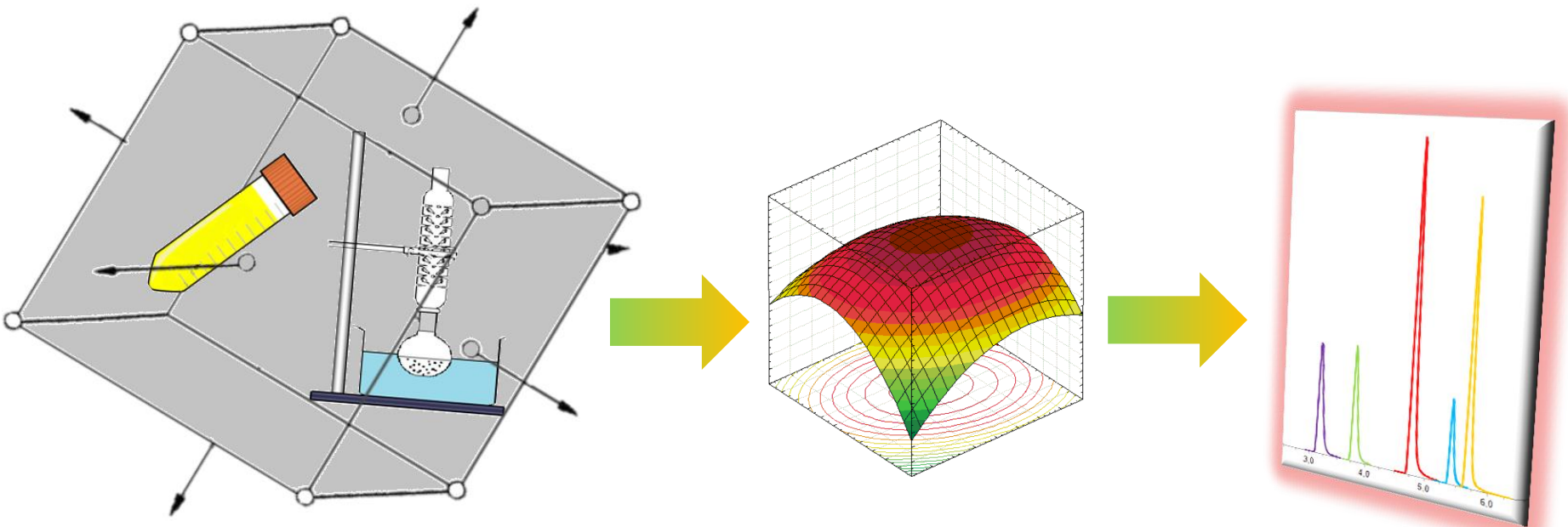


# Use of response surface methodology to optimize samples preparations in laboratory



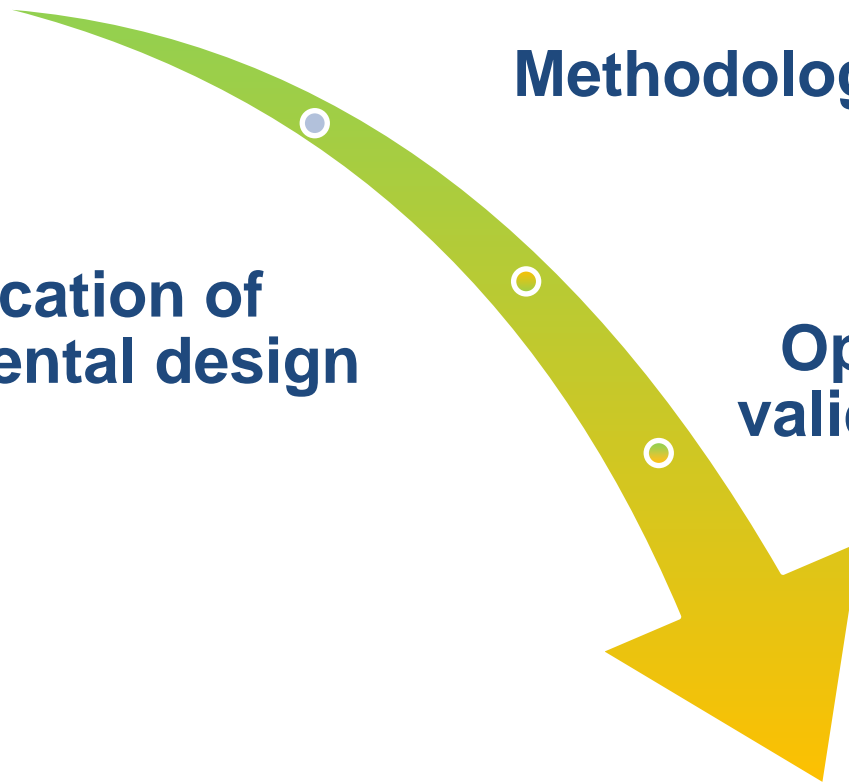
**Context**

**Methodology**

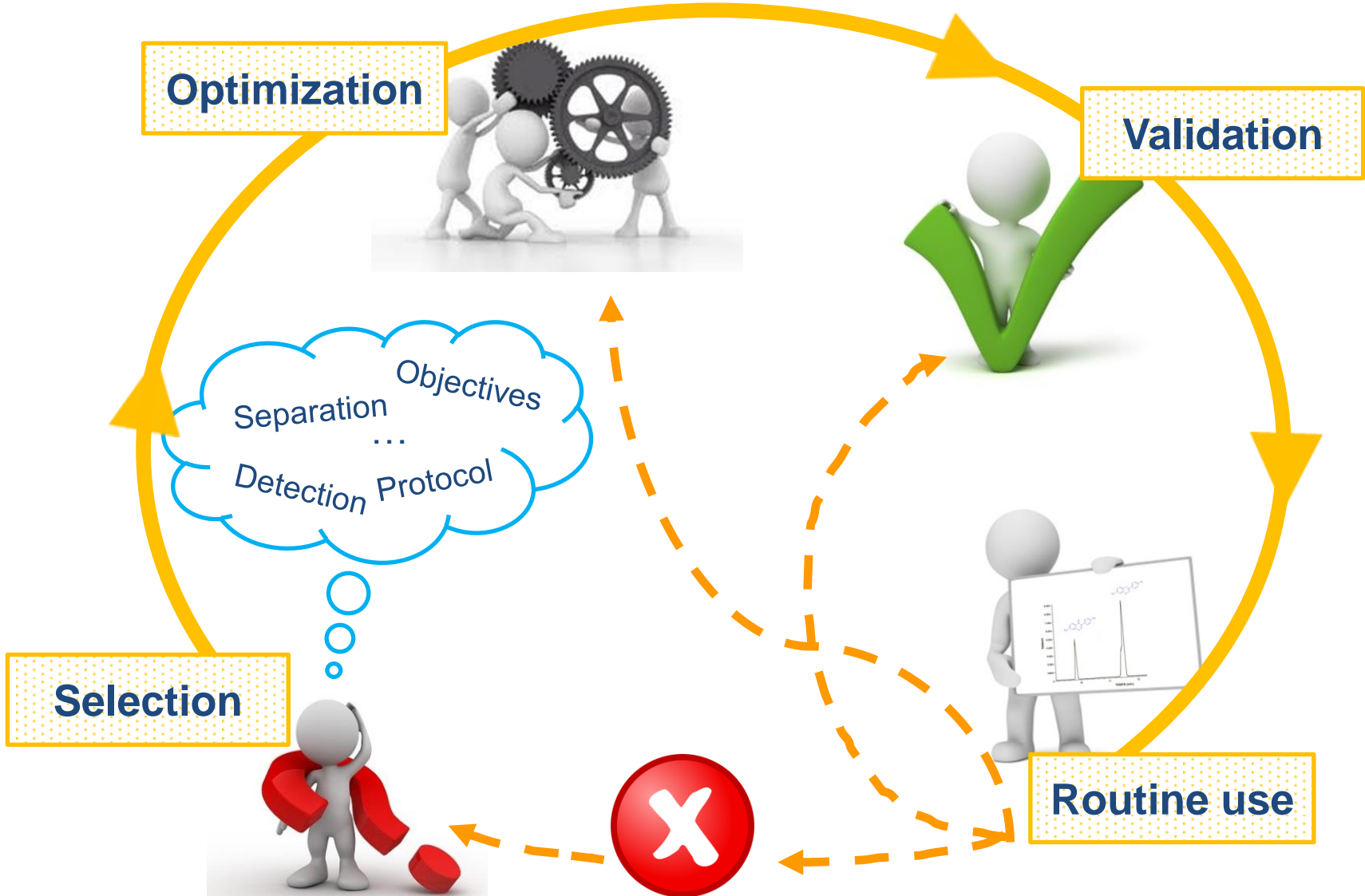
**Application of  
experimental design**

**Optimized and  
validated method**

**Conclusions**



# Context - lifecycle of an analytical method



**An Improved Method for Determination of Isoflavones in Soybean Powder by Liquid Chromatography**

1,111111 / B.C. Chen\*

Received 17 May 2007 / Revised 21 September 2007 / Accepted 21 Sept 2007

**Key Words:** Chromatography, Isoflavones, Soybean powder

**Summary:** An improved method for the determination of isoflavones in soybean powder by liquid chromatography (LC) with tandem mass spectrometry (MS) is described. The method involves the extraction of isoflavones from soybean powder with methanol, followed by cleanup with C<sub>18</sub> solid phase extraction (SPE) and detection by LC-MS/MS. The method is simple, sensitive, and accurate. The limits of detection (LOD) and limits of quantification (LOQ) for daidzein, genistein, and biochanin A are 0.1, 0.2, and 0.3 µg/g, respectively. The method was applied to the analysis of isoflavones in soybean powder, and the results were compared with those obtained by the standard method. The results showed that the proposed method is more accurate and sensitive than the standard method.

**Introduction:** Isoflavones are a class of phytoestrogens that have been shown to have various biological activities, including antioxidant, anti-inflammatory, and anticancer effects. They are found in soybeans and other legumes. The determination of isoflavones in soybean powder is important for quality control and for the study of their health benefits.

**Validation:** The method was validated for accuracy, precision, and recovery. The accuracy was evaluated by comparing the results obtained by the proposed method with those obtained by the standard method. The precision was evaluated by determining the relative standard deviation (RSD) of the results. The recovery was evaluated by spiking the sample with a known amount of isoflavones and determining the recovery percentage.

## LC Determination of Four Isoflavone Aglycones in Red Clover (*Trifolium pratense* L.)

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Online publication: 15 November 2007

### Abstract

Red clover (*Trifolium pratense* L.) is an important forage for dairy cattle. It contains isoflavone aglycones, genistein, formononetin, and biochanin A. The study aims to determine the concentration of these isoflavone aglycones in red clover. The method involves the extraction of isoflavone aglycones from red clover with methanol, followed by cleanup with C<sub>18</sub> solid phase extraction (SPE) and detection by LC-MS/MS. The method is simple, sensitive, and accurate. The limits of detection (LOD) and limits of quantification (LOQ) for daidzein, genistein, formononetin, and biochanin A are 0.1, 0.2, 0.3, and 0.4 µg/g, respectively. The method was applied to the analysis of isoflavone aglycones in red clover, and the results were compared with those obtained by the standard method. The results showed that the proposed method is more accurate and sensitive than the standard method.

### Keywords

Column liquid chromatography  
Red clover  
Isoflavones  
Validation

Full Short Communication  
DOI: 10.15663/0337-007-0459-0  
0009-5893/08/01  
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**Abstract:** Cow's milk can be used to study human milk. In order to study human milk, it is necessary to determine the concentration of isoflavone aglycones in cow's milk. The method involves the extraction of isoflavone aglycones from cow's milk with methanol, followed by cleanup with C<sub>18</sub> solid phase extraction (SPE) and detection by LC-MS/MS. The method is simple, sensitive, and accurate. The limits of detection (LOD) and limits of quantification (LOQ) for daidzein, genistein, formononetin, and biochanin A are 0.1, 0.2, 0.3, and 0.4 µg/g, respectively. The method was applied to the analysis of isoflavone aglycones in cow's milk, and the results were compared with those obtained by the standard method. The results showed that the proposed method is more accurate and sensitive than the standard method.

**A New Simplified and Stability Indicating Liquid Chromatography Method for Routine Analysis of Isoflavones in Different Complex Matrices**

Francini K. A. Yano<sup>1</sup>, Grazielle P. Ramos<sup>1</sup>, Helena F. Frê<sup>1</sup>, Grazielle P. Ramos<sup>1</sup>, Mariana C. Nogueira<sup>2</sup>, Wagner L. Bassani<sup>1</sup>

Received 17 May 2007 / Revised 21 September 2007 / Accepted 21 Sept 2007

**Abstract:** In this work, a stability indicating method for the routine analysis of isoflavones in different complex matrices was developed. The method involves the extraction of isoflavones from complex matrices with methanol, followed by cleanup with C<sub>18</sub> solid phase extraction (SPE) and detection by LC-MS/MS. The method is simple, sensitive, and accurate. The limits of detection (LOD) and limits of quantification (LOQ) for daidzein, genistein, formononetin, and biochanin A are 0.1, 0.2, 0.3, and 0.4 µg/g, respectively. The method was applied to the analysis of isoflavones in different complex matrices, and the results were compared with those obtained by the standard method. The results showed that the proposed method is more accurate and sensitive than the standard method.

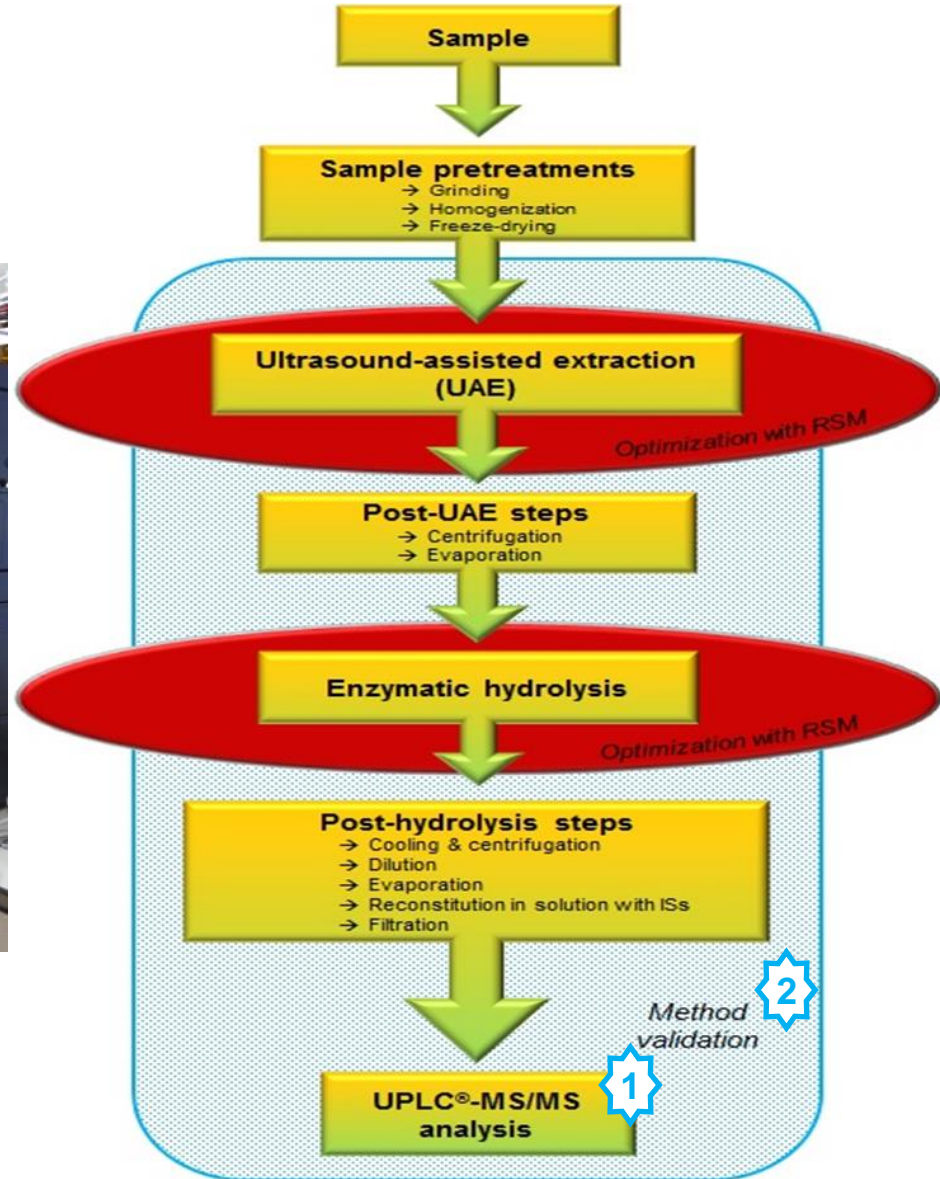
**Use of multivariate statistical techniques to optimize the separation of isoflavones by liquid chromatography**

Francini K. A. Yano<sup>1</sup>, Grazielle P. Ramos<sup>1</sup>, Helena F. Frê<sup>1</sup>, Grazielle P. Ramos<sup>1</sup>, Mariana C. Nogueira<sup>2</sup>, Wagner L. Bassani<sup>1</sup>

Received 17 May 2007 / Revised 21 September 2007 / Accepted 21 Sept 2007

**Abstract:** The use of multivariate statistical techniques to optimize the separation of isoflavones by liquid chromatography is described. The method involves the extraction of isoflavones from complex matrices with methanol, followed by cleanup with C<sub>18</sub> solid phase extraction (SPE) and detection by LC-MS/MS. The method is simple, sensitive, and accurate. The limits of detection (LOD) and limits of quantification (LOQ) for daidzein, genistein, formononetin, and biochanin A are 0.1, 0.2, 0.3, and 0.4 µg/g, respectively. The method was applied to the analysis of isoflavones in different complex matrices, and the results were compared with those obtained by the standard method. The results showed that the proposed method is more accurate and sensitive than the standard method.





## Experimental Design



### Objectives:

Researching factors of influence // Understanding the impact of factors and their possible interactions // **Finding optimal conditions** //

*"Decreasing the number of assays --> decreasing development costs"*

Number of factors



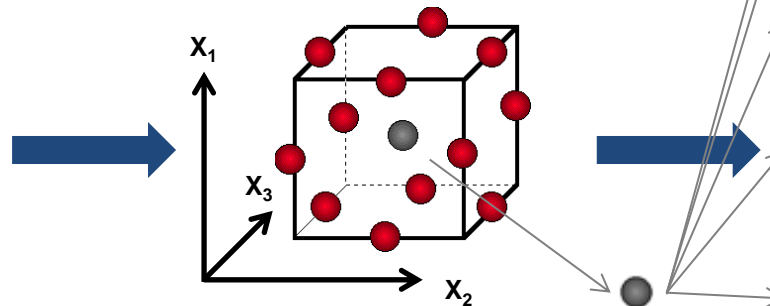
Number of central points

### Box-Behnken

$$N = 2k(k-1) + C_0$$

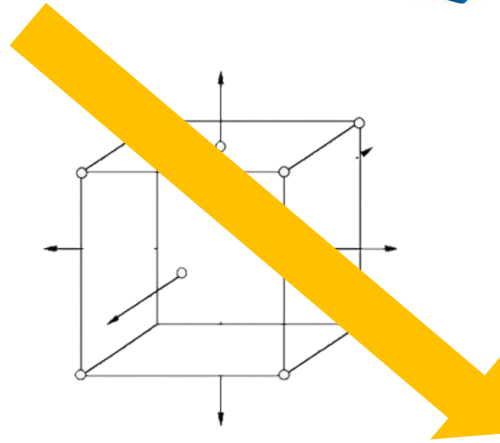
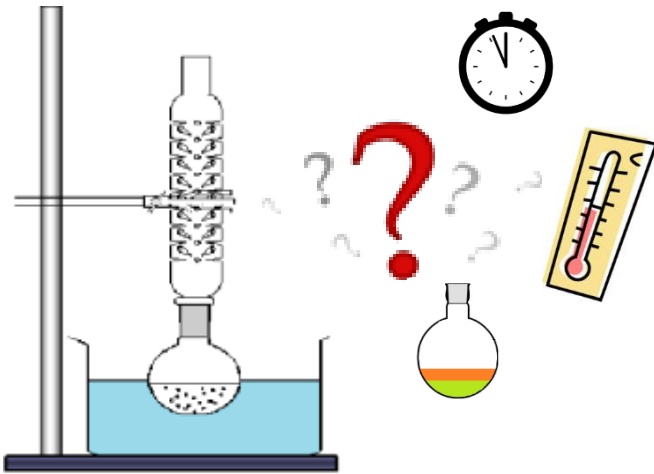
≥ 3 factors ( $X_n$ )

3 levels/factor



Assay	Temperature (°C)	Time (min.)	Solvent concentration (%)
1	0	0	0
2	0	0	0
3	1	0	1
4	0	1	1
5	-1	0	1
6	-1	1	0
7	1	1	0
8	0	0	0
9	-1	-1	0
10	1	-1	0
11	0	1	-1
12	0	0	0
13	0	-1	1
14	-1	0	-1
15	0	-1	-1
16	+1	0	-1
...	0	0	0

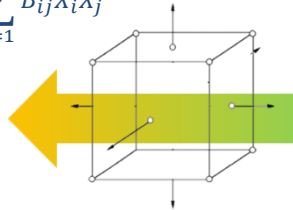
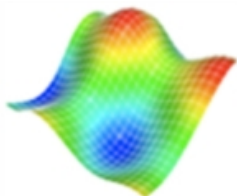
Level	Temperature (°C)	Time (min.)	Solvent concentration (%)
-1	20	10	10
0	50	50	50
1	80	90	90



Assay	Temperature (°C)	Time (min.)	Solvent concentration (%)
1	50	50	50
2	50	50	50
3	80	50	90
4	50	90	90
5	20	50	90
6	20	90	50
7	80	90	50
8	50	50	50
9	20	10	50
10	80	10	50
11	50	90	10
12	50	50	50
13	50	10	90
14	20	50	10
15	50	10	10
16	80	50	10

$$Y = \beta_0 + \sum_{i=1}^3 B_i X_i + \sum_{i=1}^3 B_{ii} X_i^2 + \sum_{i=1}^3 B_{ij} X_i X_j$$

Second-order equation



JMP - MeOH\_Box-Behnken\_Isoflavones\_2015

Fichier Édition Tableaux Lignes Colonnes Plans Analyse Graphique Outils Afficher Fe

MeOH\_Box-Behnken\_Isoflavones\_2015

MeOH\_Box-Behnken\_Iso  
Plans Box-Behnken  
Modèle

Exécuter le script  
Édition  
Supprimer

Configu ration	X1	X2	X3	Y_MeOH _Total
1 000	50	50	50	741,9
2 000	50	50	50	751,2
3 +0+	80	50	90	692,6
4 0++	50	90	90	657,6
5 -0+	20	50	90	677,3
6 --0	20	90	50	769,4
7 ++0	80	90	50	720,1
8 000	50	50	50	726
9 --0	20	10	50	776,6
10 +-0	80	10	50	830,2
11 0+-	50	90	10	286,2
12 000	50	50	50	654,2
13 0-+	50	10	90	646
14 -0-	20	50	10	238,6
15 0--	50	10	10	352
16 +0-	80	50	10	587,5

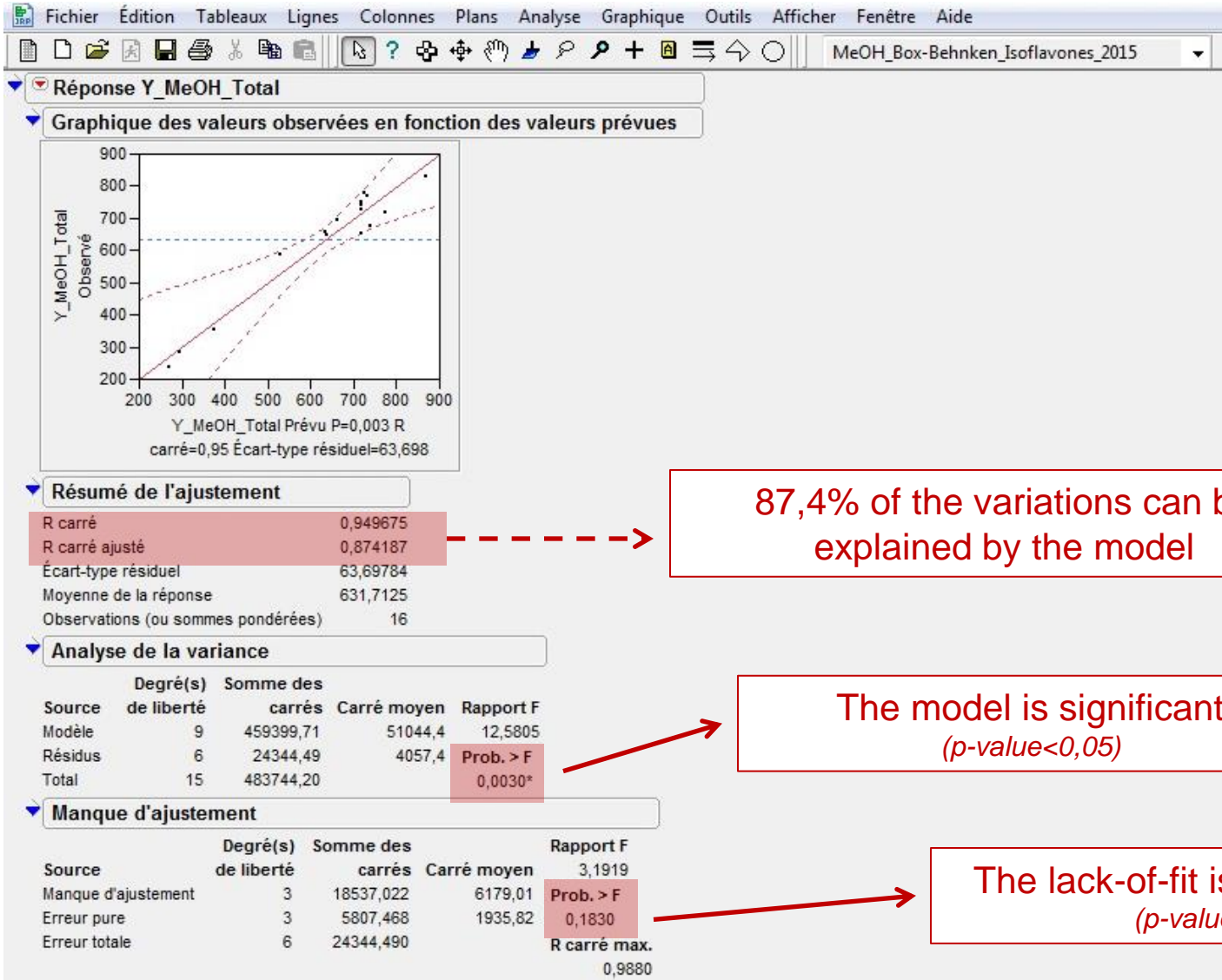
Colonnes (5/0)  
Configuration  
X1 \*  
X2 \*  
X3 \*  
Y\_MeOH\_Total \*

**Factors**  
X1 → Temperature (°C)  
X2 → Time (min.)  
X3 → Solvent concentration (%)

**Results**  
(Isoflavones concentration)

**Matrix**

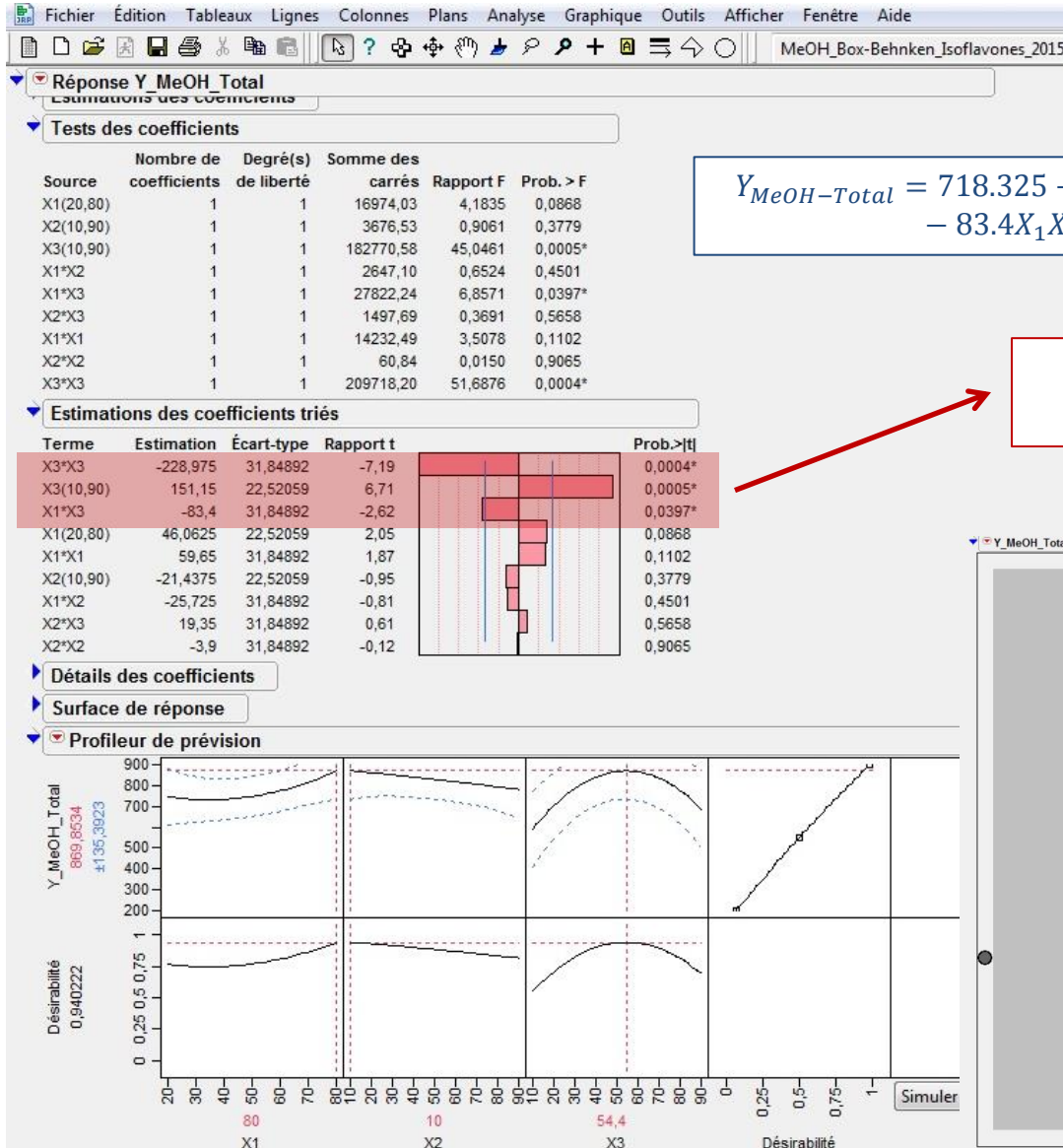




87,4% of the variations can be explained by the model

The model is significant  
(p-value < 0,05)

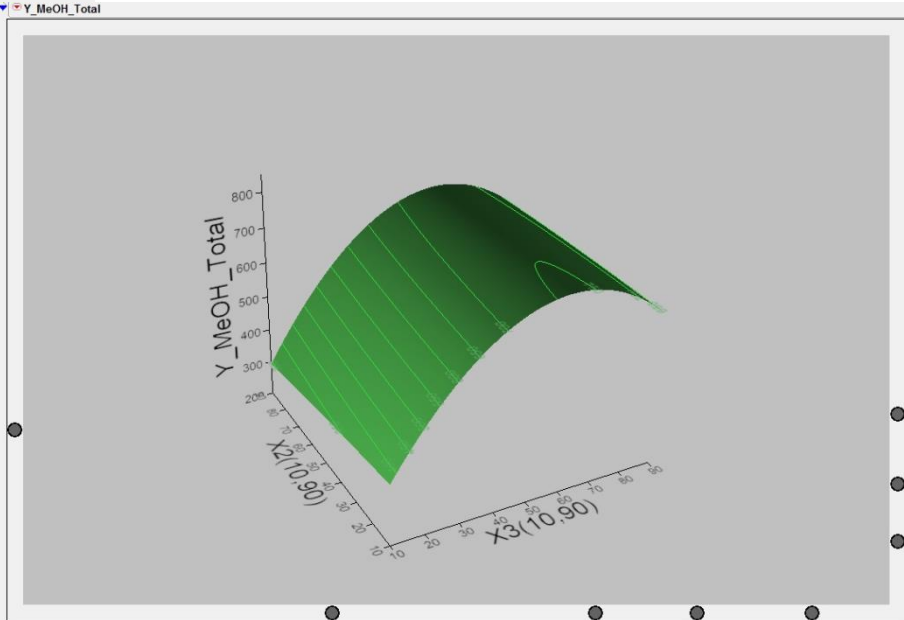
The lack-of-fit is not significant  
(p-value > 0,05)

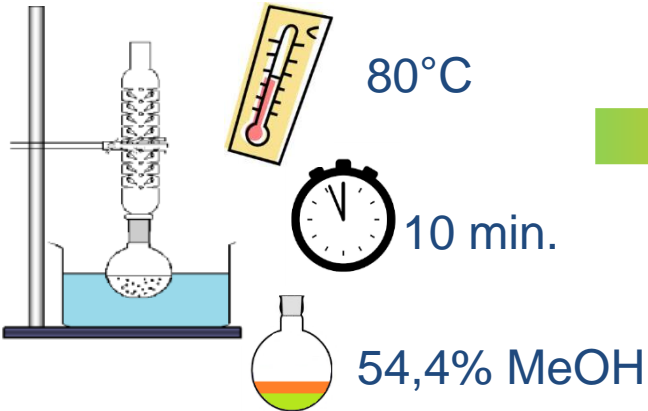


Second-order polynomial model

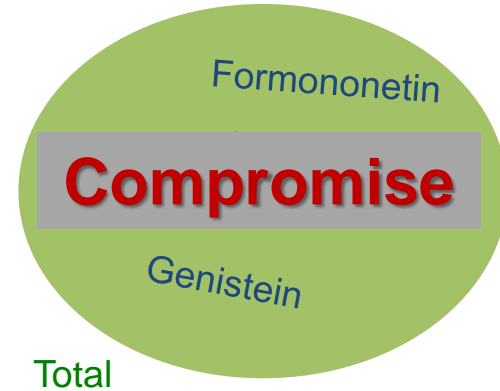
$$Y_{MeOH-Total} = 718.325 + 46.0625X_1 - 21.4375X_2 + 151.15X_3 - 25.725X_1X_2 - 83.4X_1X_3 + 19.35X_2X_3 + 59.65X_1^2 - 3.9X_2^2 - 228.975X_3^2$$

Significant impact  
(p-value < 0,05)





$869,8 \pm 135,4 \mu\text{g}\cdot\text{g}^{-1} \text{MS}$



Compound	Factors →	X <sub>1</sub> Temperature (°C)	X <sub>2</sub> Time (min., sec.)	X <sub>3</sub> Solvent concentration (%)	Y ( $\mu\text{g}\cdot\text{g}^{-1} \text{MS}$ )		
	Conditions ↓				Predicted <sup>l</sup>	Pred. Inter. <sup>m</sup>	Obtained (n=7) <sup>n</sup>
Total	Optimal	80	10	54,4	$869,8 \pm 135,4$	1036,6 – 703,1	<b><math>892,3 \pm 57,4</math></b>
	<b>Selected</b>	<b>80<sup>a</sup></b>	<b>10<sup>b</sup></b>	<b>55<sup>c</sup></b>	<b><math>869,7 \pm 135,6</math></b>	<b>1036,6 – 703,0</b>	
Formononetin	Optimal	80	19,54	50,7	$476,3 \pm 85,9$	619,1 – 333,4	<b><math>517,2 \pm 50,1</math></b>
	<b>Selected</b>	<b>a</b>	<b>b</b>	<b>c</b>	<b><math>474,2 \pm 98,6</math></b>	<b>620,4 – 328,2</b>	
Biochanin A	Optimal	80	10	57,1	$305,1 \pm 45,6$	374,5 – 235,8	<b><math>291,6 \pm 26,8</math></b>
	<b>Selected</b>	<b>a</b>	<b>b</b>	<b>c</b>	<b><math>304,9 \pm 45,4</math></b>	<b>360,8 – 249,0</b>	
Genistein	Optimal	80	90	68,4	$66,0 \pm 6,7$	73,9 – 58,1	<b><math>62,9 \pm 6,2</math></b>
	<b>Selected</b>	<b>a</b>	<b>b</b>	<b>c</b>	<b><math>63,6 \pm 6,4</math></b>	<b>71,4 – 55,7</b>	
Daidzein	Optimal	80	29,44	60,7	$19,2 \pm 2,7$	23,3 – 15,1	<b><math>20,7 \pm 2,3</math></b>
	<b>Selected</b>	<b>a</b>	<b>b</b>	<b>c</b>	<b><math>18,9 \pm 3,5</math></b>	<b>23,2 – 14,6</b>	

<sup>l</sup> Predicted content  $\pm$  half confidence interval ( $\alpha=0.05$ ), <sup>m</sup> Prediction interval of 95%, <sup>n</sup> Mean value  $\pm$  standard deviation

## Sample pretreatments

*(Grinding, homogenization & freeze-drying)*

## Ultrasound Assisted Extraction

*(80°C, 10 min & 55% MeOH)*

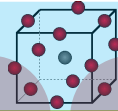
## Enzymatic hydrolysis

*(20°C, 18 h & pH 6)*

## Post-hydrolysis steps

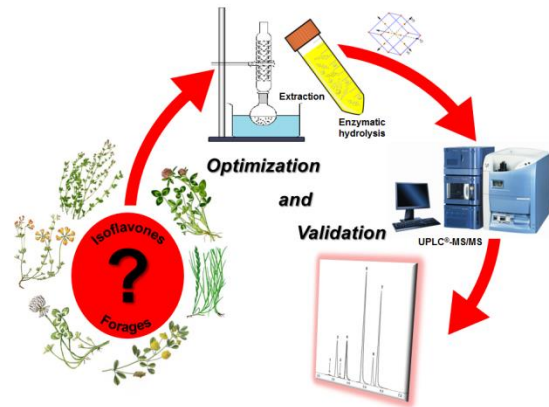
*(Dilution, evaporation, solubilization of dry residues & filtration)*

## UPLC<sup>®</sup>-MS/MS analysis



EMA VICH GL49 ([http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2011/04/WC500105053.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/04/WC500105053.pdf))

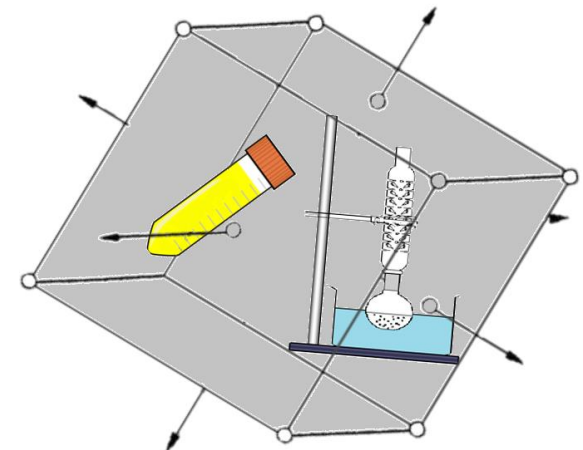


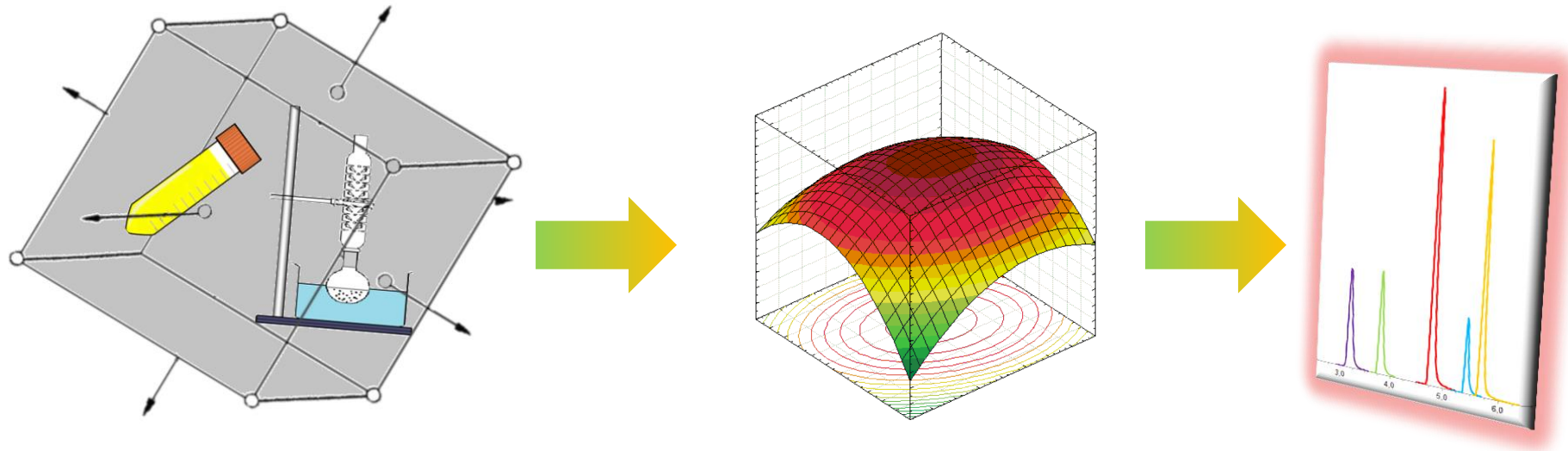


➤ This methodology allows to propose a reliable and robust analytical method for simultaneous quantification of isoflavones in forages. (“Quality”)

➤ To obtain a maximum of information with a small number of assays → allowing to find the optimal conditions for UAE and hydrolysis process among the numerous and divergent conditions proposed in literature.

Experimental design





## Acknowledgements:

- Public Service of Wallonia (PhytoHealth project, Moerman funds)
- Members of PhytoHealth project, C. Rasse (SMCS-IMMAQ, UCL) and C. Jasselette