

Spectrophotometric determination of the total flavonoid content in *Ocimum basilicum* L. (Lamiaceae) leaves

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ABSTRACT

Background: The chemical characterization is essential to validate the pharmaceutical use of vegetable raw materials. Ultraviolet spectroscopy is an important technique to determine flavonoids, which are important active compounds from *Ocimum basilicum*. **Objective:** The objective of this work was to optimize a spectrophotometric method, based on flavonoid-aluminum chloride (AlCl_3) complexation to determine the total flavonoid content (TFC) in leaves of *O. basilicum* (herbal material), using response surface methodology. **Materials and Methods:** The effects of (1) the herbal material: Solvent ratio (0.02, 0.03, 0.05, 0.07, and 0.08 g/mL), (2) stock solution volume (0.8, 2.3, 4.4, 6.5, and 8.0 mL) and (3) AlCl_3 volume (0.8, 1.0, 1.2, 1.4, and 1.6 mL) on the TFC were evaluated. The analytical performance parameters precision, linearity and robustness of the method were tested. **Results:** The herbal material: Solvent ratio and stock solution volume showed an important influence on the method response. After choosing the optimized conditions, the method exhibited a precision (RSD%) lower than 6% for repeatability (RSD%) and lower than 8% for intermediate precision (on the order of literature values for biotechnological methods), coefficient of correlation of 0.9984, and no important influence could be observed for variations of the time of complexation with AlCl_3 . However, the time and temperature of extraction were critical for TFC method and must be carefully controlled during the analysis. **Conclusion:** Thus, this study allowed the optimization of a simple, fast and precise method for the determination of the TFC in leaves of *O. basilicum*, which can be used to support the quality assessment of this herbal material.

Key words: Flavonoids, *Ocimum basilicum*, response surface, spectrophotometry

INTRODUCTION

Ocimum basilicum L. (Lamiaceae), known in the traditional medicine as Manjerição and Basil,^[1-3] has biologically active constituents associated with antiulcerogenic,^[4] antibacterial, antifungal effects.^[4-7] Flavonoids, including nevodensin, salvigenin, cirsiolol, eupatorin, apigenin, acacetin, cirsimaritin, quercetin and ladanein, are among the main secondary metabolites identified in Basil,^[8,9] besides the high essential oils content.^[1-6]

The analysis of the content of main active components in raw materials and phytomedicines is an essential step to evaluate the quality of products and validate the efficacy and safety of their therapeutic use.^[10] A widely used

technique for the quantification of flavonoids is based on its complexation with aluminum chloride (AlCl_3) and the spectrophotometric determination of the formed complex, which provides a bathochromic displacement and the hyperchromic effect. In this method, several factors are crucial for the formation of the flavonoid- AlCl_3 complexes and must be optimized to improve the method performance such as the reaction time, the complexant concentration, flavonoid: Herbal material ratio and the chemical structure of the polyphenol. Thus, a multivariate approach can be used to evaluate the effect of the critical variables on the selected response. Experimental designs are multivariate tools used for the systematic and effective evaluation of simultaneous modifications of several variables in a specific system.^[11]

Response surface methodology (RSM) is a set of mathematical and statistical techniques useful for designing experiments, building models and analyzing the effects of several independent factors. RSM comprises the evaluation of empirical models fitted to experimental data.

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The advantages of its application on the optimization of analytical procedures are widely disseminated, since the design of polynomial experiments allow testing, simultaneously, several factors with potential influence on the response, producing large amounts of information from a relatively small number of experiments.^[12]

The objective of this work was to optimize a spectrophotometric method based on flavonoid-AlCl₃ complexation to determine the total flavonoids content in leaves of *O. basilicum*, using RSM.

MATERIALS AND METHODS

Material

Plant material

The dried and ground leaves of *O. basilicum* L. of $d\varnothing \leq 0.500$ mm (herbal material) used in the experiments were obtained from specimens collected on August 2011, in Joinville, SC, Brazil. The voucher specimen deposited in the herbarium Joinvillea (number 8234) was taxonomically identified.

Chemicals and reagents

The standard of quercetin dehydrate ($\geq 98\%$ HPLC) was purchased from Sigma Co. (St. Louis, MO, USA). Absolute ethanol from Labmaster (Pinhais, PR, Brazil), methanol from Cromato Produtos Químicos (Diadema, SP, Brazil), AlCl₃ hexahydrate from Nuclear (Diadema, SP, Brazil) pro analysis grade. Distilled water was prepared with distiller Quimis Q-341 (Diadema, SP, Brazil).

Instrumentation

Spectrophotometric measurements were performed on an ultraviolet (UV)-1601PC UV/visible (UV-Vis) spectrophotometer (Shimadzu, Kyoto, Japan) equipped with 1 cm quartz cells.

Methods

Wavelength selection

For the determination of the maximum absorption, the herbal material: 60% ethanol (v/v) extract (0.08 g/mL) was subjected to a general procedure for flavonoid quantification (sub-section General method for total flavonoids content [TFC] determination).^[13] The UV-Vis spectra (300-500 nm) were obtained in a spectrophotometer after complexation with AlCl₃ 2% (w/v). The same sample without AlCl₃ was used as a blank solution.

Optimizing the determination of the TFC

Conditions for the optimal TFC were determined by RSM, which was performed using the software Design-Expert[®] 8.0.6 from Stat-Ease, Inc. (Minneapolis, MN, USA). A central composite design (CCD) was used to investigate the effects of three independent variables, which are coded as (A) the

herbal material: Ethanol ratio (w/v), (B) the volume of stock solution and (C) the AlCl₃ volume, on the dependent variable (TFC in mg/g herbal material). The data obtained from the CCD was fitted to a second order polynomial equation. The generalized nonlinear computer-generated quadratic model is given as Equation 1.

$$Y = \beta_0 + \sum_{i=0}^3 \beta_i \chi_i + \sum_{j=0}^3 \beta_{ii} \chi_i^2 + \sum_{i=0}^3 \sum_{j=0}^3 \beta_{ij} \chi_i \chi_j \quad (1)$$

where, Y is the predicted response associated with each factor level combination; β_0 is the intercept; β_i is the linear coefficient; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient; and X_i and X_j are the coded levels of independent variables. The terms $X_i X_j$ and X_i^2 represent the interaction and quadratic terms, respectively. From preliminary experiments, the levels of the independent variables were selected: (A) 0.02, 0.03, 0.05, 0.07, and 0.08 g/mL; (B) 0.8, 2.3, 4.4, 6.5, and 8.0 mL; (C): 0.8, 1.0, 1.2, 1.4 and 1.6 mL. The experimental conditions for CCD and the TFC results are presented in Table 1. The experimental design required 17 experiments, including five replicates at the center point to estimate the overall error. The experiments were performed in random order to avoid systematic error. All trials were carried out in triplicate and the data were reported as means \pm standard deviation (SD).

The adequacy of the model was determined by evaluating the lack of fit, coefficient of regression (R^2) and the Fisher

Table 1: Randomization of the experimental design for the optimization of TFC in herbal material (leaves of *O. basilicum*) and the obtained results

Independent variables			Response (TFC) ^a
A	B	C	
0.05	8.0	1.2	0.8701 \pm 0.0357
0.05	4.4	1.2	0.7307 \pm 0.0634
0.07	6.5	1.0	0.5745 \pm 0.0446
0.03	2.3	1.0	1.1195 \pm 0.0523
0.03	6.5	1.0	0.8978 \pm 0.0412
0.03	6.5	1.4	0.7437 \pm 0.2914
0.05	0.8	1.2	1.6167 \pm 0.1329
0.05	4.4	1.6	0.7120 \pm 0.0376
0.07	6.5	1.4	0.6630 \pm 0.0629
0.02	4.4	1.2	1.1302 \pm 0.0019
0.05	4.4	1.2	0.7267 \pm 0.0778
0.07	2.3	1.4	0.6968 \pm 0.0600
0.03	2.3	1.4	0.8510 \pm 0.1940
0.05	4.4	0.8	0.6633 \pm 0.0571
0.08	4.4	1.2	0.4556 \pm 0.0496
0.07	2.3	1.0	0.7101 \pm 0.0756
0.05	4.4	1.2	0.7110 \pm 0.0410

^aValues expressed in mg of flavonoids/g of herbal material (mean \pm SD, n=3). A: Herbal material; Solvent ratio; B: Stock solution volume; C: AlCl₃ volume; TFC: Total flavonoid content; SD: Standard deviation; *O. basilicum*: *Ocimum basilicum*; AlCl₃: Aluminum chloride

test value (*F*-value) obtained from the analysis of variance (ANOVA) considering $P < 0.05$ as significant. The software uses the quadratic model equation shown above to generate response surfaces. Three-dimensional response surface plots were generated by keeping one response variable at its optimal level and plotting it against two factors (independent variables).

General method for total flavonoid content determination

The herbal material was extracted under reflux conditions (80 °C) with 20.0 mL water-ethanol solution 60% (v/v) (pH = 5.06) during 60 min. The extract was cooled to room temperature and filtered. The residue was re-extracted under equivalent conditions. Both hydroalcoholic extract and re-extract were combined, and the volume was completed to 50 mL of water-ethanol solution 60% (v/v), resulting in the stock solution.

An aliquot of the stock solution was transferred to a 10.0 mL volumetric flask and made to volume with methanol, resulting in the blank solution. A second aliquot of the stock solution was transferred to another 10.0 mL volumetric flask, a volume of the 2% AlCl₃ was added and made to volume with methanol, which was named test solution. After 25 min the absorbance of the test solution was measured at 430 nm against blank solution.

TFC_{herbal material} results were calculated, as quercetin, using Equation 2, and it represents the average of three-determinations. The results were expressed as the amount of flavonoid (mg)/g of herbal material (corrected for moisture content).

$$\text{TFC}_{\text{herbal material}} = \frac{\text{TFC}_{\text{test solution}} \times 1.25 \times 50}{w - ld} \quad (2)$$

where TFC_{test solution} is the total concentration of flavonoids in the test solution (mg/mL), 1.25 corresponds to the dilution factor, 50 is the volume of the stock solution (mL), *w* is the mass of herbal material (g), and *ld* is the loss on drying of herbal material.

Calibration curve and linearity

Standard solution of quercetin in methanol (0.050 mg/mL) was prepared in triplicate and diluted to concentrations of 0.001, 0.006, 0.011, 0.016, and 0.021 mg/mL. The absorbance of diluted solutions was measured at 430 nm 25 min after adding AlCl₃ against the blank solution. The calibration curve was constructed by plotting the measurements of mean absorbance versus the concentration of the standard solutions. The results were analyzed by linear regression and the correlation coefficient (R^2) was calculated GraphPad Prism 5.00 from

GraphPad Software, Inc. (La Jolla, CA, USA). One-way ANOVA was calculated to compare the replicates of the calibration curve.

Precision

The intra-day and inter-day variabilities were studied to assess the precision of the method. Intra-day variability was determined by analyzing within 24 h. TFC was measured 6 times under the optimized method conditions and the relative standard deviation (RSD%) was considered a measure of repeatability. Inter-day variability was determined by performing the analysis in sextuplicate by two analysts in four different days (analyst A performed the analysis in days 1, 2, and 3, while analyst B performed the analysis in day 4), the RSD% being considered a measure of intermediate precision.

Robustness

Robustness was evaluated by the variation of the following conditions of the TFC method: Time of complexation with AlCl₃ solution (15 and 35 min), time of extraction of herbal material (50 and 70 min) and temperature of extraction (70 °C and 90 °C). The results were evaluated by two-way ANOVA followed by Bonferroni *post-hoc* test, with a confidence level of 95% (GraphPad Prism® 5.00).

RESULTS AND DISCUSSION

Wavelength selection

The UV-spectra for herbal material and standard quercetin after reaction with AlCl₃ are presented in Figure 1. Both UV-profiles observed for the tested samples presented a maximum at 430 nm, defining this wavelength for the TFC analysis.

Optimizing the determination of the total flavonoid content

Table 1 shows the experimental design employed and the corresponding TFC results. Figure 2 illustrates

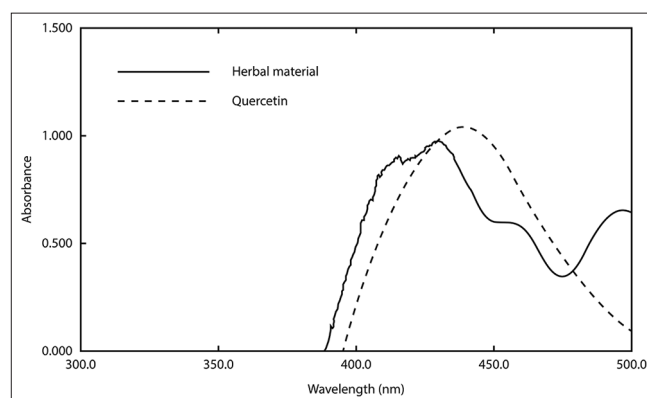


Figure 1: Spectrum for aluminum chloride-flavonoid complexes: Herbal material (leaves of *Ocimum basilicum*) and standard (quercetin)

response surface plots showing the effect of herbal material: 60% ethanol ratio and volume of stock solution on TFC response. The results of ANOVA for TFC with corresponding coefficients of multiple determination (R^2) for leaves of *O. basilicum* are shown in Table 2. The model was adequate and explained most of the observed variability. For the model fitted, the software generates model coefficients, R^2 -values, F -values and significant probabilities and hence one can justify the significance of each experimental variable. The maximum predictable response for TFC was obtained based on a total of 17 experiments required for determining 12 regression coefficients of the model [Table 2]. A plot of experimental and theoretical values indicated an excellent fit of the model ($F = 6.93$; $P = 0.126$; $R^2 = 0.9985$).

The data listed in Table 1 were converted into a second-order polynomial equation with three independent

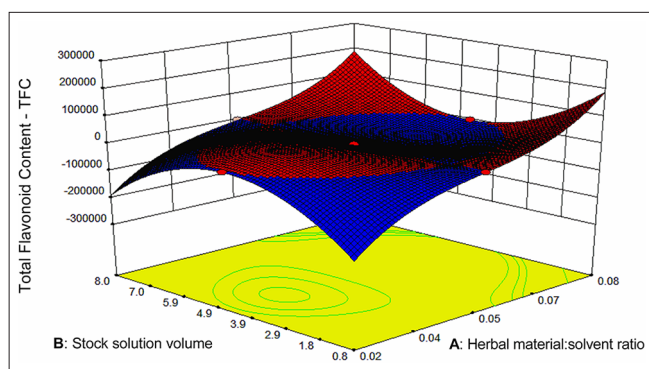


Figure 2: Response surface (three-dimension) showing the effect of factors (high aluminum chloride volume as fixed factor; (a) Herbal material: Solvent ratio; (b) Stock solution volume) added on TCF

Table 2: Analysis of variance for response surface of reduced quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F value	P>F
Model	1.14	12	0.095	221.74	<0.0001
A	0.079	1	0.079	184.30	0.0002
B	0.28	1	0.28	648.39	<0.0001
C	1.186×10 ⁻³	1	1.186×10 ⁻³	2.76	0.1720
AB	0.082	1	0.082	191.63	0.0002
AC	0.031	1	0.031	72.06	0.0011
BC	5.843×10 ⁻³	1	5.843×10 ⁻³	13.59	0.0211
A ²	0.054	1	0.054	124.64	0.0004
B ²	0.27	1	0.27	623.09	<0.0001
A ² B	0.084	1	0.084	196.54	0.0002
A ² C	0.011	1	0.011	25.85	0.0071
AB ²	0.079	1	0.079	184.30	0.0002
A ³	0.079	1	0.079	184.29	0.0002
Residual	1.719×10 ⁻³	4	4.298×10 ⁻⁴		
Lack of fit	1.502×10 ⁻³	2	7.512×10 ⁻⁴	6.93	0.1261
Pure error	2.169×10 ⁻⁴	2	1.084×10 ⁻⁴		

A: Herbal material: Solvent ratio; B: Stock solution volume; C: AlCl₃ volume; AlCl₃: Aluminum chloride

variables. Consequently, the polynomial models describing the correlation between the response (TFC) and the variables were presented as follows:

$$\begin{aligned}
 \text{TFC} = & 0.50 - 1.04 \times 10^5 A - 0.37B + 0.024C \\
 & + 1.62AB + 0.18AC + 0.076BC + 0.29A^2 \\
 & + 0.91B^2 + 0.76A^2B - 0.28A^2C \\
 & + 1.90 \times 10^5 AB^2 + 1.04 \times 10^5 A^3
 \end{aligned} \tag{3}$$

where A, B and C denoted the herbal material: 60% ethanol ratio (w/v), the volume of stock solution and the AlCl₃ volume, respectively. The values of adjusted R^2 (0.9940), a measurement for fitness of the regressed model (Equation 3), indicate a high degree of correlation between observed and predicted values. The analysis of variance (ANOVA) of the regression model was performed to determine the significance. The $F = 221.74$ implies that the model is highly significant ($P < 0.0001$), that is, there is a chance of only 0.01% of the model occur due to noise, as can be seen in Table 2. A lack of fit was not significant ($P > 0.05$) showing that the reduced quadratic model is valid for the spatial influence of variables on the response. The linear and quadratic terms of the herbal material: 60% ethanol ratio and the volume of stock solution, the cubic term of herbal material: 60% ethanol ratio, the linear interactions between these terms and between them and the AlCl₃ volume, the interactions between the square of the herbal material: 60% ethanol ratio and the other two factors, the interaction of the square of the volume of stock solution and the herbal material: 60% ethanol ratio had statistically significant effects on TFC ($P < 0.05$).

The model (Equation 3) clearly demonstrates that A and B have negative effects on TFC, while C has a positive effect on it. The absolute value for the coefficient of A is much greater than those of B and C. This indicates that the linear term influence of A is more significant than those of B and C. The response surface of the TFC model indicates that the lower the herbal material: 60% ethanol ratio (0.02 g/mL or 1:66), the higher the content of flavonoids, independent of the AlCl₃ volume, when intermediate levels of stock solution are used. These results indicate that the herbal material: Solvent ratio has a negative influence on TFC. The use of extreme volumes (0.8 or 8.0 mL) of the stock solution implies in higher TFC when herbal material: 60% ethanol ratio is higher (0.08 g/mL).

Based on the preceding analysis, and considering that higher herbal material: 60% ethanol ratio provides more precise results, the optimized conditions for the TFC method

were: An herbal material: 60% ethanol ratio of 0.08 g/mL, 8.0 mL of stock solution and 1.6 mL of AlCl₃ solution.

Calibration curve and linearity

Calibration solutions were prepared in triplicate and analyzed under the optimal conditions as described above. The one-way ANOVA calculated to compare the replicates of the calibration curve showed no significant difference between groups ($F = 0.00007$, $P > 0.99$). The calibration curve was found to be linear in the range 0.001-0.021 mg/mL for quercetin dihydrate (C₁₅H₁₀O₇) [Figure 3]. The regression equation ($y = 80.79x - 0.0734$) and correlation coefficient of 0.9984 revealed a good linearity response for the method developed, since according to the literature a coefficient of determination for standard curves >0.99 indicates that the calculated line could explain more than 99% of the experimental data.^[11]

Precision

Due to their complex nature, analytical procedures for biological and biotechnological products can present a higher inherent variation. The results of repeatability (intra-day variation) and intermediary precision (inter-day variation) showed RSD% values below 6% and below 8%, respectively [Table 3]. For intermediary precision, no statistical difference was detected when the same analyst evaluated the method on different days and when different analysts performed it on different days ($P < 0.05$). Replicates of all performed analysis included the extraction, complexation and quantification steps. Although this approach increases the sources of variation, it allows evaluating the influence of all involved steps on the precision of the method. Therefore, our data indicate an acceptable precision and they are in accordance with those found in other works for samples of the same nature.^[14,15]

Robustness

Results of TFC obtained under variation of analytical conditions of the method for robustness evaluation are

presented in the Table 4. The analysis by two-way ANOVA revealed that the response (TFC) was not affected by any significant interference when small changes were inserted in the time of complexation with AlCl₃ ($P > 0.05$). However, the statistical analysis points out that variations in the time or temperature of extraction influence the TFC results ($P < 0.05$) [Figure 4]. These results show that the time and the temperature of extraction are critical for the TFC method and should be carefully controlled during the analysis.

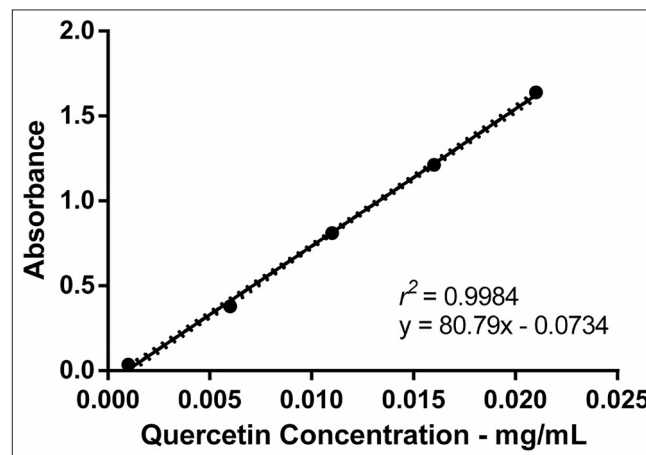


Figure 3: Calibration curve for standard quercetin-aluminum chloride complex spectrophotometrically assayed at 430 nm

Table 3: Repeatability and intermediate precision of the TFC method for leaves of *O. basilicum* (herbal material)

	Intra-day variation		Inter-day variation		Mean of analysis
	Analyst A ^a		Analyst B ^a		
	Day 1	Day 2	Day 3	Day 4	
Mean	0.62	0.64	0.73	0.66	0.66
RSD%	4.61	3.42	1.11	5.88	7.43

^aValues expressed in mg/g of herbal material; RSD%: Relative standard deviation; *O. basilicum*: *Ocimum basilicum*; TFC: Total flavonoid content

Table 4: TFC obtained under variations of the analytical conditions of the method for robustness evaluation

Replicates	TFC (mg/g of herbal material)						
	General method	Time of complexation		Time of extraction		Temperature of extraction	
		Item 2.4.1	15 min	35 min	50 min	70 min	70°C
1	0.68	0.67	0.71	0.61	0.60	0.66	0.61
2	0.68	0.69	0.70	0.61	0.60	0.64	0.62
3	0.70	0.68	0.72	0.62	0.60	0.66	0.61
Mean	0.69	0.68	0.71	0.61	0.60	0.65	0.61
RSD%	1.68	1.47	1.41	0.94	0.00	1.77	0.94

RSD%: Relative standard deviation; TFC: Total flavonoid content

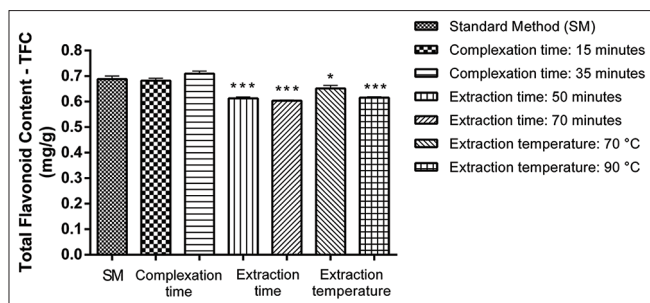


Figure 4: Robustness evaluation of the total flavonoid content (TFC) obtained under variations of analytical conditions. * $P < 0.05$ and *** $P < 0.001$ compared with the TFC obtained under general method conditions

CONCLUSION

This study allowed the optimization of a simple, fast and precise method for the determination of the TFC in leaves of *O. basilicum*, which can be used to support the quality assessment of this herbal material.

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