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Flavonoid content assay: influence of the reagent concentration and reaction time on the spectrophotometric behavior of the aluminium chloride – flavonoid complex

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The spectrophotometric assay based on aluminum chloride complex formation is one of the most commonly analytical procedures applied to flavonoid content determination. However, only a few optimization studies on the reaction conditions were done so far. The present work aims to the investigation of aluminum chloride concentration and reaction time effects on the spectrophotometric behavior of different flavonoids. The effects of both variation factors were studied by Central Composite Design and Response Surface Analysis methodology. The absorption data analysis showed that the effects of reaction time and reagent concentration on the absorption maximum are intricate and specific. A clear relationship between spectrophotometric behavior and flavonoid type or particular structure patterns could not be established.

1. Introduction

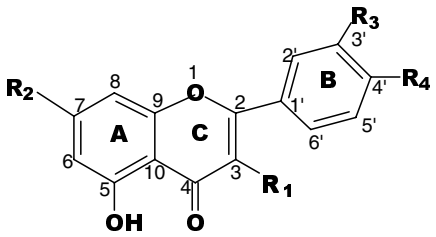
Flavonoids are phenolic substances with ubiquitous distribution in the plant kingdom. Some of them serve as markers in chemotaxonomic studies and reference substances for the quality control of phytopharmaceutical products [1]. The spectrophotometric assay based on aluminum chloride complex formation is one of the most commonly analytical procedures applied to flavonoid content determination. The reaction of aluminum chloride with the flavonoid free hydroxyl groups and its influence on the UV-VIS spectrum were formerly related to different flavonoid types [2]. The maximum wavelength displacement and the hyperchromic effect were associated with structure patterns and hydroxyl numbers. Besides that, the hyperchromic effect was also related to the flavonoid concentration [1, 2]. Those effects are the theoretical principle of certain analytical procedures, in particular the procedure proposed by Christ and Müller [3]. This one, however, was directed specifically for flavonols, as quercetin and kaempferol, and some vegetable drugs rich in glycosyl-O-flavonols. The German Pharmacopoeia [4], for instance, directed this procedure for the total flavonoid content assay of different vegetable drugs, disregarding method specificity.

This work investigates the influence of the reaction time and aluminum chloride concentration on the spectrophotometric behavior of some flavonoids, which are important factors to be considered in the development and validation of flavonoid content assay methods.

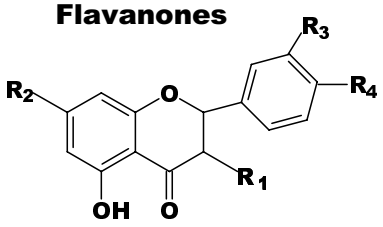
2. Investigations, results and discussion

The influence of reaction time and aluminum chloride concentration were investigated using a Central Composite Design (CCD) and a Response Surface Analysis (RSA). The advantages of both statistical methodologies are well

Flavones and flavonols



Flavanones



Flavonoid	Type	R_1	R_2	R_3	R_4
Apigenin	flavone	H	OH	H	OH
Chrysin	flavone	H	OH	H	H
Luteolin	flavone	H	OH	OH	OH
Quercetin	flavonol	OH	OH	OH	OH
Kaempferol	flavonol	OH	OH	H	OH
Rutin	flavonol	O-rutinose	OH	OH	OH
Naringenin	flavanone	H	OH	OH	OH
Sakuranetin	flavanone	H	H_3CO	H	OH

established in the pharmaceutical field [5–9]. The variation factors and experimental ranges were chosen considering earlier studies on *Passiflora* species [10, 11]. The flavonols quercetin and rutin (quercetin-3-rutinoside), the flavones apigenin, chrysin, and luteolin, as well as the flavanones sakuranetin and naringenin, selected as the reference flavonoids, represent different flavonoid types. The structure patterns considered were the C2-C3 double bond, the presence of vicinal C-3′-C-4′ hydroxyl groups attached to the flavonoid ring B and free or substituted hydroxyl groups linked to C-3 and C-7 positions. The experimental CCD matrix (2×2, provided of four star points and five central points) used throughout the work is showed in Table 1.

2.1. Flavones (apigenin, chrysin, luteolin)

For the apigenin spectrophotometric behavior, the theoretical results obtained using equation 1 showed good agreement with the experimental ones, and the mathematical model was validated considering the regression coefficient ($r^2 = 0.8498$), ANOVA test ($F_{0.05} = 7.92$) and lack-of-fit test ($F_{0.05} = 1.14$) (Table 2). The surface response and contour plot graphs (Fig. 1) showed clearly one absorption maximum related to a reaction time of 45 min and an aluminum chloride concentration of 5% (w/v). By comparison the values of the t-test, the main equation coefficients were assigned to the quadratic (b_{22}) and linear (b_2) terms, related to the aluminum chloride concentration (Table 3). On the other hand, the time reaction showed only a minor effect on absorption.

Table 1: General central composite design matrix applied to the investigation of reaction time and $AlCl_3$ concentration effects on the flavonoid absorption variation

Coded levels of variables			Levels of variables in units	
Experiment	Reaction time	$AlCl_3$ concentration	Reaction time (min)	$AlCl_3$ concentration (w/v %)
A	−1	−1	30	2.5
B	−1	+1	30	7.5
C	+1	−1	60	2.5
D	+1	+1	60	7.5
E_1	0	0	45	5.0
E_2	0	0	45	5.0
E_3	0	0	45	5.0
E_4	0	0	45	5.0
E_5	0	0	45	5.0
F	+1.414	0	66.21	5.0
G	−1.414	0	23.76	5.0
H	0	+1.414	45	8.54
I	0	−1.414	45	1.46

The results for the second flavone, chrysin were not very different from those for apigenin. In this case, however, the experimental results were described best by equation 2 ($r^2 = 0.8885$) (Table 2). The cubic equation model could also be validated, considering the regression coefficient, the ANOVA and lack-of-fit tests results (Table 2). The surface response and contour plot graphs (Fig. 2) as well as the t-Student values related to the cubic (b_{222}), quadratic (b_{22}) and linear (b_2) terms (Table 3), showed that aluminum chloride concentration was the main factor responsible for the absorption variation, instead of the reaction time factor. The chrysin reaction time and aluminum chloride concentration maximal values (55 min and 6.5%, respectively) were higher than the apigenin values. In spite of some shape similarities between both flavones, the chrysin response surface graph suggests additionally the occurrence of a second maximum located at aluminum chloride concentrations lower than 1%, indicating a secondary complex formation.

In the luteolin case, the reaction time and $AlCl_3$ concentration factors showed only a negligible influence on the absorption variation (Tables 2 and 3). Neither equation 1 nor equation 2 models could be validated, what means that the absorption variation due to the factors influence was not statistically significant ($\alpha = 0.05$).

The only difference between apigenin and chrysin structures lies in the hydroxyl group attached to the apigenin ring B, which is not present in the chrysin molecule. Thus, the absorption variation differences should be related to it. Moreover, it was expected that luteolin shows a similar behavior, since its structure resembles closely that of apigenin. However, either the aluminum chloride concentration or the reaction time had just a little effect on the absorption variation. It seems, therefore, possible that the occurrence of vicinal hydroxyl groups attached to the luteolin ring B led to more intricate interactions.

2.2. Flavonols (quercetin)

For quercetin, the experimental model was described and validated using equation 1, which was more suitable than equation 2 (table 2). The surface response and the contour plot graphs showed a clearly different behavior, if compared with the flavones apigenin and chrysin (Fig. 3). The aluminum chloride concentration showed no influence on the absorption response, while reaction time was the main variation factor (Tables 2 and 4). It was not possible to detected at what reaction time the absorption maximum occurred (Fig. 3), but a similar study using quercetin and kaempferol showed that it occurs at the first seconds after addition of the aluminum chloride reagent, followed by a sustained absorption decrease [12]. It is important to remark that querce-

Table 2: Regression data for flavones, flavonols and flavanones

Flavonoid	Class	Model	Main Factors	Validation	$F_{regression}$	r^2	$F_{lack\ of\ fit}$
Apigenin	Flavone	Equation 1	$AlCl_3$ conc. (b_{22}, b_2)	Yes	7.92**	0.8498	1.14
Chrysin	Flavone	Equation 2	$AlCl_3$ conc. (b_{222}, b_{22}, b_2)	Yes	5.69**	0.8885	0.05
Luteolin	Flavone	Equation 1	None	No	0.89	0.3897	0.12
Quercetin	Flavonol	Equation 1	None	Yes	3.44*	0.7108	1.33
Rutin	Flavonol	Equation 2	$AlCl_3$ conc. (b_2, b_{22}, b_{222})	Yes	7.25**	0.9103	13.16
Naringenin	Flavanone	312 nm: equation 1	312 nm: none	No	1.53	0.5221	3.91
		378 nm: equation 2	378 nm: interaction (b_{12}) and time (b_{111}, b_1, b_{11})	Yes	10.94**	0.9387	0.02
Sakuranetin	Flavanone	310 nm: equation 1	310 nm: none	No	0.37	0.2103	4.91
		378 nm: equation 1	378 nm: none	No	0.62	0.3073	4.40

** significant for $\alpha = 0.05$; * significant for $\alpha = 0.10$. conc. = concentration

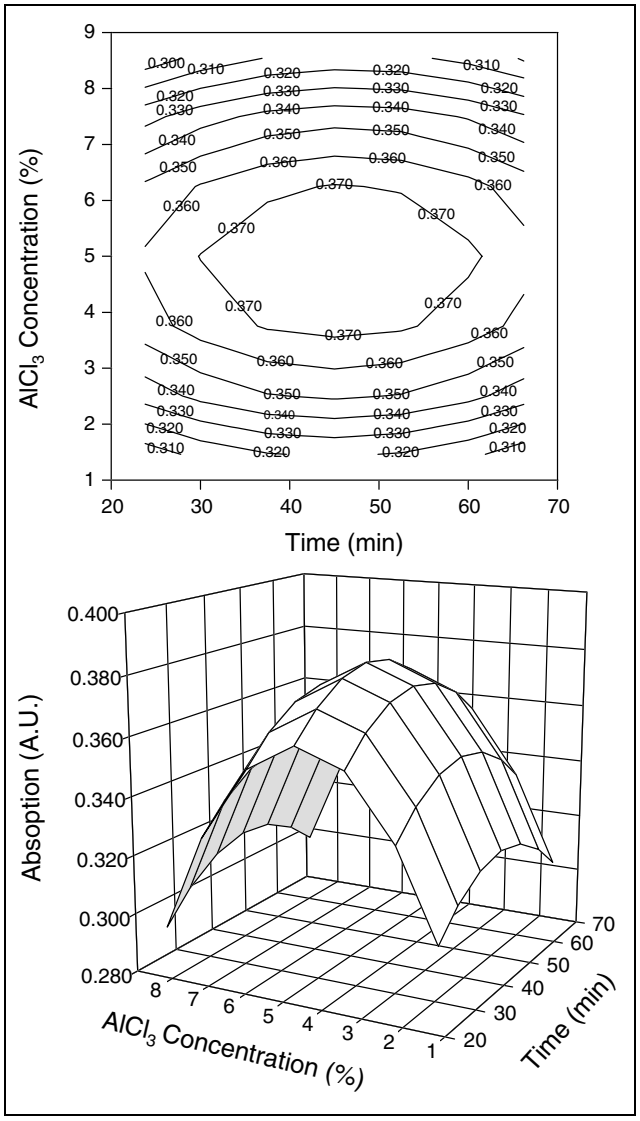


Fig. 1: Contour plot and response surface graphs for apigenin

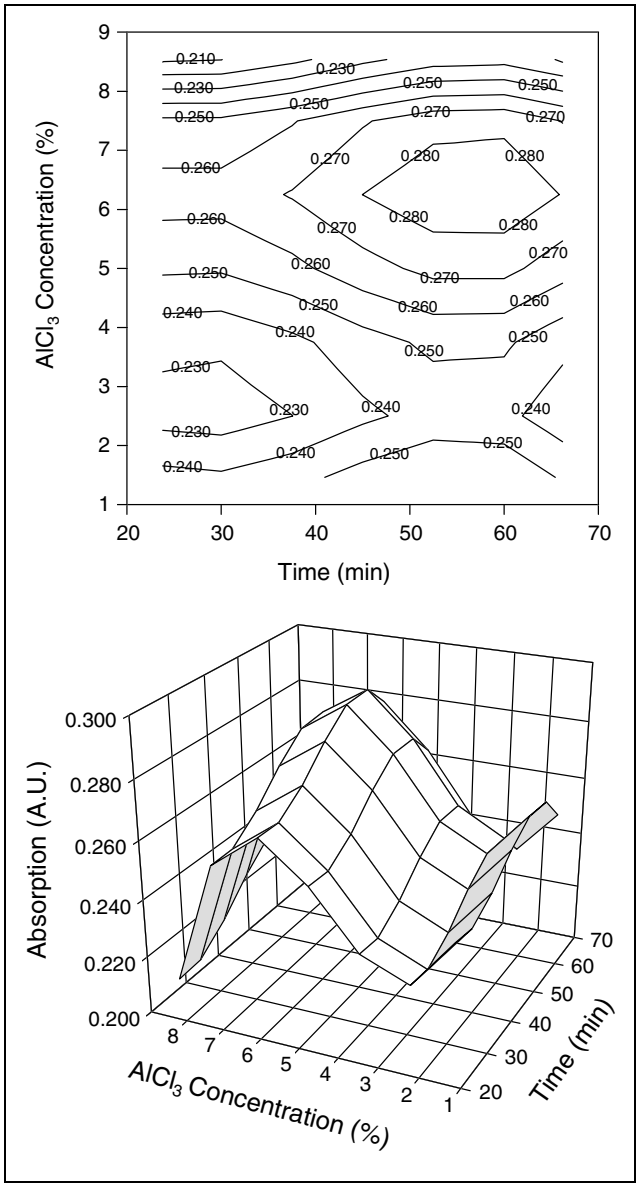


Fig. 2: Contour plot and response surface graphs for chrystin

tin differs from luteolin only in the additional free hydroxyl group in C-3 position. If we take rutin, a quercetin derivative which presents one rutinose molecule attached to the C-3 hydroxyl group, the relevance of a free hydroxyl group linked to C-3 position becomes clear (Tables 2 and 4; Fig. 4). In view of the r^2 value, the ANOVA and lack-of-fit test results, equation 2 can be considered validated for rutin (Table 2). The rutin surface response and contour plot graphs were distinctly different from the flavonol quercetin graphs. The main effect was assigned to the linear (b_2), quadratic (b_{22}) and cubic (b_{222}) terms of the equation, all related to aluminum chloride concentration (Table 4). An absorption maximum could be located at a concentration of 2%. A second maximum appears to rise at aluminum chloride concentrations higher than 3%. These facts resemble, in some aspects, the behavior observed by the flavone chrysin, to whom, the occurrence of a second maximum is strongly suggested by the analysis of its contour plot graph.

Table 3: Equation coefficients for the flavones apigenin, chrysin and luteolin

Coefficients	Apigenin equation 1	Chrysin equation 2	Luteolin equation 1
b_o	0.1858	0.4285	0.4330
t	2.90**	2.89**	11.52**
b_1	3.395×10^{-3}	-8.959×10^{-3}	-1.039×10^{-3}
t	1.53	0.85	0.80
b_2	0.0476	−0.0851	-7.649×10^{-3}
t	4.17**	3.16**	1.14
b_{12}	2.000×10^{-5}	4.667×10^{-5}	6.667×10^{-5}
t	0.11	0.40	0.64
b_{11}	-3.823×10^{-5}	-2.373×10^{-4}	8.501×10^{-6}
t	1.71	0.96	0.65
b_{22}	-4.963×10^{-3}	−0.0219	2.660×10^{-4}
t	6.20**	3.69**	0.57
b_{111}	–	-1.890×10^{-6}	–
t		1.03	
b_{222}	–	-1.593×10^{-3}	–
t		4.04**	

** Significant for $\alpha = 0.05$.

2.3. Flavanones (naringenin and sakuranetin)

The main flavanones structure features are a C2-C3 single bond and absence of hydroxyl group linked to the C-3 position. Their UV-Vis spectra recorded after treatment with aluminum chloride are quite different from those of flavones and flavonols and for this reason the absorption was recorded at 310–312 and 378 nm.

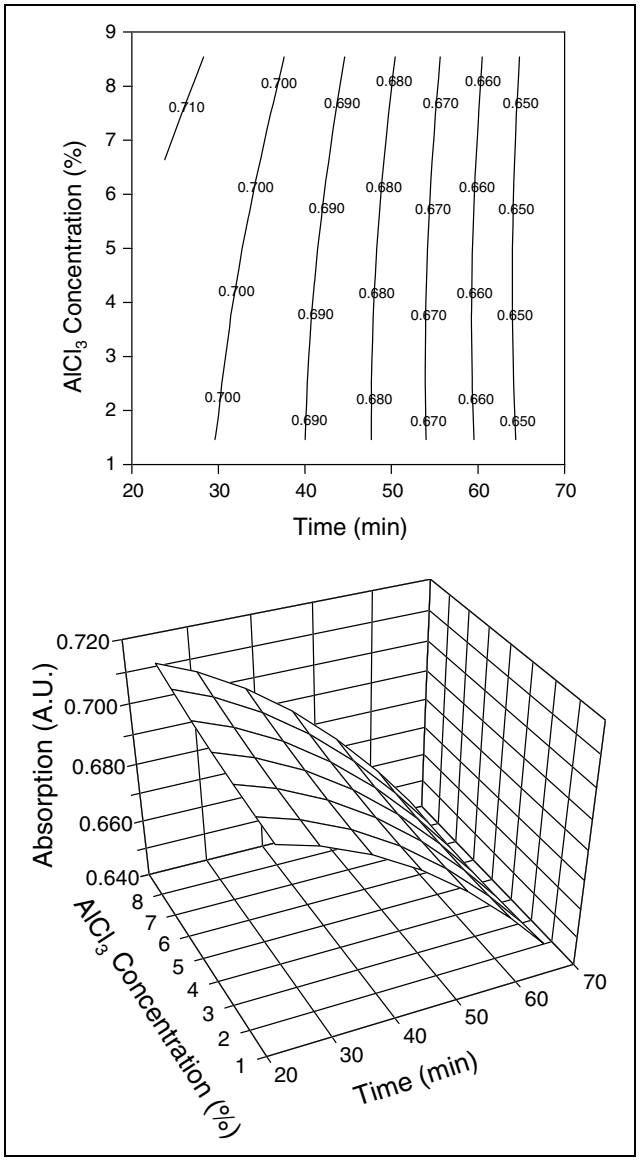


Fig. 3: Contour plot and response surface graphs for quercetin

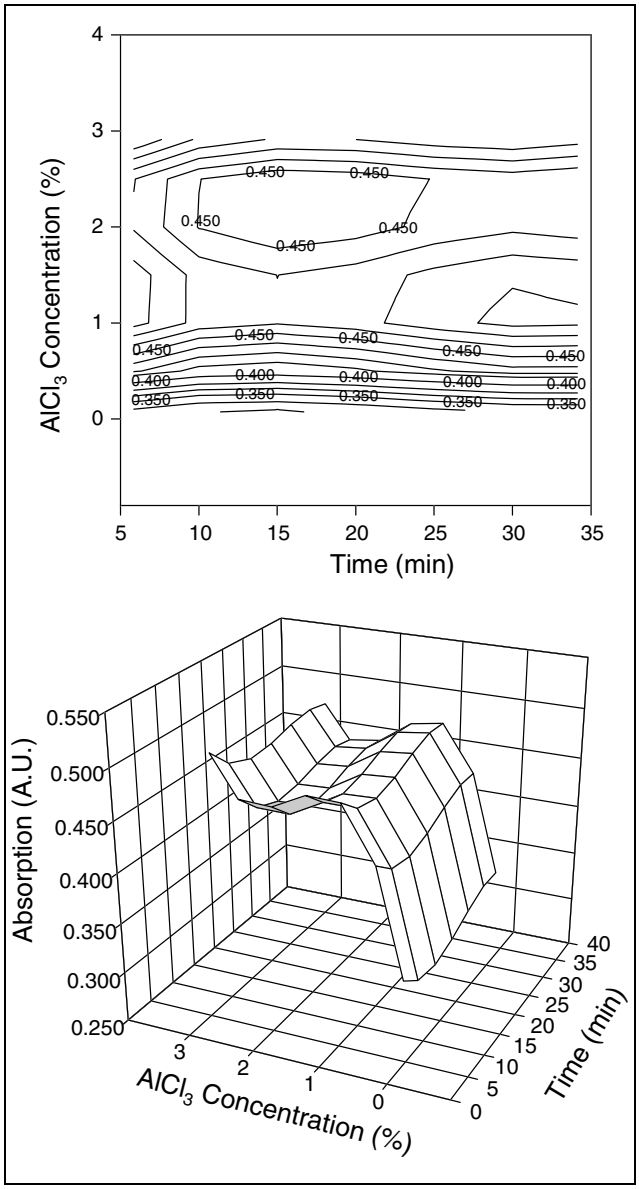


Fig. 4: Contour plot and response surface graphs for rutin

The experimental models could neither be validated for naringenin at 312 nm nor for sakuranetin at 310 and 378 nm. Consequently, the respective experimental results should be considered only as approximated approaches (Tables 2 and 5).

Table 4: Equation coefficients for flavonols quercetin and rutin

Coefficients	Quercetin equation 1	Rutin equation 2
b_o	0.7019	0.3447
t	9.42**	3.73**
b_1	5.238×10^{-4}	−0.0120
t	0.20	0.73
b_2	1.104×10^{-3}	0.3861
t	0.08	4.40**
b_{12}	-3.200×10^{-5}	-4.250×10^{-4}
t	0.16	0.40
b_{11}	-2.033×10^{-5}	6.326×10^{-4}
t	0.78	0.68
b_{22}	1.097×10^{-4}	−0.2398
t	0.12	3.43**
b_{111}	–	-9.024×10^{-6}
t		0.59
b_{222}	–	0.0458
t		2.97**

** Significant for $\alpha = 0.05$; * significant for $\alpha = 0.10$

The naringenin absorption behavior at 378 nm could be best described using equation 2 (Table 2). The experimental model explained by equation 2 was validated, considering the regression coefficient ($r^2 = 0.9387$), as well as the ANOVA and the lack-of-fit tests (table 2). The surface response and contour plot graphs allow to identify reaction time as the main effect on the absorption variation (Fig. 5), while the effect of aluminum chloride concentration was not significant (Table 5). In a different way to other flavonoids studied here, the coefficient termed for the interaction, reaction time and aluminum chloride concentration, emerged as the most important of all (b_{12} t-test value = 6.03, Table 5).

The sakuranetin spectrophotometric behavior at 310 nm could neither be described by equation 1 nor by equation 2 (r^2 values below 0.3) (Table 2). Therefore, no reliable inferences could be derived about the ring A substitution patterns. A similar condition could be observed for this flavanone at 378 nm, where the application of equations 1 and 2 led to unsatisfactory results (Table 2). Since sakuranetin and naringenin ring B substitution patterns are iden-

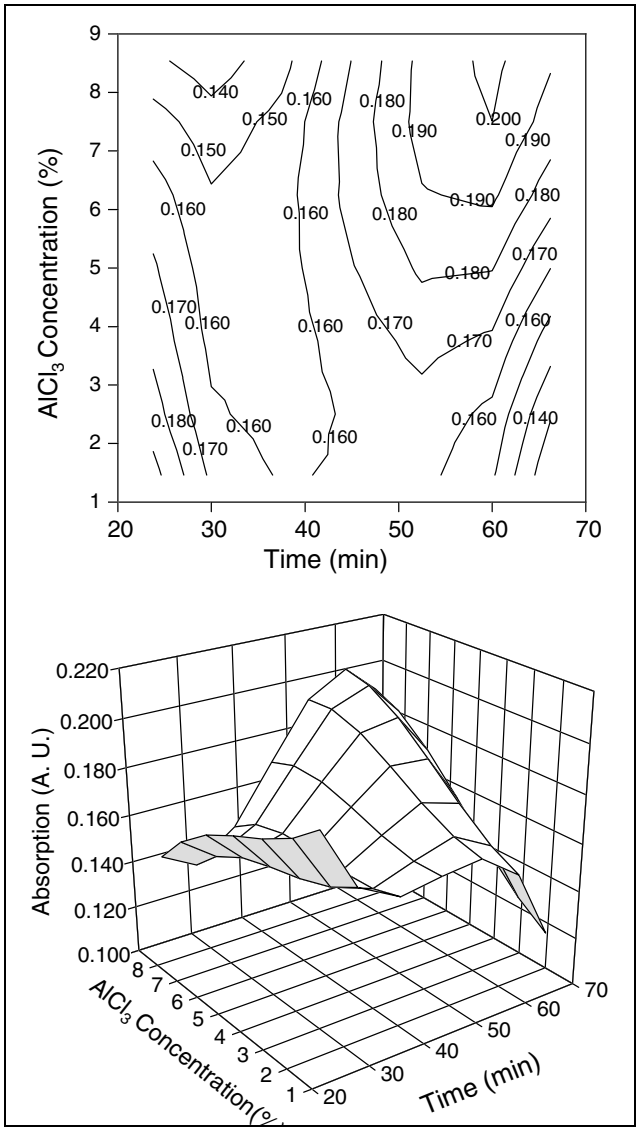


Fig. 5: Contour plot and response surface graphs for naringenin at 378 nm

tical and in both cases, ring A and B are not conjugated (absence of C2-C3 double bond) it was expected to find similar experimental results for naringenin and sakuranetin at 378 nm theoretically which, however, was not the case. The disagreement between theoretical and experimental results could be explained considering the flavonoid intermolecular interactions, in other words, the formation of high degree flavonoid-aluminum chloride-flavonoid complexes.

In general, we can conclude that all flavonoids investigated here presented dissimilar spectrophotometric behavior after reaction with aluminum chloride. This fact was observed even with flavonoids belonging to the same type or presenting close related hydroxyl group substitution patterns. Thus, the development, optimization and validation of assay methods based on aluminum chloride complex formation and directed for a single flavonoid must be considered specifically case by case, without exception. For flavonoid mixtures, the establishment of in terms of optimal assay conditions reaction time and aluminum chloride concentration appears to be therefore unattainable for vegetable raw material as well as for phytopharmaceutical products.

3. Experimental

3.1. Reagents and apparatus

Apigenin (Aldrich), chrysin (Sigma), luteolin (Sigma), quercetin (Merck), rutin (Merck), naringenin (Sigma) and sakuranetin (kindly gift from Dr. S. Bordignon, UFRGS). Aluminum chloride hexahydrate (Quimis), ethanol 96 ºGL. The aluminum chloride concentrations were calculated on its anhydrous form. UV-VIS spectrophotometer HP 8452A, provided with software HP Chem-Station.

3.2. Methods

3.2.1. Experimental design

The experimental matrix was a 2^2 factorial design augmented with 5 central points and 4 star points [5] (Table 1). The factor levels were chosen taking into account preliminary studies [12]. The time and concentration range was identical for apigenin, chrysin, luteolin, quercetin, naringenin and sakuranetin, except for rutin (Table 1). For rutin the $AlCl_3$ concentration levels were 0.5, 1.5 and 2.5% and time levels were 10, 20 and 30 min.

3.2.2. Sample preparation

Samples of 30 mg of each flavonoid were dissolved in ethanol (except for rutin which was dissolved in ethanol 40%) in a suitable way to render a final concentration of 12 µg/ml. A volume of 2 ml $AlCl_3$ ethanolic solution was added just before the final sample dilution in accordance to reagent concentration and reaction time levels designed for each experiment in the CCD matrix. Samples without $AlCl_3$ reagent were used as blank. The measure wavelengths used correspond to those λ_{max} values related to band I [2]. The flavanones absorptions were also recorded at those $\lambda_{max.}$ referred to band II [2].

Table 5: Equation coefficients for flavanones naringenin and sakuranetin related to absorption bands I and II

Naringenin			Sakuranetin	
Coefficients	312 nm (II) equation 1	378 nm (I) equation 2	310 nm (II) equation 1	378 nm (I) equation 1
b_o	0.8092	0.6077	0.3760	0.0737
t	9.33**	7.82**	5.80**	3.56**
b_1	-1.636×10^{-3}	−0.0296	3.599×10^{-4}	1.149×10^{-3}
t	0.55	5.32**	0.16	1.62
b_2	−0.0118	−0.0265	0.0134	1.281×10^{-3}
t	0.75	1.74	1.14	0.34
b_{12}	4.814×10^{-4}	3.993×10^{-4}	-8.000×10^{-5}	-4.280×10^{-5}
t	1.99	6.03**	0.44	0.74
b_{11}	-1.615×10^{-5}	6.654×10^{-4}	7.259×10^{-7}	-9.661×10^{-6}
t	0.54	5.07**	0.03	1.35
b_{22}	-8.138×10^{-4}	5.748×10^{-3}	8.280×10^{-4}	4.541×10^{-5}
t	0.74	0.82	1.01	0.17
b_{111}	–	-4.988×10^{-6}	–	–
t		5.10**		
b_{222}	–	2.017×10^{-3}	–	–
t		0.90		

** Significant for $\alpha = 0.05$; * significant for $\alpha = 0.10$

The experiment sequences were randomized and each result reported correspond to the mean value of three determinations.

3.2.3. Statistical analysis

The model equations were generated according to formerly reported methods [5, 9, 13, 14]. The mathematical models were validated conforming criteria proposed by Wherlé [13, 14]. The softwares used were Microsoft Excel® 7.0 and Sigma Stat® 1.0. The general equations applied were:

$$(\text{Quadratic}) : y = b_o + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}(x_1)^2 + b_{22}(x_2)^2, \quad (1)$$

$$(\text{Cubic}) : y = b_o + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}(x_1)^2 + b_{22}(x_2)^2 + b_{111}(x_1)^3 + b_{222}(x_2)^3 \quad (2)$$

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