Optimization of the extraction of chlorophylls in green beans (*Phaseolus vulgaris* L.) by N,N-dimethylformamide using response surface methodology

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Abstract

A method for extracting and determining chlorophyll pigments (chlorophyll *a* and *b*) in green beans (*Phaseolus vulgaris* L.) was developed. The procedure was based on the solvent extraction of pigments in vegetable samples using N,N-dimethylformamide as the extractant. Optimal conditions for extraction were determined by experimental design using response surface methodology. Central composite design "2^n + star" was used in order to optimize the following solvent extraction parameters: extraction time, homogenization time and number of extractions. The results suggest that the number of extractions is statistically the most significant factor and that the optimum values for the variables are: 90 min (extraction time), 1 min (homogenization time) and 5 (number de extractions). This work confirms the advantages of experimental design compared to traditional optimization strategies because it allows a large number of factors to be screened simultaneously, provides less ambiguous data and helps to visualize relationships between responses and factor levels. Pigments were determined using UV-Visible spectrophotometry at 647 and 664 nm. The chlorophyll content was analyzed in three green bean cultivars: "Donna", "Negrital" and "Emerite".

Keywords: Natural pigments; Food analysis; Solvent extraction; Experimental design; UV-Visible spectrophotometry.

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1. Introduction

Multiple epidemiological studies have demonstrated a relationship between a diet rich in vegetable and derivative products and the prevention of cardiovascular illnesses and some types of cancer. This fact seems to be related with their chemical composition (Lampe, 1999; Steinmetz & Potter, 1996). Green beans (*Phaseolus vulgaris* L.) are one of the most widely consumed vegetables in Europe because of their bright color, pleasant taste and low calories. The study of color-change from bright green to olive brown during storage and processing is very important because color is one of the deciding factors in whether or not a consumer acquires the product. In this sense, one of the indicators of decreased quality in green beans is the loss of chlorophyll pigments, because they can degrade to undesirable grey-brown compounds (Mangos & Berger, 1997).

Chlorophylls are the most common green pigments found in plants. As an integrated part of vegetable foodstuffs, chlorophylls have been a natural component of the human diet throughout history (Seljasen et al., 1998). In vivo, these pigments play a key role in photosynthesis (Schoefs, 2002). They have a complex four-ring structure, and the center of the molecule are coordinated with a Mg^{2+}-ion. A long hydrophobic hydrocarbon tail (phytyl) is attached to the ring structure. Chlorophyll *a* has a methyl group bound to ring II, while chlorophyll *b* has an aldehyde group in this position (Fig. 1) (Seljasen et al., 1998). Chlorophylls are susceptible to many degradation reactions caused by weak acids, oxygen, light, temperature changes and/or enzymes. Replacement of the central Mg^{2+}-ion in chloropigments with hydrogen leads to pheophytins, which are related to the color change from bright green to olive brown. Chlorophyllase catalyzes the hydrolysis of the phytyl esters of chlorophylls. Its inhibition by low

52 temperature extraction leads to less degradation of chlorophylls (Mangos & Berger,
53 1997).

54 Consumer demand of better quality vegetable products has led to an increasing
55 interest in developing accurate and specific analytical methods to analyze chlorophyll
56 content. Most of the analytical methods used to determine chlorophylls use liquid
57 chromatography (Darko et al., 2000; Monreal et al., 1999) or measure the
58 spectrophotometric response at different wavelengths and then solve equations to
59 determine the contents (Qudsieh et al., 2002; Zhuang et al., 1997).

60 To ensure that the chlorophyll analysis is effective, it is very important to
61 optimize the sample extraction when analyzing these pigments in complex samples such
62 as vegetables. It is essential to inactivate enzymes (such as chlorophyllase) which can
63 easily degrade chlorophylls during the extraction. Sodium (Monreal et al., 1999),
64 calcium (Bahçeci et al., 2005) or magnesium (Hegazi et al., 1998) carbonate has been
65 used for this purpose. Extraction methods may also differ between different vegetables
66 because of their diverse matrices (Bahçeci et al., 2005; Monreal et al., 1999; Zhuang et
67 al., 1997). For these reasons considerable caution should be exercised in the
68 employment of methods that have been developed for the analysis of specific plant
69 tissue types. A critical aspect of the analytical determination of chlorophyll pigments in
70 green beans that has not been studied in detail is how differences in vegetable matrices
71 effect the extraction of these pigments. There is evidence that chlorophylls are more
72 thoroughly extracted with methanol (Bahçeci et al., 2005; Wright & Shearer, 1984) or
73 dimethylsulfoxide (Shoaf & Lium, 1976). However, Mantoura & Llewellyn (1983)
74 found that methanol led to the formation of chlorophyll *a* derivative products, whereas
75 90% acetone did not (Monreal et al., 1999; Qudsieh et al., 2002). N,N-
76 Dimethylformamide (N,N-DMF) is a very convenient solvent for chlorophyll extraction

(Corcuff et al., 1996; Inskeep & Bloom, 1985; Zhuang et al., 1997) since the pigments are stable in this solvent for up to 20 d when stored at 4ºC in the dark (Moran & Porath, 1980). Other variables that influence the chlorophyll extraction are extraction or homogenization time and the number of extractions. Extraction times between 30 min (Tan et al., 2000) and 24 h (Corcuff et al., 1996; Sibley et al., 1996) and homogenization times between 1 and 3 min (Bahçeci et al., 2005; Cano et al., 1998; Moran & Porath, 1980) have been used. To ensure complete extraction samples have been extracted between 1 and 3 times (Bahçeci et al., 2005; Cano et al., 1998; Corcuff et al., 1996). Experimental designs are used to identify influential factors, optimize conditions and evaluate how those factors affect the samples being analyzed. In traditional strategies only one variable is changed while all the others remain constant. This approach requires a large number of experiments and does not allow the study of changes in the response that may occur when two or more factors are modified simultaneously. Experimental design is an alternative to this strategy. It allows a large number of factors to be screened simultaneously and can provide less ambiguous data. Furthermore, experimental designs combined with response surface methodology (RSM) help to visualize relationships between responses and factor levels to locate the region of highest response values (Montgomery, 1991).

The main objective of this study was to optimize the extraction of chlorophylls *a* and *b* from green beans using solvent extraction by N,N-DMF before determining chlorophylls via spectrophotometry. Central composite design was used to optimize three variables (extraction time, homogenization time and number of extractions) in order to determine an optimal set of operational conditions.

2. Materials and methods

2.1. Plant material and chemicals

Green beans (*Phaseolus vulgaris* L., cvs. “Donna”, “Negrital” and “Emerite”) were harvested from fields located in Arafo in southern Tenerife (Canary Islands, Spain) in May of 2005. The pods were selected, eliminating those that showed mechanical injuries and/or rotting. For chlorophyll determination, 3 kg of pods were sliced, frozen in liquid nitrogen and stored at -80ºC until the analyses were carried out.

Chlorophyll *a* and chlorophyll *b* standards were supplied by Sigma (Madrid, Spain). Stock standard solutions containing 0.2 mg/ml of chlorophyll *a* or chlorophyll *b* were prepared in acetone and stored in glass stoppered bottles at -20ºC in the dark. Solutions of variable concentrations were prepared by diluting the stock standard solution in N,N-dimethylformamide (N,N-DMF). Acetone and N,N-DMF were purchased from Panreac (Madrid, Spain). Sodium carbonate (Na_2CO_3), used to avoid chlorophyll degradation, was obtained from Merck (Darmstadt, Germany).

2.2. Solvent extraction method and chlorophyll determination

Frozen pulverized green bean samples were accurately weighed at 2 g and mixed with 3 ml of N,N-DMF (Sibley et al., 1996). Sodium carbonate (0.15 g) was added to prevent the chlorophylls from degrading to pheophytins. The mixture was homogenized in a Politron PT 6000 (Kinematica AG, Lucerne, Switzerland) high speed blender at 18000 g in ice and darkness (homogenization time varied depending on the particular experiment; ranging between 1 and 20 min). Then pigments present in the sample were extracted (extraction time varied depending on the particular experiment; ranging between 1 and 90 min) in a water bath at 4ºC, darkness and under agitation in sealed vessels. The sample was centrifuged at 9000 g (refrigerated at 4ºC) for 20 min in a

Jouan CR 312 centrifuge (Thermo Electron Corporation, Madrid, Spain). Depending on the experiment, this procedure was repeated (ranging between 1 and 5 times) and the resulting supernatants were mixed together. Determination of pigments was done in triplicate.

Spectrophotometric measurements of chlorophyll content were made on a Shimadzu UV–visible 160A double-beam recording spectrophotometer (Kyoto, Japan) equipped with a Hellma (Jamaica, USA) cell (path length 10^{-2} m). The slit width of the monochromator was fixed at 2 nm. The absorbance was recorded in triplicate for each sample at 647 and 664 nm simultaneously in order to determine chlorophyll *a* and *b* content, which was expressed as mg/100 g fresh weight.

2.3. Experimental design

Green beans (*Phaseolus vulgaris* L., cv. "Donna") were used to optimize chlorophyll extraction. Statgraphics Plus version 4.1 (Statistical Graphics, Rockville, USA) was employed to generate design and regression analysis and to obtain the plot. The optimization procedure is divided into the following steps: (a) selection of independent variables that affect the extraction of pigments, the level of these variables and the response variable, (b) selection of the type of experimental design and (c) mathematical solution of the second order equation that relates the response to the independent variables. The entire experiment was executed in two phases using a central composite design (CCD). The objective of the first phase was to evaluate the use of sodium carbonate to inhibit the effect of chlorophyllase. In the second phase a CCD "2^3 + star" was projected on a face-centered star design with two center points. The variables (number of extractions, extraction time and homogenization time) were set at

three separate coded levels. The unknown function was assumed to be approximated by a second-order polynomial equation such as:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot x_i + \sum_{i=1}^{k} \beta_{ii} \cdot x_i^2 + \sum_i \sum_j \beta_{ij} \cdot x_i \cdot x_j + \varepsilon \quad (i < j)$$

where y is the response; β_0 (center point of system), β_i (coefficient of linear effects), β_{ii} (coefficient of quadratic effects) and β_{ij} (coefficient of interactive effects) are the different constant coefficients of the model; x_i and x_j are levels of independent variables; and ε is the error of the model.

Grubbs' test was applied to detect outliers in the data set (Miller & Miller, 2000). Analysis of variance was used to evaluate the effect of cultivar on chlorophyll content. Fisher's Least-Significant-Difference test (LSD), at the 5% significance level, was applied to experimental results to assess intra-pair significant differences (Hsu, 1996).

3. Results and discussion

3.1. Spectrophotometric determination of chlorophylls a and b: analytical features

Chlorophyll determination was done using UV-visible spectrophotometry at two characteristic wavelengths, 647 and 664 nm, which are the maximum absorption wavelengths for chlorophyll *b* and chlorophyll *a*, respectively. Calibration graphs were obtained by using multiple linear regression and constructed by plotting the signal obtained for the two pigments (in absorbance units) against the analyte concentration at seven concentration levels. Each concentration level was analyzed in triplicate. According to this calibration graphs, the following formulas were obtained for quantifying chlorophyll *a* and *b*: Chlorophyll *a* (mg/l) = $8.9 \cdot \text{Abs}_{664} - 1.9 \cdot \text{Abs}_{647} - 0.11$

and Chlorophyll *b* (mg/l) = 30 · Abs_{647} - 7.1 · Abs_{664} - 0.98. In order to quantify the total chlorophyll in green beans the chlorophyll *a* and chlorophyll *b* content were added.

Quality parameters for the spectrophotometric determination of the chlorophylls are reported in Table 1. The detection limit was defined as three times the standard deviation of the background noise, determined using N,N-dimethylformamide (N,N-DMF), divided by the slope (at the most sensitive wavelength) of each calibration graph. The repeatability of the procedure, expressed as relative standard deviation (RSD), was checked on eleven consecutive analyses of a standard solution containing 5 mg/l of chlorophyll *a* or 15 mg/l of chlorophyll *b*.

3.2. Optimization of the chlorophylls extraction

The use of Na_2CO_3 to inhibit the effect of chlorophyllase, normally present in plants, on the chlorophylls has been described as a way of preventing pigment oxidation (Cano et al., 1998; Monreal et al., 1999). The effect of adding 0.15 g of Na_2CO_3 to plant material before the extraction was evaluated. The addition of Na_2CO_3 to green beans increases the extraction of chlorophyll *a* by 45% and chlorophyll *b* by 50%. Due to the results obtained, the addition of carbonate is recommended before the extraction of chlorophylls from green beans to prevent the oxidation of these pigments.

The use of an experimental design to explore the variables that affect the solvent extraction of chlorophyll pigments gives a clear idea of the overall number of experiments and the effects that the interaction between the variables may have on the extraction. In accordance with our previous experience in the treatment of green bean samples, three variables that can potentially affect extraction efficiency were chosen: extraction time, number of extractions and homogenization time. A central composite design (CCD) superimposed on a face-centered star design, "2^3 + star", with two center

points was used, resulting in 16 randomized runs, doing each experiment in triplicate (n = 48). Although chlorophylls have been extracted from green beans, the analytical method used was not optimized for this specific vegetable (Cano et al., 1998; Monreal et al., 1999). In order to optimize chlorophyll extraction specifically from green beans, the minimum and maximum levels were chosen based on the experience of other authors in the pretreatment of different types of plant materials (Bahçeci et al., 2005; Cano et al., 1998; Corcuff et al., 1996; Tan et al., 2000). The lowest and highest values given to each factor were: 0 and 90 min (for the extraction time), 1 and 5 (for the number of extractions) and 1 and 20 min (for the homogenization time). Higher extraction times were not chosen because chlorophylls from green bean are degraded when exposed to high extraction times (3-24 h), as was observed in previous experiments. This result indicates that long extraction times are not needed to extract chlorophylls from green beans, in contrast to other vegetable matrices such as broccoli florets (Corcuff et al., 1996) and red maple (Sibley et al., 1996). Other factors implicated in the extraction were kept constant: amount of green bean cv. “Donna” (2 g), volume of extractant (3 ml) and final volume of the extract (10-ml).

Table 2 shows the design matrix, which includes the factors that influence chlorophylls extraction and the amounts (expressed as mg/100 g fresh weight) of chlorophyll *a*, chlorophyll *b* and total chlorophyll obtained in the different experiments carried out. The sequential listing of the experimental design parameters represents the statistically randomized order in which the experimental treatments were undertaken. Variance analysis (ANOVA) was used to estimate the statistical significance of the factors that had the greatest effect on the extraction and interactions between them (Fig. 2). It should be noted that modification of the experimental conditions used for the extraction does not affect all of the pigments equally. The number of extractions is the

225 factor that has the greatest influence on the extraction of both types of chlorophylls.
226 Thus, when the number of extractions is increased the extraction efficiency increases as
227 well. The Pareto chart (Fig. 2a) for chlorophyll *a* shows that the interaction between the
228 number of extractions and the homogenization time has a significant influence on the
229 efficiency of the extraction. However, homogenization time and the interaction between
230 extraction time and homogenization time affect the extraction of chlorophyll *b* (Fig. 2b).
231 The response-surface graphs obtained using the experimental design are shown
232 in Fig. 3, Fig. 4 and Fig. 5. The number of extractions and homogenization time are
233 represented in Fig. 3. When the number of extractions increases the extraction of both
234 chlorophylls is higher. This effect is also noticed in Fig. 4 which represents extraction
235 time versus number of extractions. Both graphs show that the optimum number of
236 extractions is 5 for both pigments. In Fig. 5 extraction time versus homogenization time
237 is represented. It can be observed that the optimum extraction efficiency is reached at
238 high extraction times and low homogenization times for the extraction of chlorophyll *a*;
239 however, the highest extraction of chlorophyll *b* is obtained with low extraction times in
240 conjunction with high homogenization times (Fig. 4 and Fig. 5). When the
241 homogenization time is low, the highest extraction of chlorophyll *b* is obtained with
242 high extraction times. This can also be seen in the Pareto chart for chlorophyll *b* (Fig.
243 2b) which shows that the interaction between extraction time and homogenization time
244 increases the efficiency of the extraction of chlorophyll *b* as long as one factor is high
245 and the other is low. The decrease in extraction efficiency when extraction time is
246 increased might indicate a degradation of chlorophyll *b* over time. The degradation of
247 chlorophylls can be caused by various factors. Although these factors are assumed to be
248 mainly biotic, such as the action of enzymes, abiotic factors such as temperature, light
249 and oxygen are also likely to be involved (Kowalewska & Szymczak, 2001). The effect

of the degradation of chlorophyll *b* in green bean extracts is more pronounced with long homogenization times (which imply an aeration of the sample) than for short times. If it is assumed that chlorophyllase was inhibited by Na_2CO_3 it can be supposed that oxygen is involved in chlorophyll *b* degradation. Kowalewska & Szymczak (2001) and Schoefs (2002) described how extracts containing chlorophylls react easily with oxygen upon illumination resulting in the formation of activated oxygen species.

It can be concluded that in order to determine both chlorophylls in the same extract a pragmatic approach must be used when selecting the extraction conditions. Using 5 extractions, a homogenization time of 20 min and an extraction time of 0 min, a recovery of 75% of chlorophyll *a* and of 100% of chlorophyll *b* are obtained. However, using a homogenization time of 1 min and an extraction time of 90 min (and 5 extractions), the recovery for chlorophyll *a* is 100% and of 70% for chlorophyll *b*. These last extraction conditions were selected as optimum because high homogenization times could produce an aeration of the sample and mechanical injury in the high-speed blender. For both chlorophylls 96% of the extraction takes place between the second and the fifth extraction. The amount of chlorophyll recovered in each of these extractions (2^{nd} – 5^{th}) was higher than 12%. The maximum recovery of chlorophyll *a* and *b* takes place in the second (35 ± 2%) and fifth (38 ± 3%) extractions, respectively.

3.3. Analysis of green bean chlorophylls

Considering that green bean cultivar influences chlorophyll content notably, chlorophyll *a* and *b* and total chlorophyll were determined in three green bean cultivars. Because different cultivars of green beans have similar matrices, the calibration graphs obtained for quantifying chlorophylls and the extraction conditions selected as optimum were used to determine the chlorophylls in the different cultivars (Table 3).

The conditions used for chlorophyll extraction (5 extractions, homogenization time of 1 min and extraction time of 90 min) are optimal for chlorophyll *a* extraction but only achieve 70% chlorophyll *b* extraction. A more accurate quantity for chlorophyll *b* was calculated using a correction factor (f = 1.44). This correction was estimated from the equation derived from the experimental design used to optimize chlorophyll *b* extraction. It is defined as the ratio between mg Chl *b*/100 g obtained in optimal conditions for extracting chlorophyll *b* and mg Chl *b*/100 g obtained in selected conditions for extracting chlorophyll *a* and chlorophyll *b*.

Chlorophyll *a* content is higher in the "Emerite" cultivar than in the other two cultivars ("Negrital" and "Donna"). Moreover, chlorophyll *b* and total chlorophyll contents in "Donna" are lower than in "Negrital" and "Emerite" (nearly 1.70 times).

4. Conclusions

Chlorophyll pigments found in green beans can be efficiently extracted using an adequate selection of the experimental conditions optimized in this study. The most important factor among the optimized conditions is the number of extractions, although high extraction times and low homogenization times contribute notably to the extraction efficiency. Optimum values of the variables that have the greatest influence on chlorophyll extraction from green beans are: 5 extractions, a 90 min extraction time and a 1 min homogenization time. However, to optimize all factors that could affect chlorophyll extraction it would be necessary to apply RSM to extraction with acetone and compare the results with those obtained in this work. The results of this work confirm the advantages of experimental design compared to traditional optimization strategies because it allows a large number of factors to be screened simultaneously,

299 provides less ambiguous data and helps to visualize relationships between responses and
300 factor levels.
301
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Figure captions

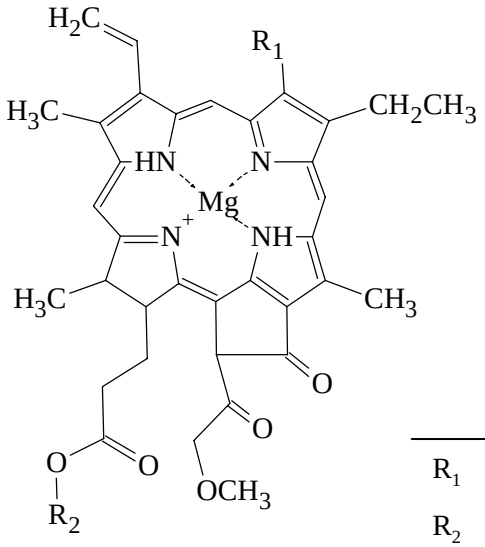
Fig. 1. Chemical structure of chlorophyll *a* and chlorophyll *b*.

Fig. 2. Pareto charts for the standardized main effects in the central composite design (2^3+ star) experiment for (a) chlorophyll *a* and (b) chlorophyll *b*, where the vertical line indicates the statistical significance of the effects.

Fig. 3. Estimated response surfaces in the central composite design (2^3+ star) experiment obtained by plotting the number of extractions and homogenization time for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (Chl *t*).

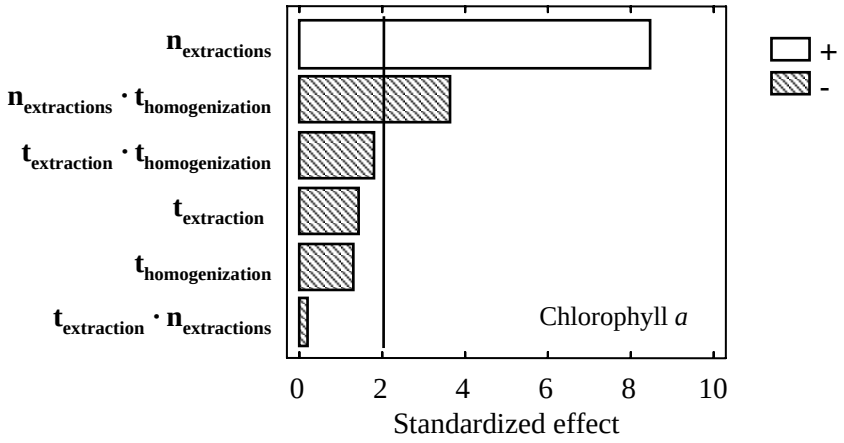
Fig. 4. Estimated response surfaces in the central composite design (2^3+ star) experiment obtained by plotting extraction time and the number of extractions for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (Chl *t*).

Fig. 5. Estimated response surfaces in the central composite design (2^3+ star) experiment obtained by plotting extraction time and homogenization time for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (Chl *t*).

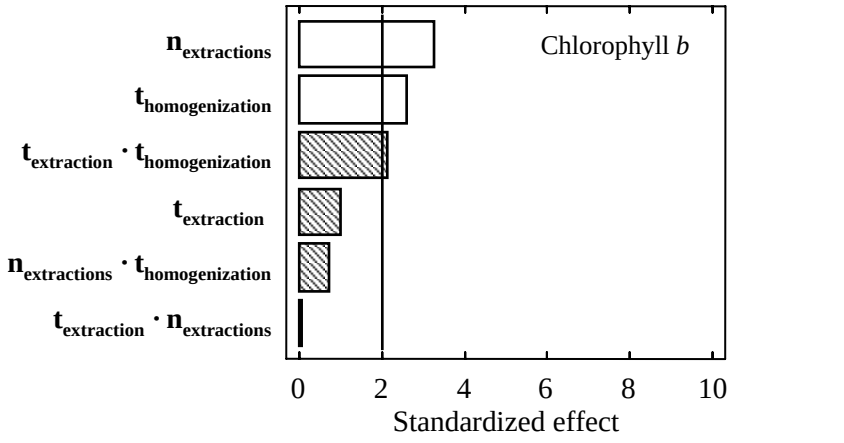


	Chlorophyll *a*	Chlorophyll *b*
R_1	CH_3	CHO
R_2	$C_{20}H_{39}$	$C_{20}H_{39}$

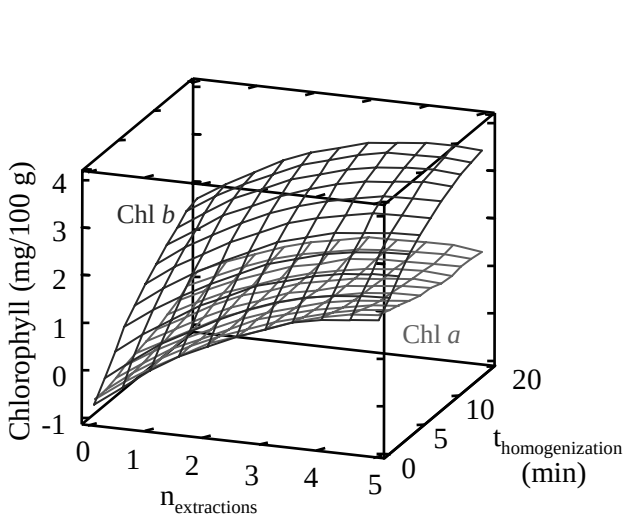
a

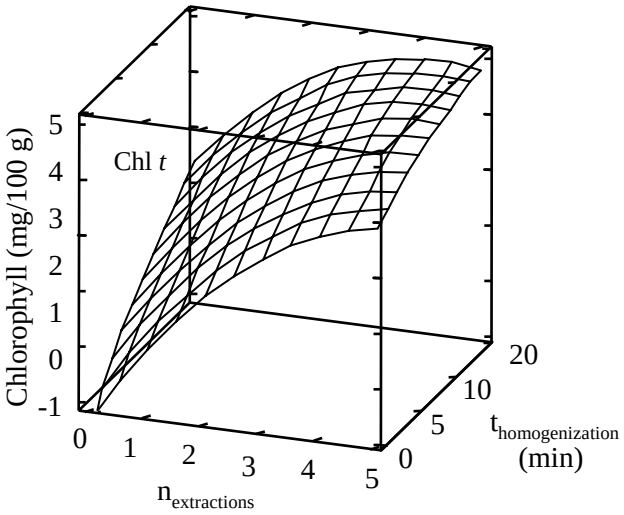

$n_{extractions}$
$n_{extractions} \cdot t_{homogenization}$
$t_{extraction} \cdot t_{homogenization}$
$t_{extraction}$
$t_{homogenization}$
$t_{extraction} \cdot n_{extractions}$
Chlorophyll *a*
+
-
0
2
4
6
8
10
Standardized effect

b

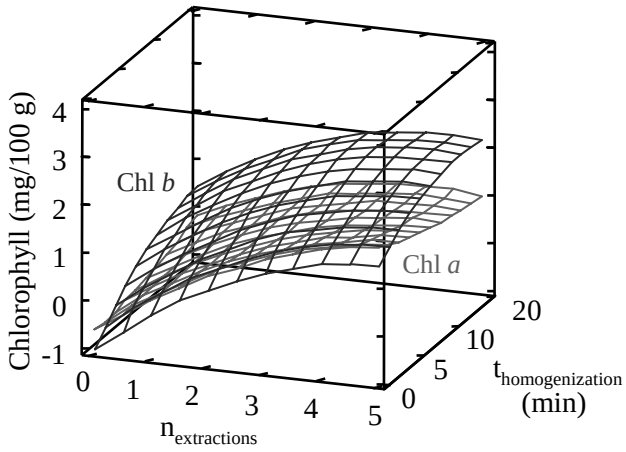

$n_{extractions}$
$t_{homogenization}$
$t_{extraction} \cdot t_{homogenization}$
$t_{extraction}$
$n_{extractions} \cdot t_{homogenization}$
$t_{extraction} \cdot n_{extractions}$
Chlorophyll *b*
0
2
4
6
8
10
Standardized effect

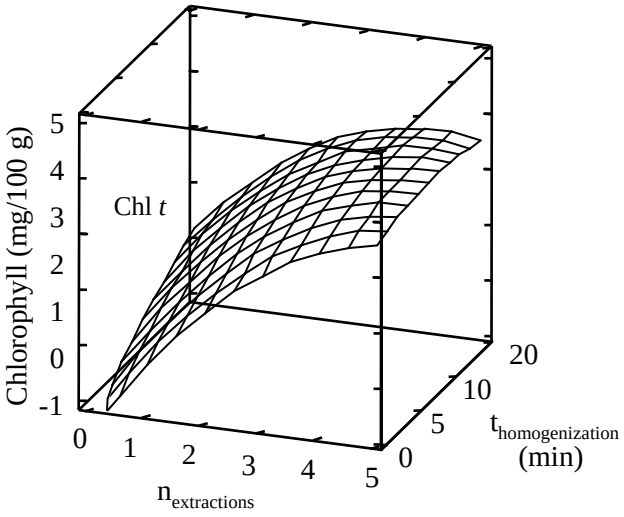
(a) fixing extraction time at 0 min



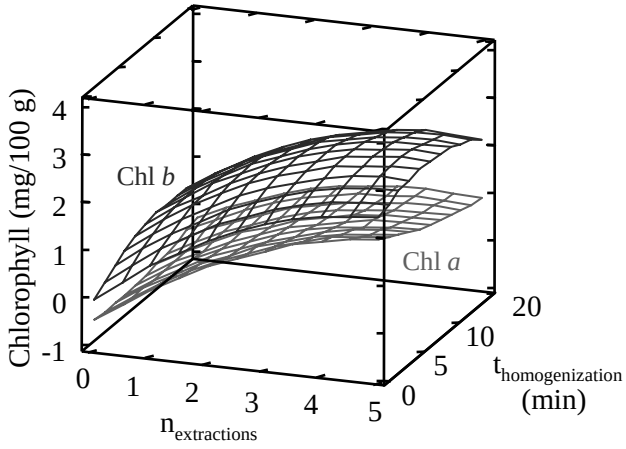


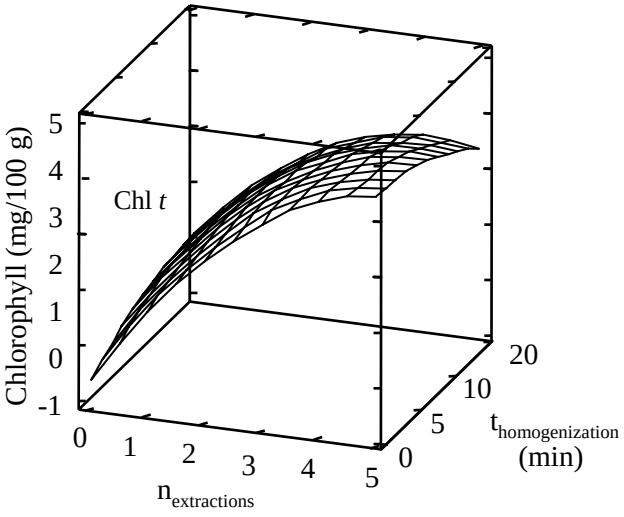
(b) fixing extraction time at 45 min



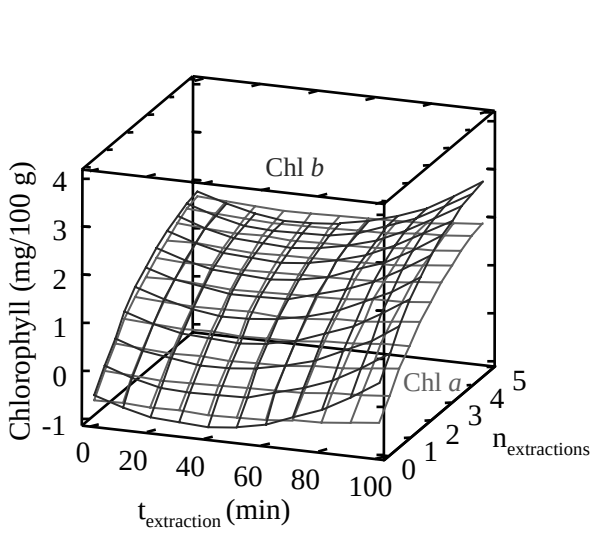


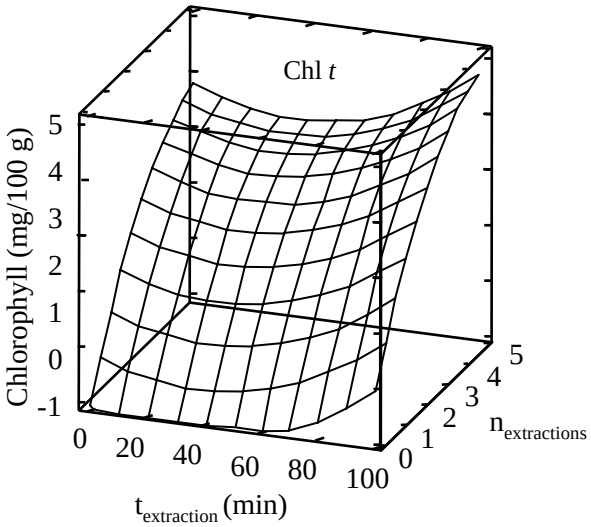
(c) fixing extraction time at 90 min



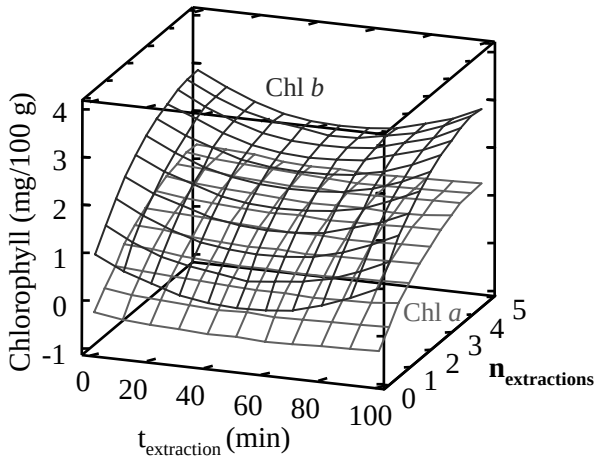


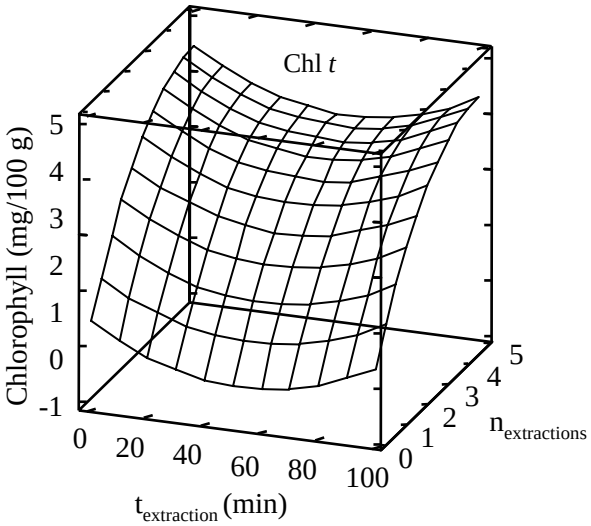
(a) fixing homogenization time at 1 min



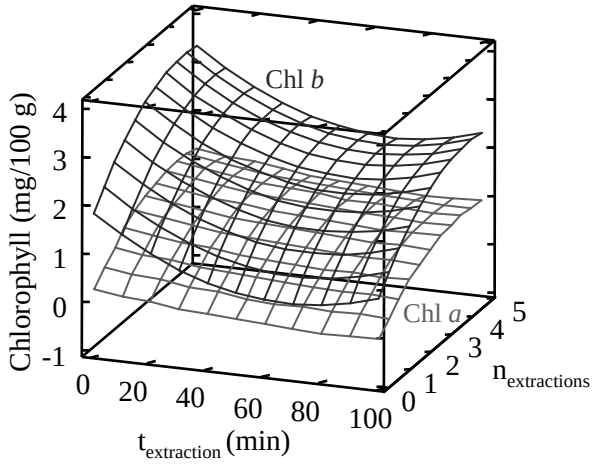


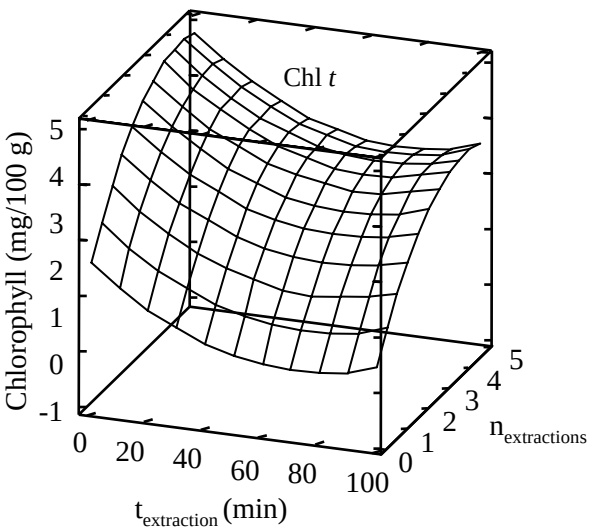
(b) fixing homogenization time at 10.5 min



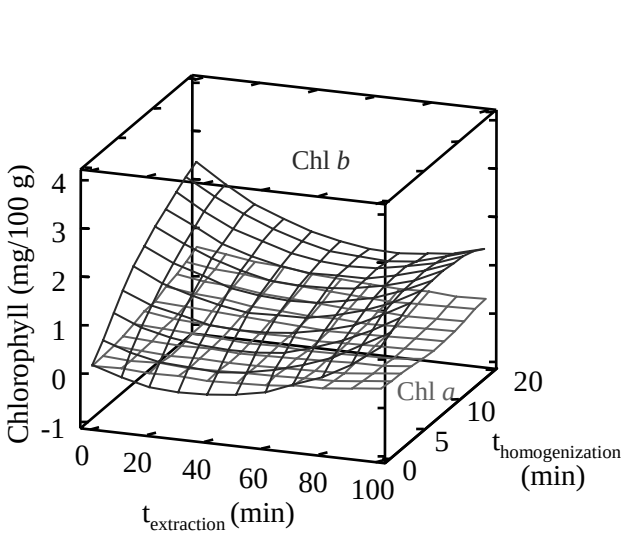


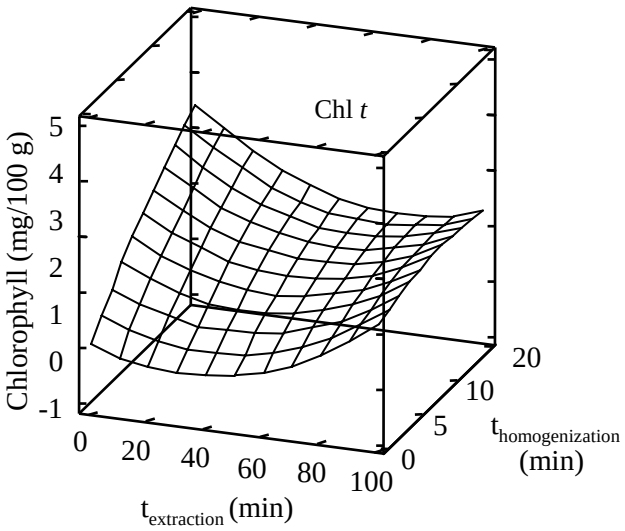
(c) fixing homogenization time at 20 min



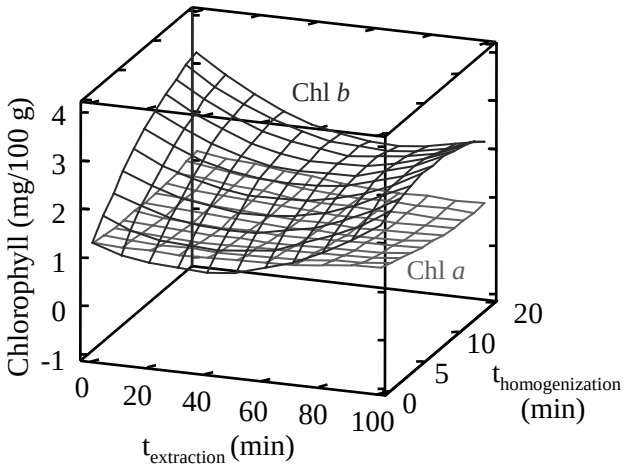


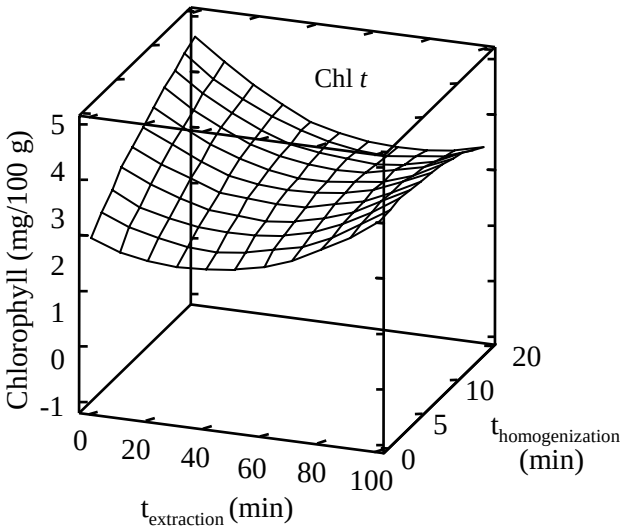
(a) fixing the number of extractions at 1



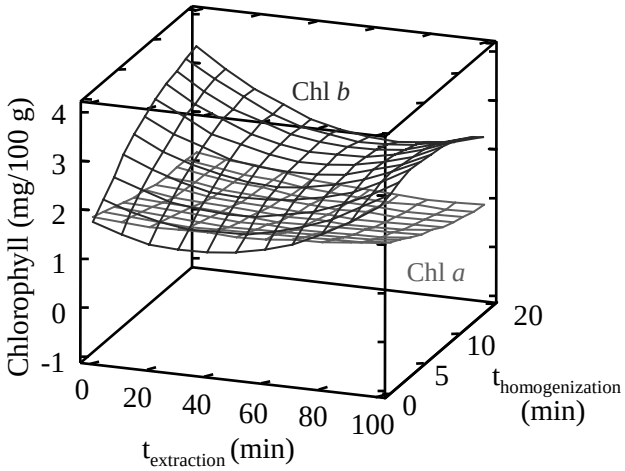


(b) fixing the number of extractions at 3





(c) fixing the number of extractions at 5



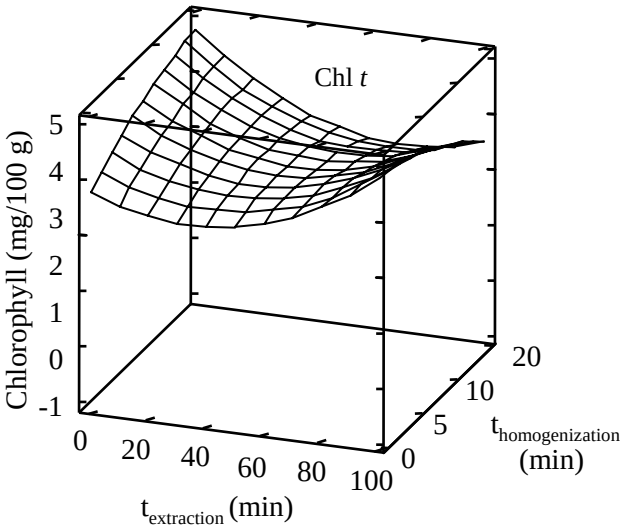


Table 1

Quality parameters for the spectrophotometric determination of chlorophyll *a* and chlorophyll *b*

	Chlorophyll *a*		Chlorophyll *b*	
	λ = 647 nm	λ = 664 nm	λ = 647 nm	λ = 664 nm
slope[a] ($\cdot 10^2$)	2.8 ± 0.2	11.8 ± 0.8	3.5 ± 0.3	0.84 ± 0.07
intercept[a] ($\cdot 10^3$)	3.2 ± 0.1	13.5 ± 0.5	3.4 ± 0.3	1.6 ± 0.1
r^2	0.997	0.999	0.993	0.995
linear range[b] (mg/l)	0.35 - 10.0		1.00 - 35	
detection limit[b] (mg/l)	0.07		0.23	
RSD (%)	7.0		9.2	

[a] Values are the mean ± standard deviation obtained by using seven concentration levels (n = 3 determinations). [b] The linear range and detection limit correspond to the most sensitive wavelength.

Table 2

Design matrix in the central composite design (2^3 + star) and response values of the chlorophyll pigments from green bean

run	$n_{extractions}$	$time_{extraction}$ (min)	$time_{homogenization}$ (min)	mg/100 g green bean		
				Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll
1	1	0	1	0.16 ± 0.03	0.49 ± 0.13	0.65 ± 0.16
2	3	45	20	0.68 ± 0.06	1.14 ± 0.18	1.82 ± 0.25
3	3	45	10.5	1.35 ± 0.56	3.3 ± 1.6	4.7 ± 2.2
4	1	90	1	0.07 ± 0.01	0.16 ± 0.04	0.23 ± 0.06
5	5	45	10.5	1.02 ± 0.23	1.64 ± 0.32	2.7 ± 0.5
6	3	90	10.5	0.99 ± 0.25	2.5 ± 0.9	3.5 ± 1.1
7	3	45	1	1.48 ± 0.13	1.77 ± 0.16	3.2 ± 0.3
8	5	90	1	1.81 ± 0.10	2.5 ± 0.7	4.3 ± 0.8
9	1	90	20	0.50 ± 0.28	1.59 ± 0.93	2.1 ± 1.2
10	5	0	20	1.54 ± 0.17	4.0 ± 0.9	5.6 ± 1.0
11	5	90	20	0.93 ± 0.04	1.95 ± 0.26	2.9 ± 0.3
12	3	45	10.5	0.66 ± 0.04	0.69 ± 0.12	1.34 ± 0.08
13	5	0	1	1.59 ± 0.28	1.45 ± 0.43	3.1 ± 0.7
14	3	0	10.5	1.13 ± 0.31	2.2 ± 0.7	3.3 ± 1.0
15	1	0	20	0.71 ± 0.19	2.3 ± 0.7	3.0 ± 0.9
16	1	45	10.5	0.39 ± 0.38	1.22 ± 1.23	1.60 ± 1.60

Values are the mean ± standard deviation of $n = 3$ determinations.

Table 3

Quantification of chlorophyll *a*, chlorophyll *b* and total chlorophyll in green beans of the cultivars “Donna” (flat pod) and “Negrital” and “Emerite” (round pod) under optimal extraction conditions

Cultivar[a]	Chlorophyll *a* (mg/100 g)	Chlorophyll *b* (mg/100 g)	Total chlorophyll (mg/100 g)
Donna	2.9 ± 0.7 b	4.3 ± 0.8 b	7.1 ± 1.5 b
Negrital	4.2 ± 1.1 b	7.7 ± 1.9 a	11.9 ± 2.9 a
Emerite	6.4 ± 0.2 a	6.8 ± 0.5 a	13.3 ± 0.7 a

Values are the mean ± standard deviation of $n = 3$ determinations.

Within a column (a-b), different letters denote significant differences ($p < 0.05$) between cultivars.