Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **26** (6): 904-8 (2007)

Notas técnicas

Recibido el 14 de abril de 2007
Aceptado el 11 de agosto de 2007

Use of Central Composite Design to Evaluate the Robustness of a LC-Method

Luiz Alberto Lira SOARES [1]*, Tatiane Pereira DE SOUZA [1],
George GONZÁLEZ ORTEGA [2] & Pedro Ros PETROVICK [2]

[1] *PPGCF - Departamento de Farmácia - Universidade Federal do Rio Grande do Norte, Avenida General Cordeiro de Farias s/n, 59010-180, Natal, RN, Brasil.*

[2] *PPGCF - Faculdade de Farmácia - Universidade Federal do Rio Grande do Sul, Avenida Ipiranga 2752, 90610-000, Porto Alegre, RS, Brasil.*

SUMMARY. The purpose of this work was to evaluate the preliminary robustness of a RP-LC methodology developed and validated for separation and quantification of catechin and epicatechin. The robustness was investigated by a central composite design, where the concentration of acetic acid solution and the initial proportion of acetonitrile were the independent variables, the dependent variables were the retention times, capacity factor and resolutions of both peaks. The analysis was performed using a C_{18} column with acid acetic-acetonitrile mobile phase employing gradient elution and detection by UV at 280 nm. Except for the resolution of epicatechin other responses showed a similar performance. While the initial proportion of acetonitrile significantly influences all dependent variables decreasing the responses as the concentration of acetonitrile is higher„ the acid concentration has a minor influence.

RESUMEN. "Utilización del Diseño Central Compuesto para Evaluar la Robustez de un Método de CLAR". El objetivo del presente trabajo fue evaluar la robustez del método CLAR-fase invertida, previamente validado, para separación y cuantificación de catequina y epicatequina. La robustez fue investigada por medio de un diseño central compuesto, las variables independientes fueron concentración de ácido acético y proporciones iniciales de acetonitrila y las variables dependientes fueran el tiempo de retención, el factor de capacidad y la resolución de ambos picos. El ensayo fue realizado utilizando una columna C_{18} y ácido acético-acetonitrilo como fase móvil, sistema de elución gradual y detección UV en 280 nm. Todas las respuestas mostraron un comportamiento similar, excepto la resolución de la epicatequina. La proporción de acetonitrilo influenció todas las variables dependientes significantemente, disminuyendo las respuestas en el caso de altos niveles. La concentración de ácido acético presentó menor influencia en las repuestas.

INTRODUCTION

The *robustness/ruggedness* of an assay method can be described as the degree of reproducibility of the results obtained by analyses of the same sample under a variety of normal test conditions, such as different laboratories, analysts, equipment, lots of reagents, temperatures, and days [1]. This kind of test was initially developed to avoid variations in interlaboratory studies and to identify the responsible factors. Therefore, the robustness tests have been performed at a late stage of the method validation, since the interlaboratory study is the last step. However, when the method is found to be not robust it should then be redesigned and optimized in order to assure accurate results. Thus, a preliminary evaluation of the robustness of a method should be performed at the early stage of its validation or at the end of its development [2-4].

The factorial designs are widely used tools for the systematic and effective evaluation of influences from differences among variables such as proportion of excipients in formulations [5], operational parameters [6] or reactional conditions [7]. Among the optimization designs, the central composite design (CCD) is the most employed second-order design to evaluate and optimize experimental conditions [8]. Additionally, with CCD it is possible to create response surfaces, which allow for the ranking of each variable according to its significance in the studied responses [9]. These characteristics suggest the ability of this kind of design for the evaluation and

KEY WORDS: HPLC, Catechin, Composite Central Design, Epicatechin, Robustness.
PALABRAS CLAVE: Catequina, CLAR, Diseño Central Compuesto, Epicatequina, Robustez.

* Author to whom correspondence should be addressed. *Email:* phtech@uol.com.br

ISSN 0326-2383

quantification of method variations in the robustness test.

There are many variables that can influence the performance of a LC analysis, such as the pH of the mobile phase; amount of organic modifier; buffer concentration; salt concentration or ionic strength; concentration of additives (ion pairing agents, competing amine); flow rate; column temperature; elution gradient (initial and final mobile phase composition, slope of gradient); column factor (batch, manufacturer, age of the column); detector factor (wavelength); and integration factor (sensitivity) [4].

Thus, the purpose of this work was to use a central composite design to evaluate the influence of the mobile phase composition in the responses of the RP-LC method for separation and quantification of catechin and epicatechin.

METHOD

Chemicals and Solvents

(+)-catechin and (-)-epicatechin were purchased from Sigma (St. Louis, MO). The mobile phase was prepared with LC grade solvents. It was composed by acetonitrile (Merck, Darmstat, Germany), water (Milli-Q system, Millipore, Bedford, MA) and acetic acid (ExtraSyntese, São Paulo, Brazil).

LC system

The analysis was carried out in a LC-10A Shimadzu liquid chromatograph equipped with a LC-10AD pump, a SPD-10A UV/VIS-detector, a SIL-10A auto sampler and CLASS-LC10 software (Shimadzu, Kyoto). A Nova-Pak C_{18} RP-column 150 mm x 39 mm i.d., 60 Å (Waters, Milford, MA) protected by a Shimadzu pre-column (10 mm x 4 mm i.d.) packed with Bondapak C_{18} 125 Å (Waters, Milford, MA) was used throughout this study. The peaks were detected at 280 nm. After filtration (0.44 µm, Millipore, Bedford, MA) and degassing with helium, an elution gradient was performed utilizing a dual valve system (FVC-10AL, Shimadzu, Kyoto) varying the proportion from solvent A (acetonitrile:water:solution of acetic acid; 50/49.7-48.3/0.3-1.7; v/v/v) to solvent B (solution of acetic acid - 0.3 to 1.7%) at a flow rate of 1.5 ml/min. The gradient program is summarized in Table 1.

Time (min)	Solvent A (%)	Solvent B (%)
0	8.97 - 16.4	4.48 - 8.02
5	20.0	10.0
8	30.0	15.0
17	45.0	22.5
20	initial	initial

Table 1. Gradient elution program. (**A** = aceto-nitrile:water:solution of acetic acid; 50/49.7-48.3/0.3-1.7; v/v/v) (**B** = solution of acetic acid - 0.3 to 1.7%).

Method Evaluation

Calibration curves

Aqueous solutions of catechin and epicatechin were prepared in the following concentrations: 16, 24, 32, 40, 50 and 80 µg/ml, and 32, 48, 64, 80, 100 and 160 µg/ml, respectively. The solutions were filtered through a 0.45 µm membrane (Millipore-HVHP, Bedford, MA). The calibration curves were analyzed by linear regression and the results represented the average of three curves performed by three injections of each concentration [10].

Linearity

The linearity of the curves was estimated by regression using the least square method. The slope, intercept (with respective confidence intervals) and coefficient of determination (R^2) were calculated and evaluated [11].

Statistical analysis

The individual data were grouped after each experiment. The mean with the respective deviation was used as a measurement of the central tendency and dispersion (relative standard deviation - RSD %) [11].

Experimental design

The experimental matrix was a 2^2 factorial design augment with 3 central points and 4 star points (Table 2). The factors selected in this study were acetic acid concentration and the initial proportion of acetonitrile in the mobile phase (solvent A). The gradient program was kept and, the response variables were the retention time (RT), resolution (RES) and capacity factor (K') for catechin and epicatechin peaks. The central composite design was adjusted to a polynomial second-order (**Eq. 1**) by the PLS method [8,9,12]. The response surfaces were obtained using the STATISTICA 6.0 software (Statsoft, USA).

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}(x_1)^2 + b_{22}(x_2)^2 \quad \textbf{(Eq. 1)}$$

where: y is the response (retention time, resolution or K'), and b_0 to b_{22} are the regression coefficients.

Experiments (%)	Acetonitrile * (%)	CH_3COOH (%, v/v)	Acetonitrile * (%)	CH_3COOH (%, v/v)
1	-1	-1	5.00	0.5
2	-1	+1	5.00	1.5
3	+1	-1	7.50	0.5
4	+1	+1	7.50	1.5
5	0	0	6.25	1.0
6	0	0	6.25	1.0
7	0	0	6.25	1.0
8	- 1.414	0	4.48	1.0
9	0	- 1.414	6.25	0.293
10	+ 1.414	0	8.02	1.0
11	0	+ 1.414	6.25	1.707

Table 2. Central composite design matrix (coded and natural variables). *Final concentration after mixture of the phases.

RESULTS

The chromatogram obtained for both peaks of catechin and epicatechin are presented in Fig. 1.

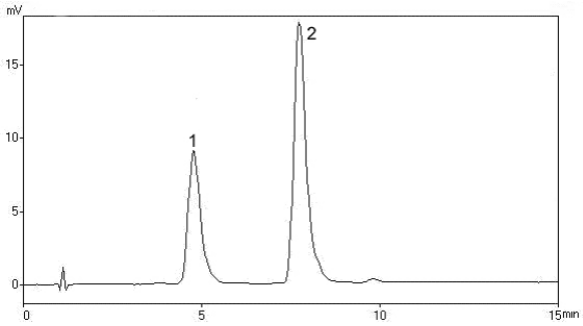

Figure 1. Chromatogram of peaks from catechin (**1**) and epicatechin (**2**) detected at 280 nm.

The regression analysis was performed for each calibration curve and the resulting parameters are summarized in Table 3. The coefficients of determination for standard curves were higher than 0.999. Thus, the calculated straight line could explain more than 99% of the experimental data. The confidence intervals for both intercept points included zero. Therefore, the result confirms the absence of constant systematic errors and the capacity of the method.

Regression parameters	(+)-Catechin	(-)-Epicatechin
Intercept	-22361.33	-20701.10
(Confidence intervals)	(-45945.83 to 1223.17)	(-41850.10 to 447.85)
Slope	8884.74	7894.64
R^2	0.9996	0.9999

Table 3. Linear regression analysis data for both catechin and epicatechin. Reprinted from Soares *et al*. [10] with permission from Elsevier.

The results for retention time, resolution and capacity factor obtained from the central composite design for both catechin and epicatechin are shown in Table 4.

Experiments	Acetonitrile* (%)*	CH_3COOH (%, v/v)	Catechin RT	Catechin RES	Catechin K'	Epicatechin RT	Epicatechin RES	Epicatechin K'
1	5.00	0.5	6.57	13.77	5.07	9.49	5.34	7.74
2	5.00	1.5	4.85	9.81	3.95	8.04	5.26	7.22
3	7.50	0.5	4.33	8.84	3.11	7.01	4.40	5.65
4	7.50	1.5	3.24	6.99	2.35	5.52	3.92	4.47
5	6.25	1.0	4.46	9.35	3.56	7.40	9.35	6.56
6	6.25	1.0	4.46	9.38	3.56	7.39	9.38	6.56
7	6.25	1.0	4.50	9.58	3.61	7.41	9.58	6.59
8	4.48	1.0	5.84	13.88	4.89	8.59	5.24	7.67
9	6.25	0.293	5.43	12.11	4.36	8.36	5.25	7.26
10	8.20	1.0	3.51	7.15	2.60	6.23	4.03	6.23
11	6.25	1.071	4.15	8.69	2.89	7.01	4.61	5.57
Peak area (RSD %)			207693.89 (3.8 %)			411075.00 (3.93%)		

Table 4. Results for retention times (RT), resolution (RES) and capacity factor (K') for catechin and epicatechin, respectively. *Final concentration after mixture of the phases.

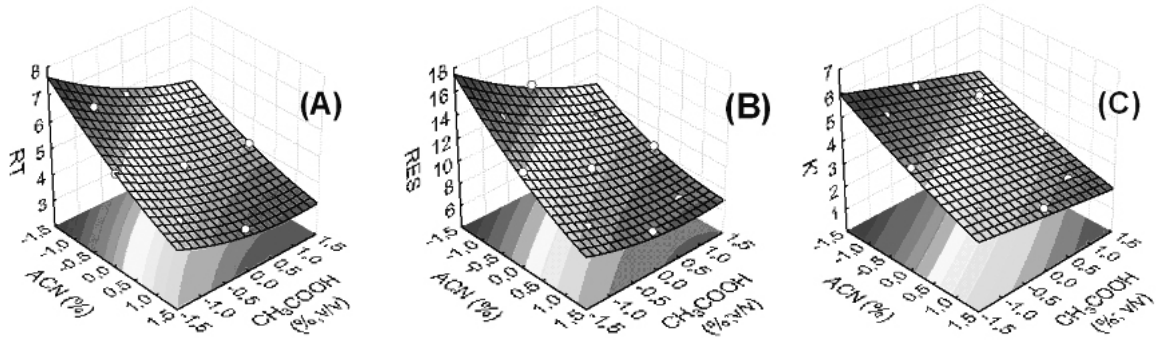

Figure 2. Response surfaces for retention times (**A**); resolution (**B**) and capacity factor (**C**) for the peak of catechin.

	Retention times (min)		Resolution		Capacity factor	
Variables	**Coeff.**	**t-test**	**Coeff.**	**t-test**	**Coeff.**	**t-test**
b_0	4.47333	42.6574*	9.43667	32.4015*	3.57667	82.8165*
b_1	-1.78628	-13.9081*	-4.31691	-12.1025*	-1.69964	-32.1328*
b_{11}	0.20917	1.3683	0.77583	1.8274	0.13583	2.1576
b_2	-1.15505	-8.9933*	-2.66165	-7.4619*	-0.98972	-18.7114*
b_{22}	0.32417	2.1206	0.66083	1.5565	0.01583	0.2515
b_{12}	0.31500	1.7343	1.05500	2.0914	0.18000	2.4063
R^2	0.982	-	0.976	-	0.996	-

Table 5. Summary of the regression results for the peak of catechin. *Significant for α = 0.05.

Catechin

The proposed mathematical models showed good multiple correlation coefficients (R^2). For all studied responses the calculated multiple correlation coefficients indicated that more than 95% of the experimental variance could be explained by the proposed equations. Concerning the response behavior (retention times, resolution and capacity factor), similarities among profiles (surface responses) were observed (Fig. 2).

A decrease on the studied responses occurred when the proportions of acetonitrile and acetic acid increases in the mobile phase. The statistical analysis of the factors showed that only linear terms were important (Table 5). However, at a higher concentration of acetic acid the influence of acetonitrile mixture decreases.

Epicatechin

According to the multiple correlation coefficients (R^2), the mathematical models used to explain the epicatechin peak profiles were adequate. However, in this case the independent variables showed different profiles for each studied response (Fig. 3). Thus, while the retention time of epicatechin showed a profile similar to catechin (Fig. 2), which decrease with the increased concentrations of acetonitrile and acetic acid; the resolution clearly showed an optimum condition. On the other hand, the capacity factor showed a different behavior if compared with the other one. This response undergoes lower influence of both independent variables. However, a decrease in the response could be observed when both variables were at higher levels (Table 6).

CONCLUSION

The evaluation of the robustness of the LC method studied on this work was performed successfully using a central composite design and response surface analysis. This technique seems to be a helpful statistical tool to explore, evaluate and quantify the main factors that could induce variations in this analytical method. To conclude, the variations in both mobile phase components in the peaks parameters were satisfactorily evaluated and the proportion of acetonitrile mixture was the main factor.

Acknowledgement. This work was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil).

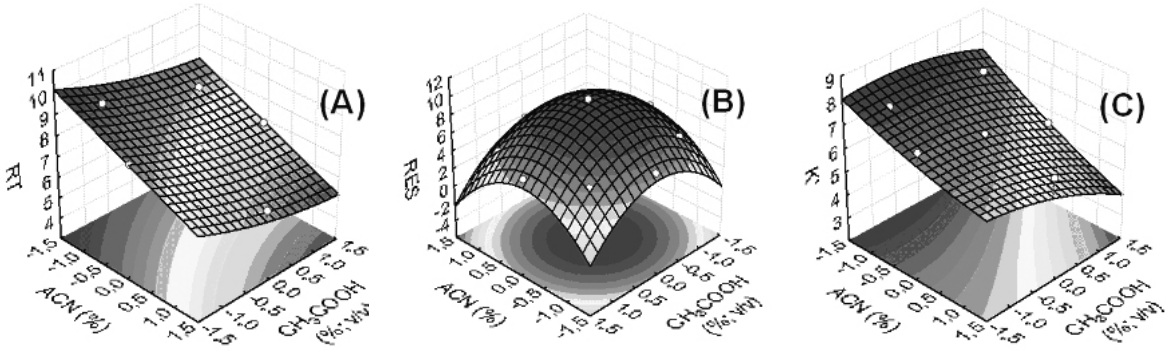

Figure 3. Response surfaces for retention times (**A**); resolution (**B**) and capacity factor (**C**) for the peak of epicatechin.

	Retention times (min)		Resolution		Capacity factor	
Variables	**Coeff.**	**t-test**	**Coeff.**	**t-test**	**Coeff.**	**t-test**
b_0	7.400	41.341*	9.437	120.425*	6.570	21.638*
b_1	-2.085	-9.508*	-0.998	-10.397*	-1.719	-4.623*
b_{11}	-0.007	-0.024	-4.828	-42.265*	0.174	0.393
b_2	-1.212	-5.529*	-0.366	-3.816*	-1.023	-2.749*
b_{22}	0.269	1.030	-4.533	-39.682*	-0.361	-0.816
b_{12}	-0.020	-0.065	-0.200	-1.474	-0.330	-0.627
R^2	0.961	-	0.998	-	0.860	-

Table 6. Summary of the regression results for the peak of epicatechin. *Significant for $\alpha = 0.05$.

REFERENCES

1. Van der Heyden, Y., M. Jimidar, E. Hund, N. Niemeijer, R. Peeters, J. Smeyers-Verbeke, D.L. Massart & J. Hoogmartens (1999) *J. Chromatogr. A* **845**: 145-154.
2. Chow, S.C. & J.P. Liu (1995) *"Statistical Design and Analysis in Pharmaceutical Science: Validation, Process Controls and Stability",* Marcel Dekker, New York.
3. Nijhuis, A., H.C.M.Van Der Knaap, S. De Jong & B.G.M. Vandeginste (1999) *Anal. Chim. Acta* **391**: 187-202.
4. Van der Heyden, Y., A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste & D.L. Massart (2001) *J. Pharm. Biom. Anal.* **24**: 723-53.
5. Soares, L.A.L., G. González Ortega, P.R. Petrovick, & P.C. Schmidt (2005) *AAPS PharmSciTech* **6**: E367-71.
6. Soares, L.A.L., Schmidt, P.C., González Ortega, G. & P.R. Petrovick (2003) *Acta Farm. Bonaerense* **22**: 147-54.
7. Petry, R.D. , G. González Ortega & W.B. Silva (2001) *Pharmazie* **56**: 465-70.
8. Box, G., W. Hunter & J. Hunter (1978) *"Statistics for Experimenters: an Introduction to Design, Data Analysis and Model Building",* Wiley, New York.
9. Myers, R.H. & D.C. Montgomery (1995) *"Response Surface Methodology: Process and Product Otimization Using Designed Experiments",* Wiley, New York.
10. Soares, L.A.L., A.L. Oliveira, G. González Ortega & P.R. Petrovick (2004) *J. Pharm. Biom. Anal.* **36**: 787-790.
11. Kromidas, S (1999) *"Validierung in der Analytik".* Wiley-VCH, Weinheim.
12. Otto, M. (1999) *"Chemometrics: Statistics and Computer Application in Analytical Chemistry",* Wiley-VCH, Weinheim.