid (2mg/L)

	Var.: Ferulic Acid (2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	9,210998E+08	1	9,210998E+08	2,5446	0,123760
Column Temp. (ºC) (Q)	1,016832E+09	1	1,016832E+09	2,8090	0,106716
Acetonitrile (%) (L)	**5,154178E+10**	**1**	**5,154178E+10**	**142,3858**	**0,000000**
Acetonitrile (%) (Q)	2,816514E+08	1	2,816514E+08	0,7781	0,386481
Flux (ml/min) (L)	**4,574960E+10**	**1**	**4,574960E+10**	**126,3847**	**0,000000**
Flux (ml/min) (Q)	**4,234315E+09**	**1**	**4,234315E+09**	**11,6974**	**0,002244**
1L by 2L	1,350416E+09	1	1,350416E+09	3,7306	0,065317
1L by 3L	**2,081002E+09**	**1**	**2,081002E+09**	**5,7488**	**0,024635**
2L by 3L	1,742645E+07	1	1,742645E+07	0,0481	0,828185
Error	8,687684E+09	24	3,619868E+08		
Total SS	1,147919E+11	33			

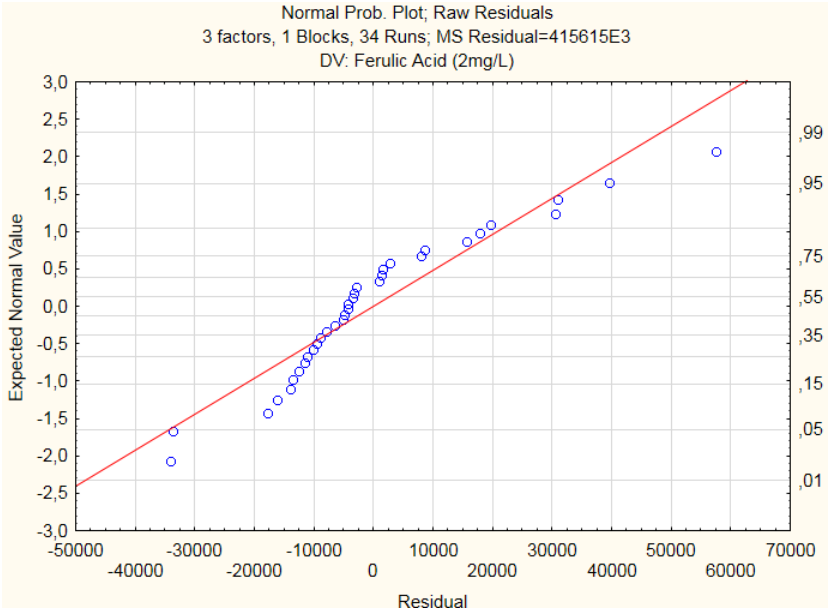


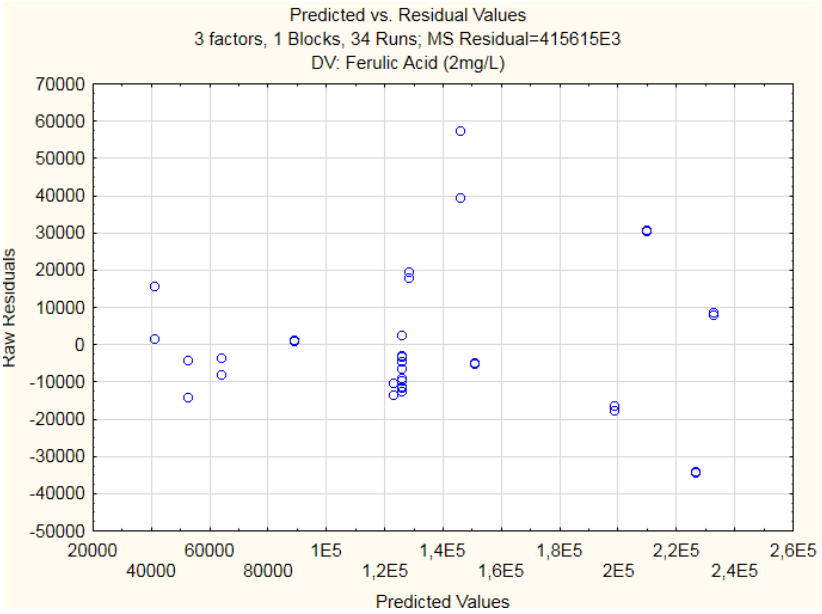

Fitted Surface; Variable: Ferulic Acid (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=415615E3
DV: Ferulic Acid (2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0
Column Temperature (ºC)
> 200000
< 200000
< 180000
< 160000
< 140000
< 120000
< 100000
< 80000
< 60000
< 40000

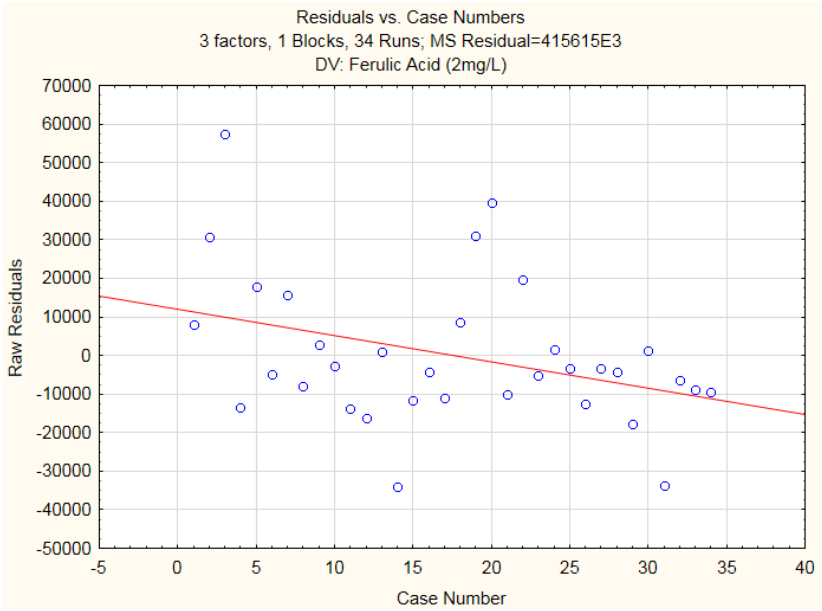

Fitted Surface; Variable: Ferulic Acid (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=415615E3
DV: Ferulic Acid (2mg/L)
Ferulic Acid (2mg/L)
350000
300000
250000
200000
150000
100000
50000
0
Column Temperature (ºC)
Flux (ml/min)
> 250000
< 250000
< 200000
< 150000
< 100000
< 50000



	Regress. Coeff.	Std.Err.	t(29)	p	-95,%	+95,%
Mean/Interc.	125498,4	4745,240	26,4472	0,000000	115793,3	135203,5
% Acetonitrile(L)	-43439,9	3900,809	-11,1361	0,000000	-51417,9	-35461,8
Flux (ml/min)(L)	-40926,3	3900,809	-10,4917	0,000000	-48904,4	-32948,3
Flux (ml/min)(Q)	11366,5	3993,692	2,8461	0,008042	3198,5	19534,5
1L by 3L	11404,5	5096,661	2,2376	0,033092	980,7	21828,3

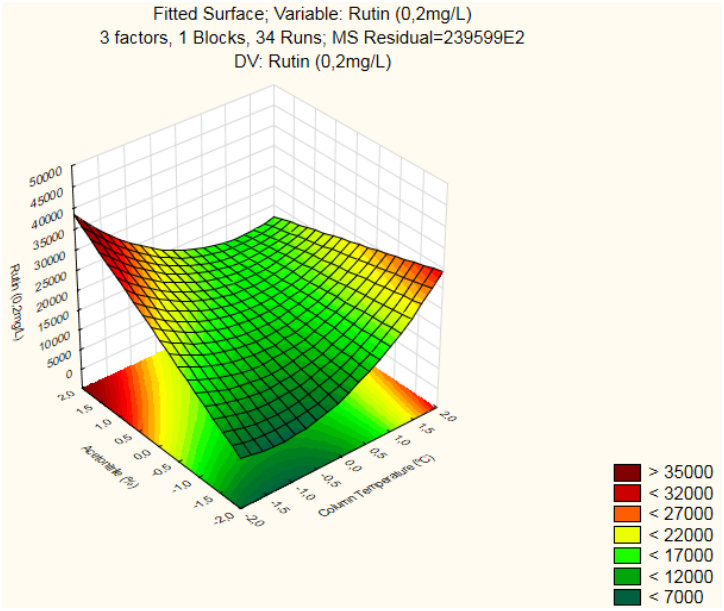


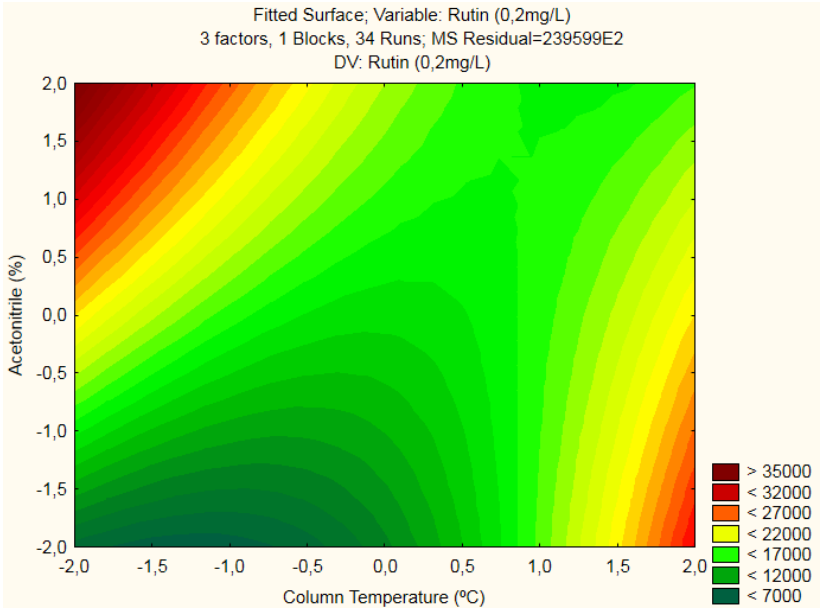

Predicted vs. Residual Values
3 factors, 1 Blocks, 34 Runs; MS Residual=415615E3
DV: Ferulic Acid (2mg/L)
Raw Residuals
70000
60000
50000
40000
30000
20000
10000
0
-10000
-20000
-30000
-40000
-50000
20000
40000
60000
80000
1E5
1,2E5
1,4E5
1,6E5
1,8E5
2E5
2,2E5
2,4E5
2,6E5
Predicted Values

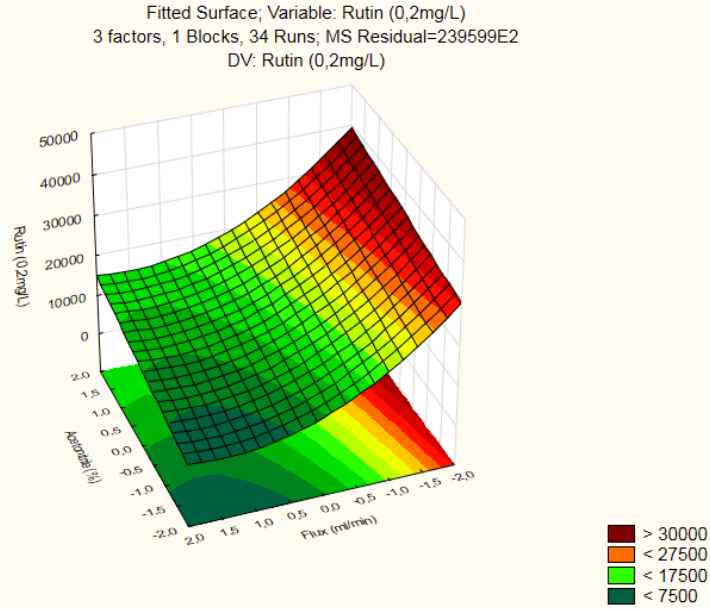

Residuals vs. Case Numbers
3 factors, 1 Blocks, 34 Runs; MS Residual=415615E3
DV: Ferulic Acid (2mg/L)
Raw Residuals
70000
60000
50000
40000
30000
20000
10000
0
-10000
-20000
-30000
-40000
-50000
-5
0
5
10
15
20
25
30
35
40
Case Number

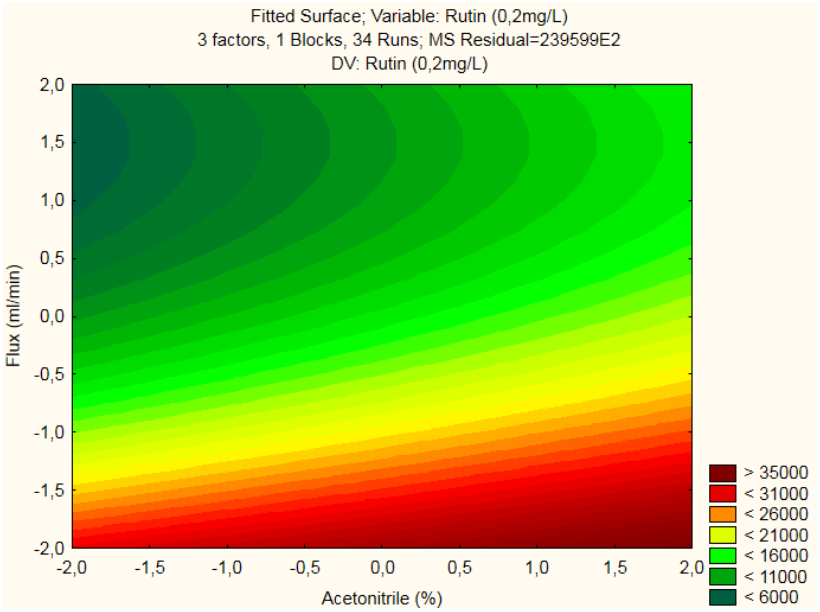
Rutin (0,2mg/L)

	Var.: Rutin (0,2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	6,517876E+07	1	6,517876E+07	3,23233	0,084791
Column Temp. (ºC) (Q)	7,744838E+07	1	7,744838E+07	3,84080	0,061731
Acetonitrile (%) (L)	**1,477172E+08**	**1**	**1,477172E+08**	**7,32555**	**0,012319**
Acetonitrile (%) (Q)	2,640438E+07	1	2,640438E+07	1,30944	0,263781
Flux (ml/min) (L)	**1,029884E+09**	**1**	**1,029884E+09**	**51,07368**	**0,000000**
Flux (ml/min) (Q)	6,754137E+07	1	6,754137E+07	3,34949	0,079674
1L by 2L	**1,164349E+08**	**1**	**1,164349E+08**	**5,77420**	**0,024352**
1L by 3L	3,375029E+07	1	3,375029E+07	1,67373	0,208072
2L by 3L	6,159110E+07	1	6,159110E+07	3,05441	0,093303
Error	4,839520E+08	24	2,016467E+07		
Total SS	2,144565E+09	33			

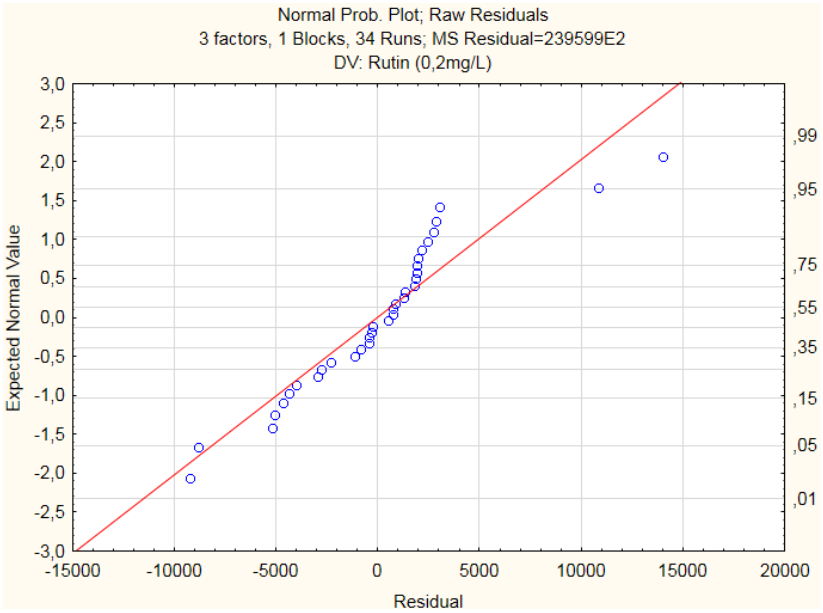


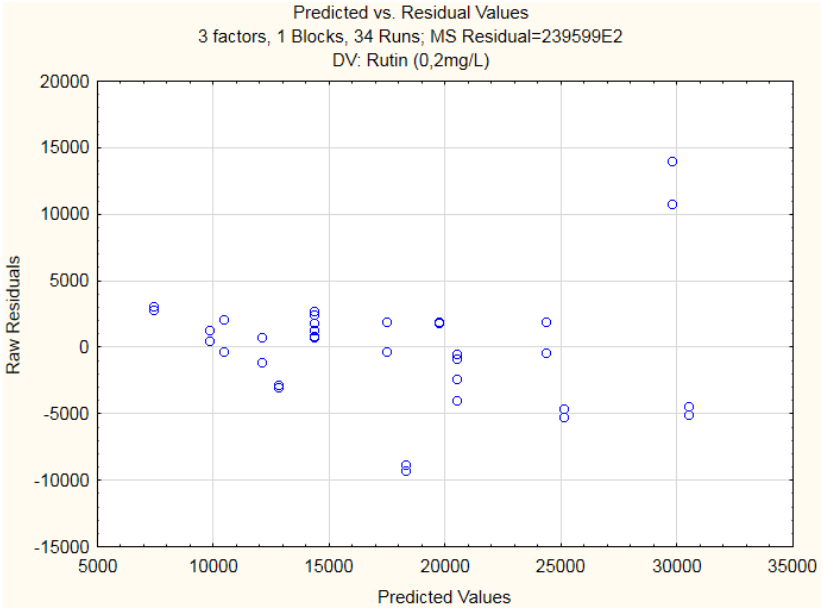

Fitted Surface; Variable: Rutin (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=239599E2
DV: Rutin (0,2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0
Column Temperature (ºC)
> 35000
< 32000
< 27000
< 22000
< 17000
< 12000
< 7000

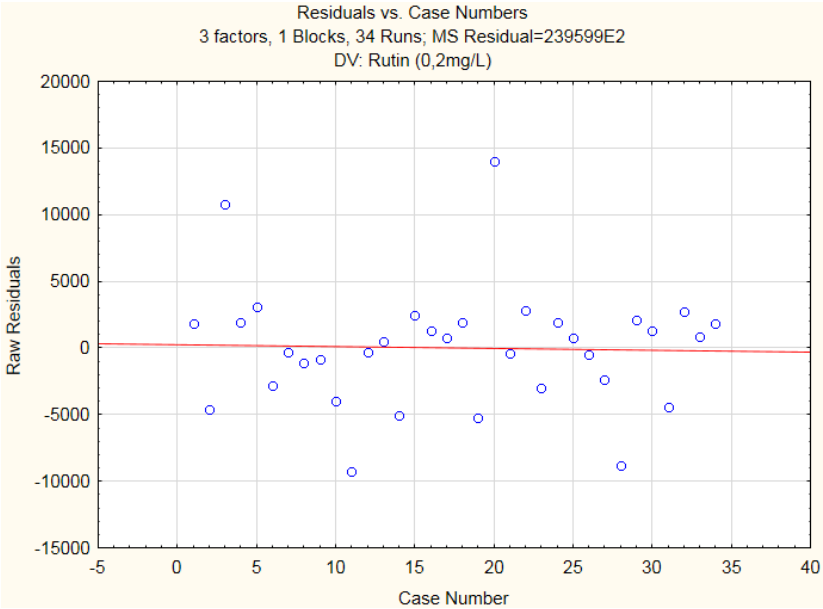

Fitted Surface; Variable: Rutin (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=239599E2
DV: Rutin (0,2mg/L)
Rutin (0,2mg/L)
50000
40000
30000
20000
10000
0
Acetonitrile (%)
Flux (ml/min)
> 30000
< 27500
< 17500
< 7500



	Regress. Coeff.	Std.Err.	t(29)	p	-95,%	+95,%
Mean/Interc.	14360,52	1497,366	9,59052	0,000000	11293,31	17427,74
Column Temperature (ºC)(Q)	2173,05	984,846	2,20649	0,035720	155,69	4190,42
% Acetonitrile(L)	2325,54	936,594	2,48298	0,019289	407,02	4244,07
Flux (ml/min)(L)	-6140,49	936,594	-6,55619	0,000000	-8059,01	-4221,96
Flux (ml/min)(Q)	2050,46	984,846	2,08201	0,046598	33,09	4067,82
1L by 2L	-2697,63	1223,721	-2,20445	0,035879	-5204,30	-190,95

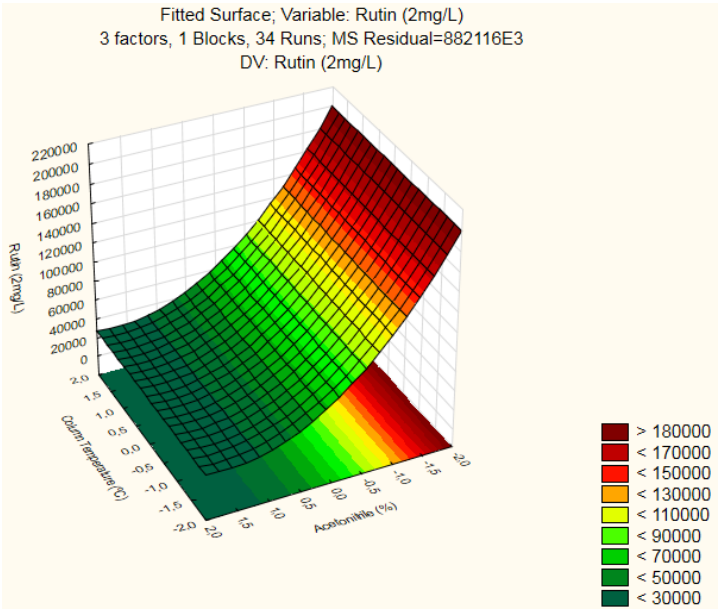


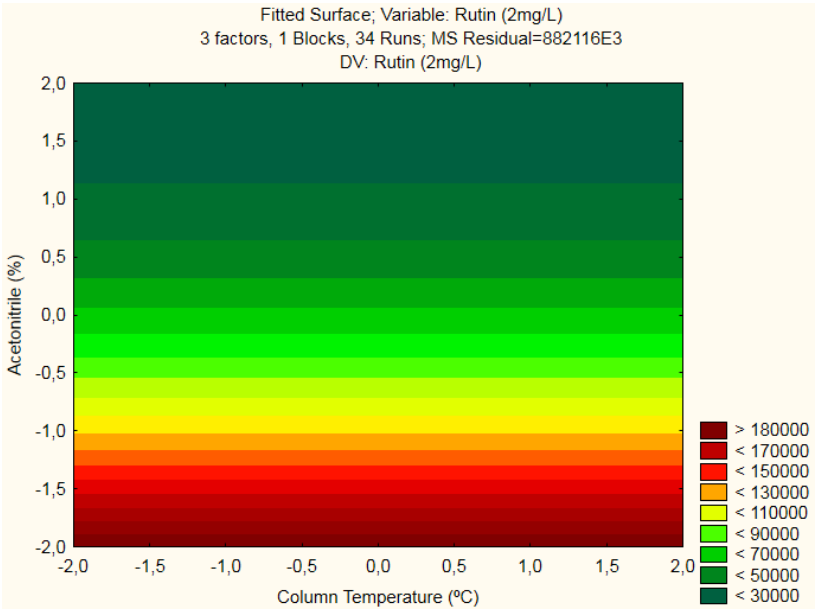


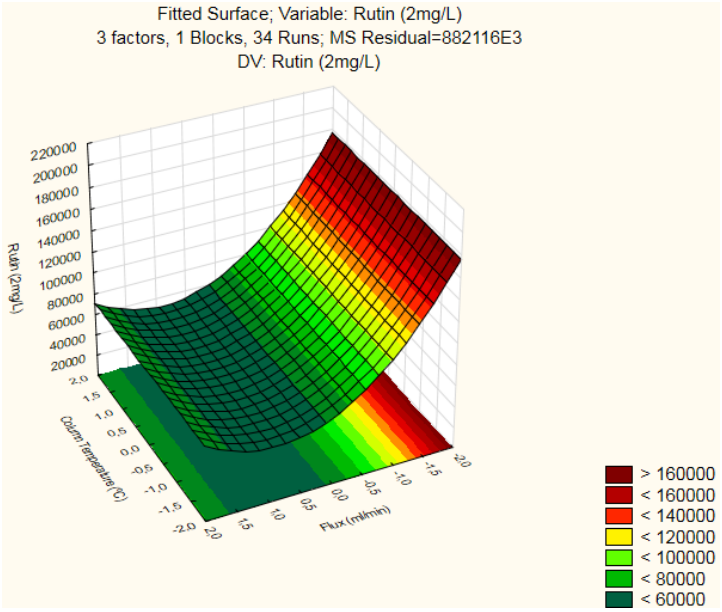


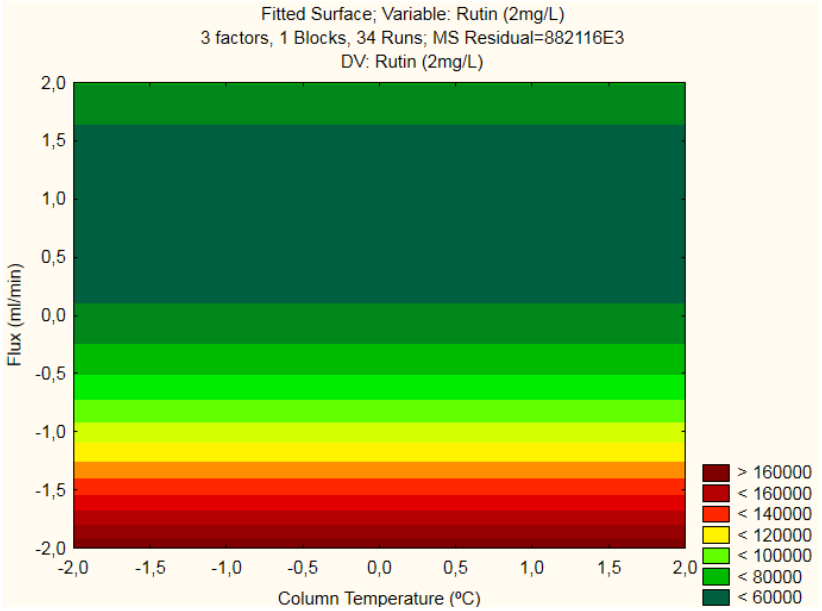
Rutin (2mg/L)

	Var.: Rutin (2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	1,490160E+09	1	1,490160E+09	1,91660	0,178970
Column Temp. (ºC) (Q)	2,194489E+09	1	2,194489E+09	2,82248	0,105925
Acetonitrile (%) (L)	**5,069848E+10**	**1**	**5,069848E+10**	**65,20680**	**0,000000**
Acetonitrile (%) (Q)	**5,561337E+09**	**1**	**5,561337E+09**	**7,15282**	**0,013259**
Flux (ml/min) (L)	**1,911991E+10**	**1**	**1,911991E+10**	**24,59143**	**0,000046**
Flux (ml/min) (Q)	**7,497519E+09**	**1**	**7,497519E+09**	**9,64307**	**0,004825**
1L by 2L	8,271520E+08	1	8,271520E+08	1,06386	0,312620
1L by 3L	1,388773E+09	1	1,388773E+09	1,78620	0,193924
2L by 3L	1,020723E+09	1	1,020723E+09	1,31282	0,263181
Error	1,866007E+10	24	7,775030E+08		
Total SS	1,034505E+11	33			

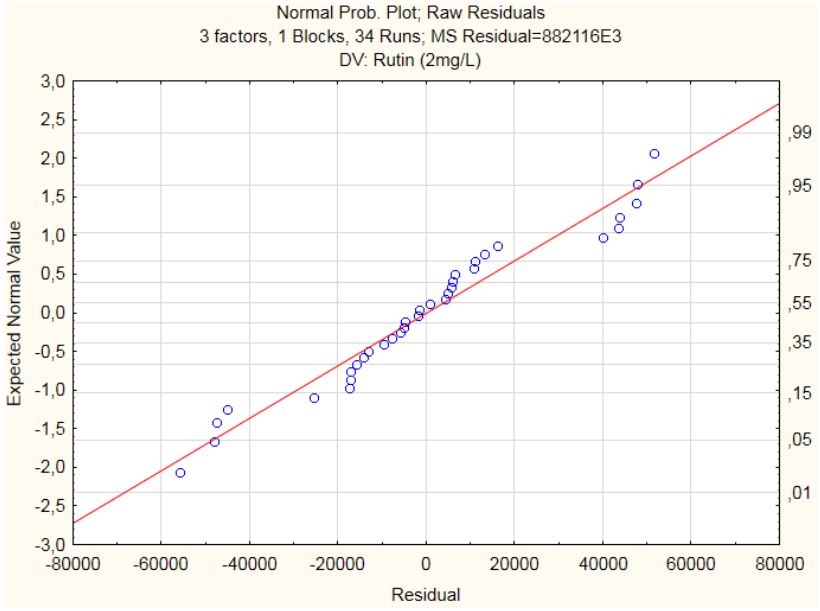

Fitted Surface; Variable: Rutin (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=882116E3
DV: Rutin (2mg/L)
Rutin (2mg/L)
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Column Temperature (ºC)
Acetonitrile (%)
> 180000
< 170000
< 150000
< 130000
< 110000
< 90000
< 70000
< 50000
< 30000

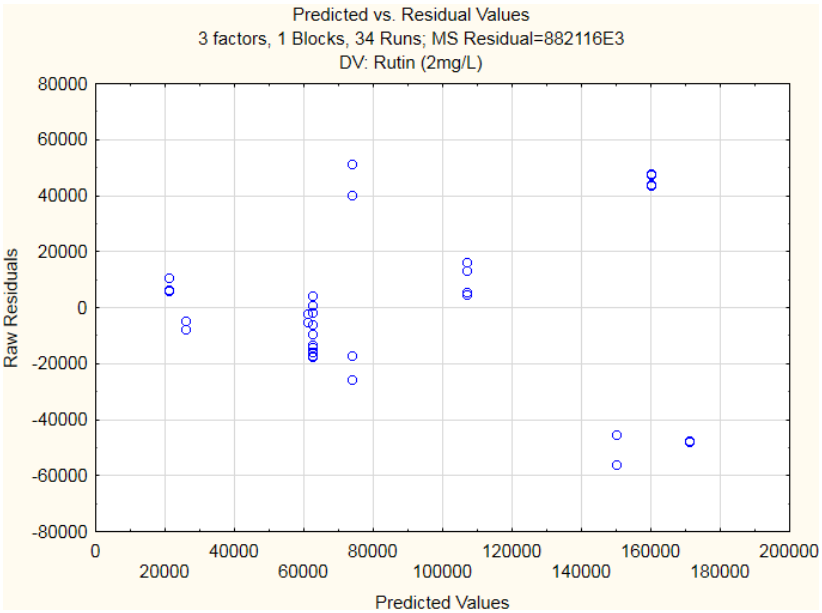

Fitted Surface; Variable: Rutin (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=882116E3
DV: Rutin (2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
Column Temperature (ºC)
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
2,0
> 180000
< 170000
< 150000
< 130000
< 110000
< 90000
< 70000
< 50000
< 30000

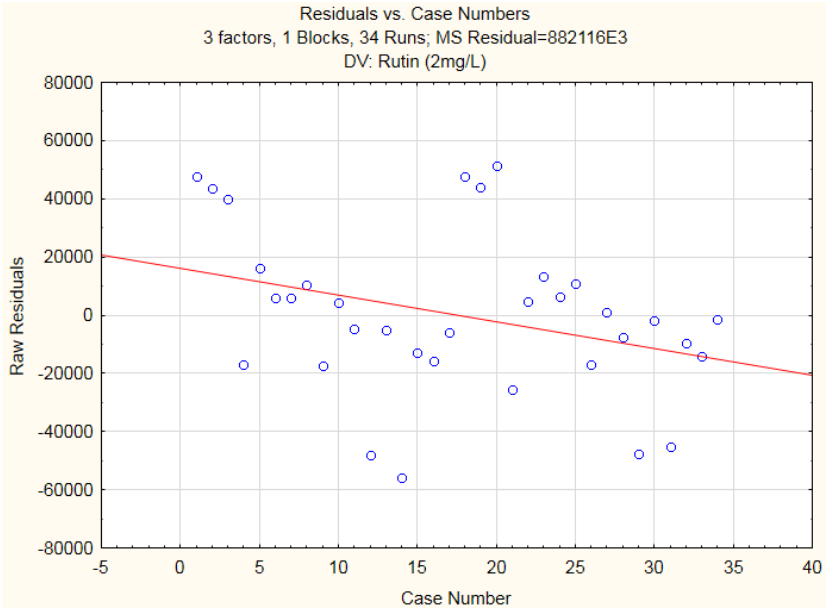




	Regress. Coeff.	Std.Err.	t(29)	p	-95,%	+95,%
Mean/Interc.	62308,4	9085,497	6,85800	0,000000	43726,5	80890,3
Acetonitrile (%)(L)	-43083,0	5682,923	-7,58114	0,000000	-54705,9	-31460,2
Acetonitrile (%)(Q)	12790,8	5975,700	2,14047	0,040856	569,1	25012,5
Flux (ml/min)(L)	-26457,7	5682,923	-4,65565	0,000066	-38080,6	-14834,8
Flux (ml/min)(Q)	15320,9	5975,700	2,56387	0,015798	3099,2	27542,6

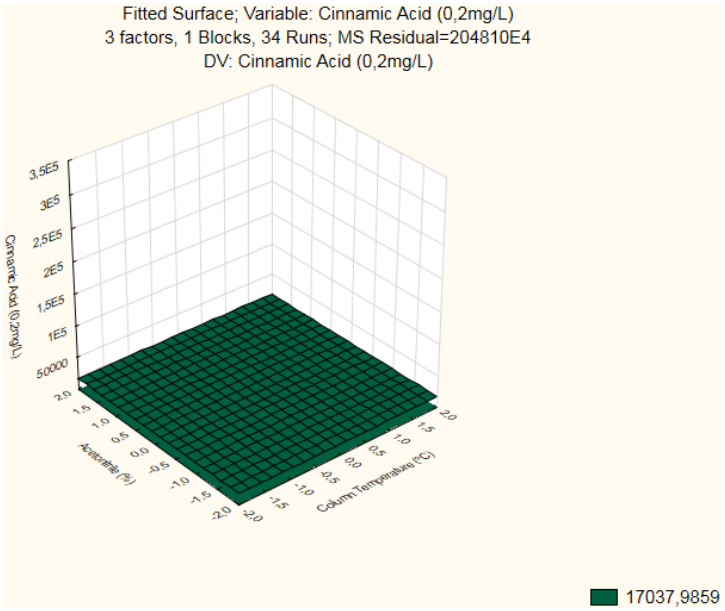

Normal Prob. Plot; Raw Residuals
3 factors, 1 Blocks, 34 Runs; MS Residual=882116E3
DV: Rutin (2mg/L)
Expected Normal Value
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
,99
,95
,75
,55
,35
,15
,05
,01
-80000
-60000
-40000
-20000
0
20000
40000
60000
80000
Residual

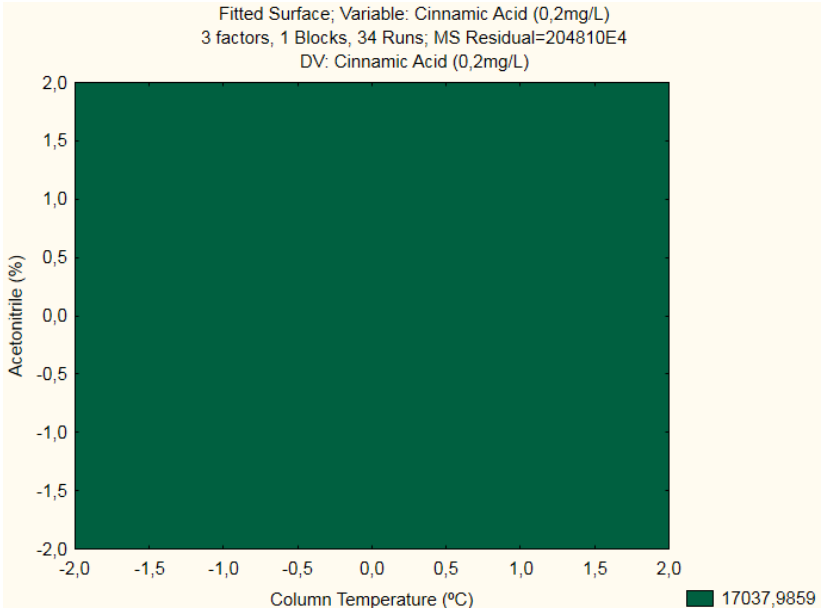

Predicted vs. Residual Values
3 factors, 1 Blocks, 34 Runs; MS Residual=882116E3
DV: Rutin (2mg/L)
Raw Residuals
80000
60000
40000
20000
0
-20000
-40000
-60000
-80000
0
20000
40000
60000
80000
100000
120000
140000
160000
180000
200000
Predicted Values

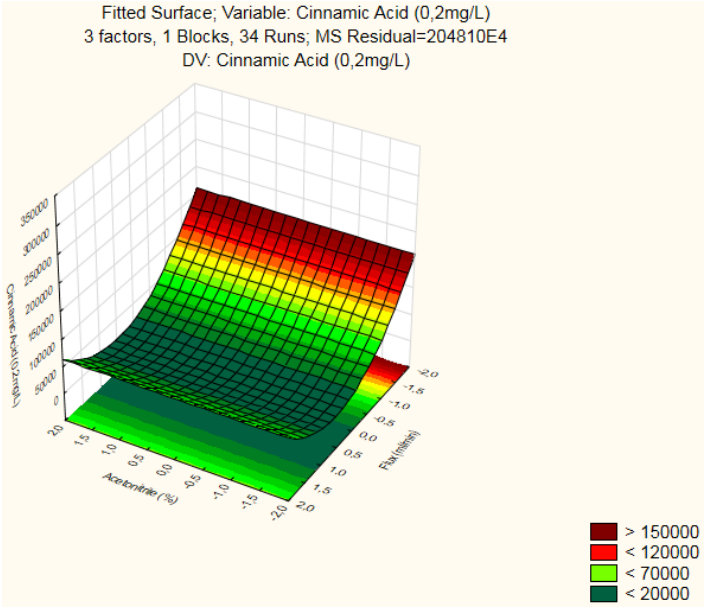

Residuals vs. Case Numbers
3 factors, 1 Blocks, 34 Runs; MS Residual=882116E3
DV: Rutin (2mg/L)
Raw Residuals
80000
60000
40000
20000
0
-20000
-40000
-60000
-80000
-5
0
5
10
15
20
25
30
35
40
Case Number

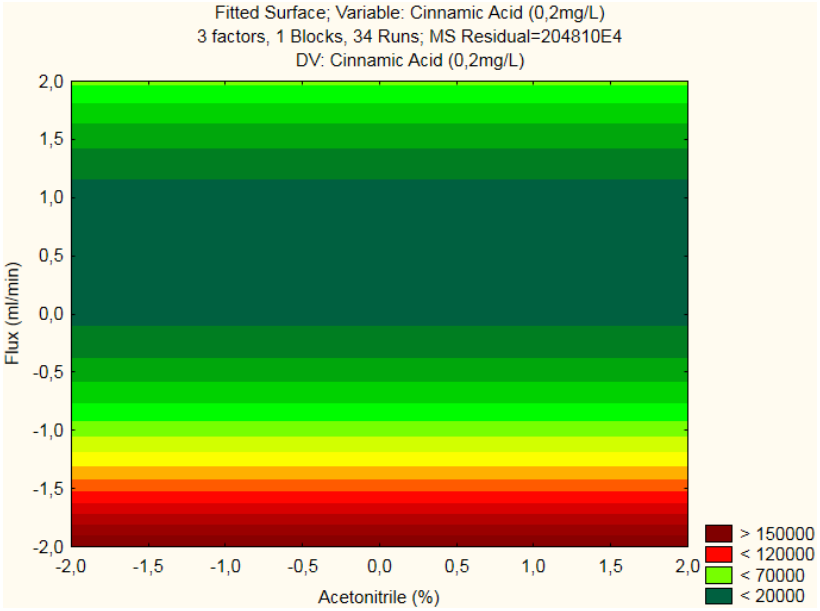
Cinnamic Acid (0,2mg/L)

	Var.: Rutin (2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	5,487303E+07	1	5,487303E+07	0,021569	0,884467
Column Temp. (ºC) (Q)	1,072390E+09	1	1,072390E+09	0,421517	0,522346
Acetonitrile (%) (L)	3,954560E+08	1	3,954560E+08	0,155439	0,696872
Acetonitrile (%) (Q)	1,307662E+09	1	1,307662E+09	0,513994	0,480334
Flux (ml/min) (L)	**1,651076E+10**	**1**	**1,651076E+10**	**6,489780**	**0,017675**
Flux (ml/min) (Q)	9,320630E+09	1	9,320630E+09	3,663601	0,067611
1L by 2L	1,758276E+06	1	1,758276E+06	0,000691	0,979244
1L by 3L	1,083056E+08	1	1,083056E+08	0,042571	0,838275
2L by 3L	3,090804E+07	1	3,090804E+07	0,012149	0,913150
Error	6,105881E+10	24	2,544117E+09		
Total SS	9,456611E+10	33			




Fitted Surface; Variable: Cinnamic Acid (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=204810E4
DV: Cinnamic Acid (0,2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,0 -1,5 -1,0 -0,5 0,0 0,5 1,0 1,5 2,0
Column Temperature (ºC)
17037,9859

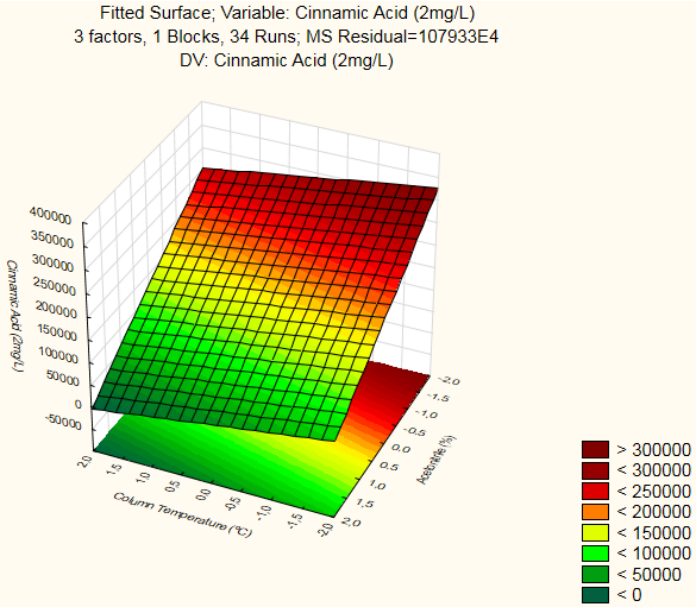

Fitted Surface; Variable: Cinnamic Acid (0,2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=204810E4
DV: Cinnamic Acid (0,2mg/L)
> 150000
< 120000
< 70000
< 20000

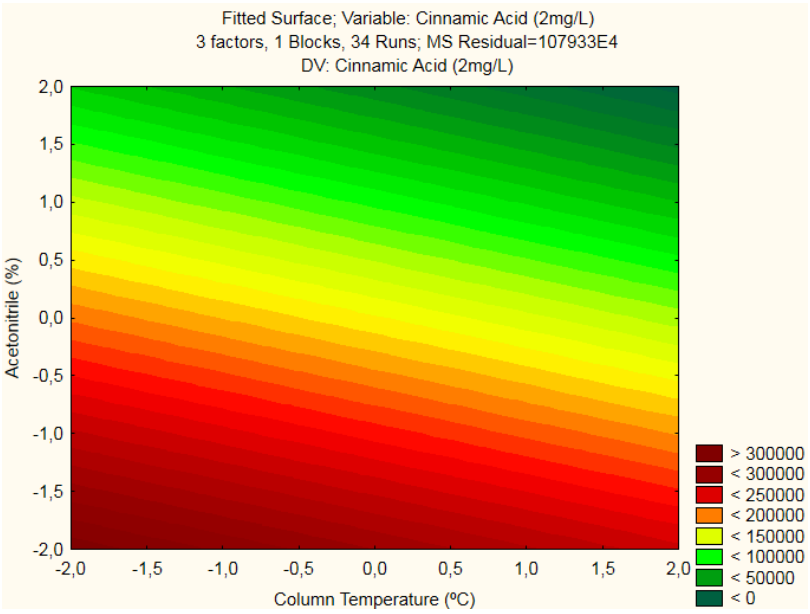


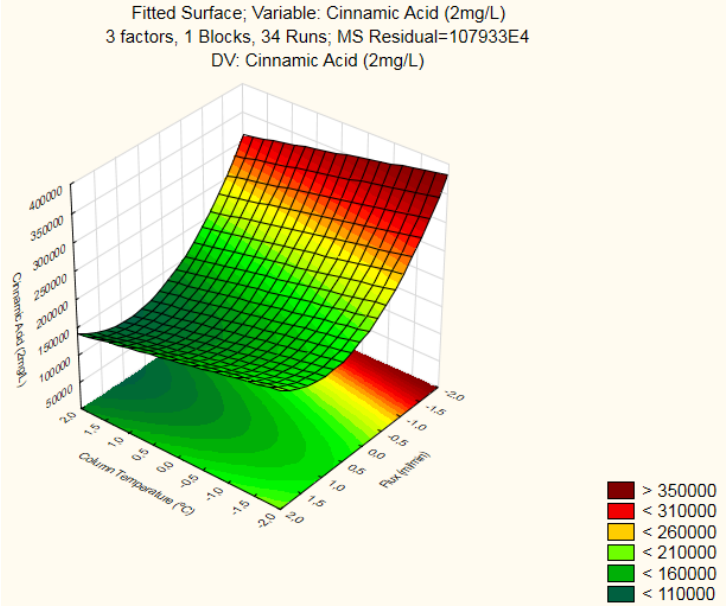
	Regress. Coeff.	Std.Err.	t(24)	p	-95,%	+95,%
Mean/Interc.	17038,0	10533,88	1,61745	0,115914	-4446,0	38521,97
Flux (ml/min)(L)	-24586,3	8659,34	-2,83928	0,007912	-42247,1	-6925,42
Flux (ml/min)(Q)	23641,3	8865,53	2,66666	0,012064	5560,0	41722,69

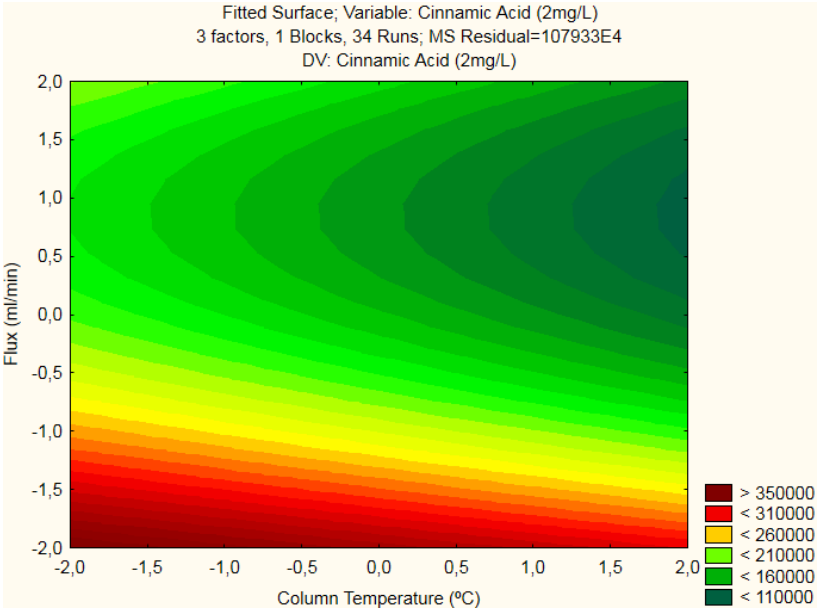
Cinnamic Acid 2

	Var.: Rutin (2 mg/L)				
	SS	df	MS	F	p
Column Temp. (ºC) (L)	**9,120554E+09**	**1**	**9,120554E+09**	**10,8944**	**0,003007**
Column Temp. (ºC) (Q)	1,843674E+09	1	1,843674E+09	2,2022	0,150826
Acetonitrile (%) (L)	**1,131537E+11**	**1**	**1,131537E+11**	**135,1605**	**0,000000**
Acetonitrile (%) (Q)	5,155829E+08	1	5,155829E+08	0,6159	0,440269
Flux (ml/min) (L)	**5,021313E+10**	**1**	**5,021313E+10**	**59,9789**	**0,000000**
Flux (ml/min) (Q)	**1,483320E+10**	**1**	**1,483320E+10**	**17,7181**	**0,000310**
1L by 2L	3,514622E+09	1	3,514622E+09	4,1982	0,051553
1L by 3L	3,326607E+09	1	3,326607E+09	3,9736	0,057705
2L by 3L	1,150957E+09	1	1,150957E+09	1,3748	0,252496
Error	2,009233E+10	24	8,371802E+08		
Total SS	2,196567E+11	33			

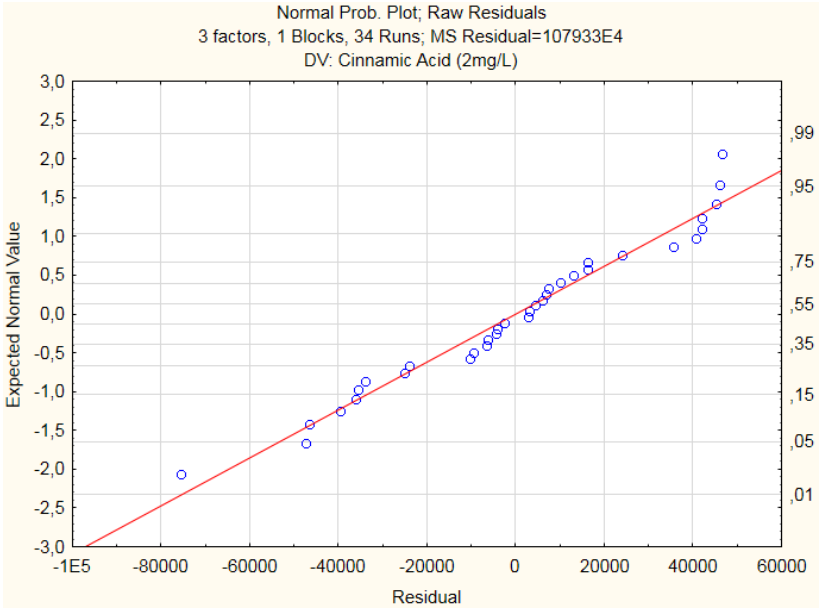

Fitted Surface; Variable: Cinnamic Acid (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=107933E4
DV: Cinnamic Acid (2mg/L)
Cinnamic Acid (2mg/L)
400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
Column Temperature (°C)
Acetonitrile (%)
> 300000
< 300000
< 250000
< 200000
< 150000
< 100000
< 50000
< 0

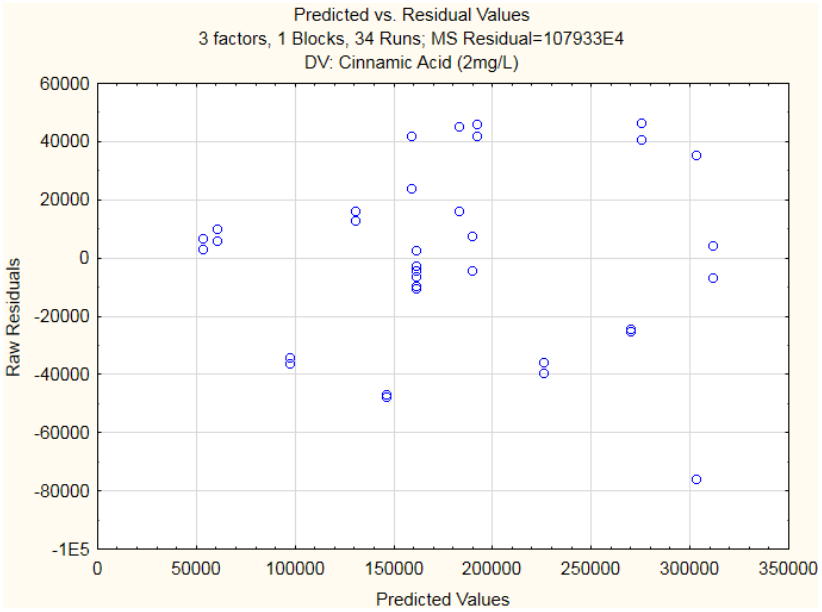

Fitted Surface; Variable: Cinnamic Acid (2mg/L)
3 factors, 1 Blocks, 34 Runs; MS Residual=107933E4
DV: Cinnamic Acid (2mg/L)
Acetonitrile (%)
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
Column Temperature (ºC)
> 300000
< 300000
< 250000
< 200000
< 150000
< 100000
< 50000
< 0

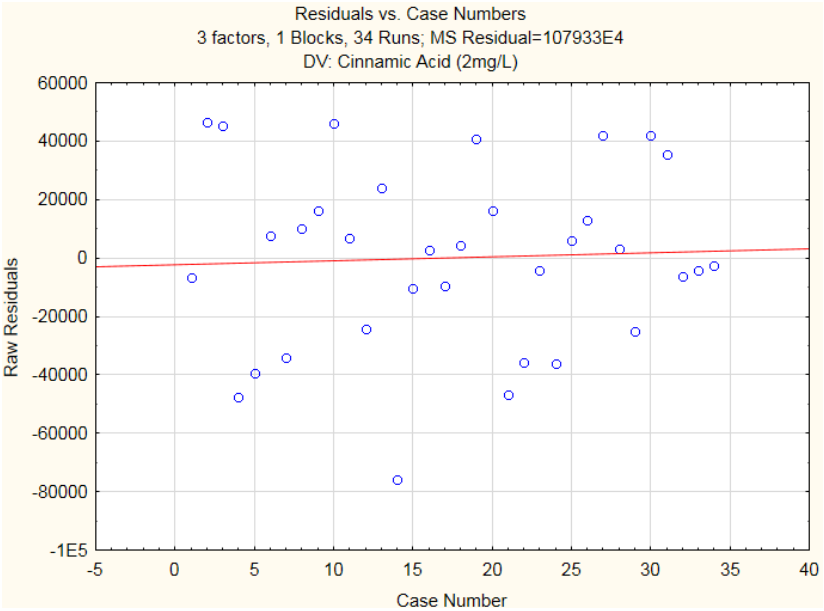




	Regress. Coeff.	Std.Err.	t(24)	p	-95,%	+95,%
Mean/Interc.	161333,5	7646,961	21,0977	0,000000	145693,8	176973,3
Column Temp. (ºC)(L)	-18273,4	6286,159	-2,9069	0,006927	-31130,0	-5416,8
% Acetonitrile(L)	-64364,0	6286,159	-10,2390	0,000000	-77220,7	-51507,4
Flux (ml/min)(L)	-42876,3	6286,159	-6,8208	0,000000	-55733,0	-30019,7
Flux (ml/min)(Q)	24677,5	6435,840	3,8344	0,000626	11514,7	37840,2


Normal Prob. Plot; Raw Residuals
3 factors, 1 Blocks, 34 Runs; MS Residual=107933E4
DV: Cinnamic Acid (2mg/L)
Expected Normal Value
3,0
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
,99
,95
,75
,55
,35
,15
,05
,01
-1E5
-80000
-60000
-40000
-20000
0
20000
40000
60000
Residual


Predicted vs. Residual Values
3 factors, 1 Blocks, 34 Runs; MS Residual=107933E4
DV: Cinnamic Acid (2mg/L)
Raw Residuals
60000
40000
20000
0
-20000
-40000
-60000
-80000
-1E5
0
50000
100000
150000
200000
250000
300000
350000
Predicted Values


Residuals vs. Case Numbers
3 factors, 1 Blocks, 34 Runs; MS Residual=107933E4
DV: Cinnamic Acid (2mg/L)
Raw Residuals
60000
40000
20000
0
-20000
-40000
-60000
-80000
-1E5
-5
0
5
10
15
20
25
30
35
40
Case Number