



Article

Optimization of the Enzymatic Extraction of Naringenin from Pink Grapefruit Pulp (*Citrus × paradisi* Macfad.)

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Abstract: Naringenin is one of the main phenolic compounds found in grapefruit (*Citrus × paradisi* Macfad.). This compound is known for its therapeutical properties as an antioxidant, antidiabetic, antihyperlipidemic and antineoplastic agent. In order to enable the development of drugs based on this compound, an appropriate extraction method needs to be developed. For this study, enzymatic extraction was chosen, as it is a cheap and green extraction method. Optimal extraction conditions (pH, temperature, agitation, solvent composition, sample-to-solvent ratio and enzyme-to-sample ratio) were determined through a Plackett–Burman and a Box–Behnken design, resulting in pH 6.0, 40 °C, 50 rpm, 20% EtOH, 0.2 g sample per 15 mL solvent and 1000 U/g. Once extraction conditions were determined, a single-factor experiment was performed under optimal conditions to determine extraction time, which resulted in 10 min per extraction. Finally, repeatability and intermediate precision were evaluated through naringenin quantification. Good values were obtained for both parameters (1.80% and 2.05%, respectively). Furthermore, extracts presented significant amount of naringenin (0.18 ± 0.02 mg/g).

Keywords: *Citrus × paradisi* Lam.; enzymatic extraction; Plackett–Burman; Box–Behnken; naringenin



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1. Introduction

One of the most common strategies when searching lead compounds for drug development is the screening of natural sources as Natural Products (NPs), which are structurally diverse chemical compounds [1]. Furthermore, their structure usually resembles some endogenous compounds [2], which enables them to interact with endogenous receptors, triggering or inactivating some signaling pathways and therefore causing a physiological effect over the organism.

NPs can come from animal [3], microbial [4], vegetal [5,6] and marine sources [7]. The study of vegetal sources of NPs is gaining relevance currently, e.g., aspirin, a widely used analgesic and antipyretic developed from salicin, an NP isolated from the white willow (*Salix alba*) [8].

One of the principal groups belonging to vegetal NPs are phenolic compounds, which are known to have great therapeutical potential due to their antioxidant properties, which make them good candidates for treating illnesses related to oxidative stress such as diabetes, cancer [9] or cardiovascular diseases [10].

Phenolic compounds are awarded this name due to the presence of at least one phenolic group (an aromatic ring with a hydroxyl group) in their structure. This group

is divided into flavonoids, with two aromatic rings in their structure bounded by a 3-carbon bridge [11] and non-flavonoids, a structurally heterogeneous group formed by non-carboxylic phenols and phenolic acids [12].

Citrus fruits flavonoids act as dietary antioxidants by inhibiting the generation of Reactive Oxygen Species (ROS) in the organism. Therefore, they can be used to palliate illnesses related to oxidative stress such as cancer, hypertension, allergies and diabetes [13].

Naringenin (Figure 1) (IUPAC Name: (S)-5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one) [14] is one of the main flavonoids produced by pink grapefruit [15,16], known to have antioxidant and anti-inflammatory properties [17,18] due to its free-radical scavenging ability and its affinity towards some endogenous receptors (Glucose Transporter Type 4 (GLUT4) and Peroxisome Proliferator-Activated Receptor γ (PPAR γ)).

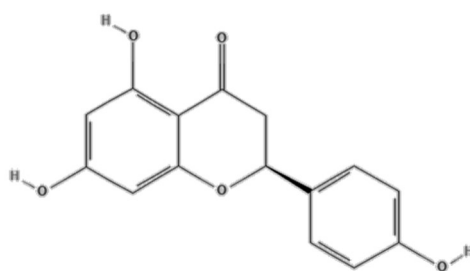


Figure 1. Chemical structure of naringenin [14].

The composition of an extract is highly variable according to the extraction method and the sample employed for its obtention [19]. Therefore, an adequate method needs to be developed before approaching massive extraction.

Enzymatic Extraction (EE) is an efficient method for obtaining bioactive compounds and preserving their integrity. It is based on the catalytic breakage of cell walls and membranes, releasing the cell internal environment to the solvent [20].

Other techniques such as Microwave-Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE) need to be performed under severe conditions, leading to higher resources and energy consumption. Furthermore, high temperatures and extreme pH values can threaten the integrity of phenolic compounds [21] (e.g., naringenin). EE also leads to high extraction yields [22] and can be complemented with other techniques such as UAE [23].

Cellulases and pectinases are the main enzymes used for EE, as cellulose and pectin are the most representative components of vegetal cell walls [24,25]. Apart from the type and amount of enzyme employed, other factors need to be considered for designing the extraction method (solvent pH and composition, temperature, agitation, sample-to-solvent ratio or extraction time) [26].

Considering all the previously mentioned, the aim of this study is to develop an efficient enzymatic extraction method for the obtainment of extracts from *C. × paradisi* containing significant amounts of naringenin.

2. Materials and Methods

2.1. Biological Samples

Pink grapefruits (*Citrus × paradisi*) were acquired from local commerce. Peel and pulp were separated and then lyophilized by means of a LYOALPHA freeze drier (Azbil Telstar Technology, Terrassa, Spain). Afterwards, they were ground with a ZM200 knife mill (Retsch GmbH, Haan, Germany) down to <40 μm particles. Finally, lyophilized samples were stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

2.2. Solvents and Reagents

The solvents used for the extraction were mixes of variable percentages of absolute ethanol (0–60%) EssentQ[®] (Scharlab, Barcelona, Spain) and citrate/phosphate buffers at

pH 4.0, 5.0 and 6.0. Buffers were prepared by solving citric acid pure, pharma grade ($C_6H_8O_7$; 99.5–100.5%) (Panreac S.L.U., Castellar del Vallés, Spain), and sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$; 99–102%) (Panreac S.L.U., Castellar del Vallés, Spain) in Milli-Q water, obtained by filtration through a MilliPore system (Bedford, MA, USA). Solvents were prepared by mixing a determined amount of ethanol, ranging from 0 to 60% solvent volume, and flushed with a citrate/phosphate buffer. Firstly, citrate and sodium dihydrogenphosphate solutions were prepared. Afterwards, buffers were made by combining them and, finally, they were mixed with the corresponding volume of ethanol.

For pH 4.0, buffer was made of 62% volume 0.1 M citrate and 38% 0.2 M sodium dihydrogenphosphate, both solved in Milli-Q water. For pH 5.0, the percentages were 49% and 51%, respectively, and for pH 6.0, 37.4% and 62.5%. Solvents were stored in darkness at room temperature up to two weeks after preparation.

For the EE, a glycerol solution of poly-(1,4- α -D-galacturonide) glycanohydrolase (P4716) from *Aspergillus niger* (25KU; 55 units/mg; Batch SLBT6394) (Merck KgaA, Darmstadt, Germany) was used. The enzymatic solution was stored at 4 °C.

Two solvents were used for the chromatographic separation: HPLC-grade acetonitrile (Panreac S.L.U, Castellar del Vallés, Spain) and Milli-Q water, both acidified with 2% glacial acetic acid (>99.7%; Reag. USP, Ph. Eur.) (Panreac S.L.U, Castellar del Vallés, Spain). To determine the amount of naringenin present in each extract based on the chromatograms obtained, naringenin ($\geq 95\%$; Merck KgaA, Darmstadt, Germany) was used as standard.

2.3. Enzymatic Extraction

2.3.1. Extraction Equipment

To approach the EE, a Nahita LNB001 (Auxilab S.L., Navarra, Spain) incubator was used. The incubator was controlled through a screen, where several parameters (time, temperature and agitation) could be set for each run.

2.3.2. Extraction Procedure

Several factors were considered for the design of the extraction method: solvent composition (0–60% ethanol) and pH (4.0–6.0), agitation (50–200 rpm), temperature (40–60 °C), sample-to-solvent ratio (0.1–0.2 g in 15 mL solvent), enzyme-to-sample ratio (100–1000 U/g) and extraction time (10–60 min).

Firstly, samples were weighted in an analytical balance. Afterwards, they were mixed with the corresponding solvent and amount of enzyme. Then, temperature, time and agitation were introduced into the incubator and extraction was started. After the extraction was completed, extracts underwent centrifugation twice ($1702 \times g$, 5 min). The supernatant was collected, made up to 25 mL and conserved in Falcon tubes until further analysis at -80 °C.

2.4. Naringenin Quantification

An ACQUITY UPLC[®] H-Class System (Waters Corporation, Milford, MA, USA) was used to separate naringenin. It consists of a quaternary elution system (Quaternary Solvent Manager) coupled to a photodiode array detector (PAD eλ Detector). For the separation, an Acquity UPLC[®] BEH C18 (1.7 μ m, 2.1 $\times$ 100 mm, Waters, Milford, MA, USA) column was utilized. The software to control the chromatography equipment was the EmpowerTM 3 (Feature Release 5) (Waters Corporation, Milford, MA, USA) software. The volume of sample injected for each separation was 3.0 μ L and the mobile phase flowed through the column at 0.6 L min⁻¹.

The eluent consisted of a mix of Milli-Q water and HPLC-grade acetonitrile, both acidified with 2% glacial acetic acid. The gradient used was previously described by Yeren-Prieto et al. [27]: 0 min, 0% B; 1 min, 5% B; 2 min, 10% B; 3 min, 15% B; 4 min, 20% B; 5 min, 30% B; 7 min, 35% B; 8 min, 40% B; 10 min, 75% B and 12 min, 0% B. The total analysis time was 15.0 min, including the return to the initial conditions and re-equilibration.

The compounds were quantified at $\lambda = 280$ nm, based on a calibration curve ($y = 14,048x - 10,521$; $R^2 = 0.9943$) made with 1, 2, 4, 5, 10, 20 mg L⁻¹ of naringenin solved in methanol. Results are therefore expressed in terms of milligrams of naringenin per gram of dry pulp.

2.5. Extraction Method

2.5.1. Plackett–Burman Experimental Design

Plackett–Burman Experimental Design is a screening design that allows researchers to determine whether each factor has or not a significant effect over the response variable reducing the number of experiments that are necessary for evaluating the effect of each factor and their significance [28].

It also enables us to reduce the number of factors to be optimized. In the case of this study, it was necessary to perform 12 experiments instead of the 128 (2^7) experiments that would correspond to a factorial design determined by 7 factors and 2 levels (−1.0, lower and 1.0 higher) (Table 1), and only 4 factors needed to be optimized instead of the 7 factors initially considered in the experimental design.

Table 1. Experimental and predicted values for standardized areas based on the Plackett–Burman screening design for the enzymatic extraction.

Run	Factors							Response	
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	Experimental	Predicted
1	1.0	−1.0	1.0	1.0	−1.0	1.0	−1.0	2.90229×10^6	817,103
2	−1.0	1.0	−1.0	−1.0	−1.0	1.0	1.0	9.83132×10^6	8.24124×10^6
3	1.0	−1.0	1.0	−1.0	−1.0	−1.0	1.0	709,272	1.95956×10^6
4	1.0	1.0	1.0	−1.0	1.0	1.0	−1.0	1.72793×10^6	915,971
5	−1.0	1.0	1.0	−1.0	1.0	−1.0	−1.0	364,552	1.17652×10^6
6	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	3.7762×10^6	2.67595×10^6
7	−1.0	1.0	1.0	1.0	−1.0	1.0	1.0	5.09436×10^6	6.47762×10^6
8	−1.0	−1.0	−1.0	1.0	1.0	1.0	−1.0	123,423	1.16689×10^6
9	−1.0	−1.0	1.0	1.0	1.0	−1.0	1.0	744,092	195,729
10	1.0	1.0	−1.0	1.0	1.0	−1.0	1.0	3.87769×10^6	1.94254×10^6
11	1.0	1.0	−1.0	1.0	−1.0	−1.0	−1.0	627,383	2.76935×10^6
12	1.0	−1.0	−1.0	−1.0	1.0	1.0	1.0	537,270	1.97731×10^6

As mentioned in Section 2.3.2, the factors selected for this study were time (X₁, expressed in minutes), pH (X₂), temperature (X₃, expressed in °C), agitation (X₄, expressed in revolutions per minute) solvent composition (X₅, expressed in terms of ethanol percentage), sample-to-solvent ratio (X₆, expressed as grams sample per 15 mL solvent) and enzyme-to-sample ratio (X₇, expressed as units of enzyme per gram sample).

2.5.2. Box–Behnken Experimental Design

Once the factors with the most significant effect were identified, it was necessary to optimize each factor. To accomplish that, a Box–Behnken design determined by 4 factors (temperature, solvent composition, solvent pH and enzyme-to-sample ratio) at 3 levels (−1.0, lower; 0, intermediate; 1.0, higher) (Table 2) was performed.

Through a statistical algorithm, the Box–Behnken design can reduce the number of experiments needed from the 81 (3^4) experiments that would have been needed from the equivalent factorial design to 27. Therefore, the information can be obtained with a relatively reduced number of experiments [29].

Table 2. Experimental and predicted values for standardized areas based on the Box–Behnken surface-response design for the enzymatic extraction.

Run	Factors			Response	
	X ₂	X ₅	X ₇	Experimental	Predicted
1	0.0	0.0	0.0	168,505	225,425
2	1.0	−1.0	0.0	168,483	68,885.1
3	1.0	0.0	1.0	143,378	246,866
4	0.0	−1.0	−1.0	174,563	295,075
5	0.0	1.0	1.0	904,389	783,877
6	0.0	1.0	−1.0	500,997	504,887
7	1.0	1.0	0.0	498,376	515,400
8	−1.0	0.0	1.0	404,093	425,007
9	1.0	0.0	−1.0	189,237	168,323
10	−1.0	0.0	−1.0	397,036	293,548
11	0.0	0.0	0.0	245,588	225,425
12	−1.0	1.0	0.0	504,772	604,370
13	0.0	0.0	0.0	262,182	225,425
14	0.0	−1.0	1.0	229,975	226,086
15	−1.0	−1.0	0.0	300,306	283,282

In this design, the distance from the experimental points to the central ones is ($\alpha = \sqrt{2}$), avoiding the performance of experiments under extremely severe conditions [30], which becomes highly relevant when factors can threaten sample's integrity, as it is the case of thermolabile samples when they undergo extreme temperatures [31]. Due to this fact, this design is called a spherical design.

$$Y = \beta_0 + \sum_{i=1}^k (\beta_i X_i + \beta_{ii} X_i^2) + \sum_{i < j}^n \sum_{i=1}^k \beta_{ij} X_i X_j + r, \quad (1)$$

Box–Behnken design also generates a polynomial model (Equation (1)), useful to predict values for the response variable when a determinate combination of factors is given. In this polynomial, β_i is the coefficient assigned to the main effects, β_{ij} to the interaction effects, β_{ii} to the quadratic factors, x_i and x_j correspond to each factor and r is the residual value.

The R^2 coefficient indicates the fit of the values predicted by the model to the experimental values, and the statistical significance of each factor can be evaluated through an analysis of variance (ANOVA). All experiments were analyzed with the statistical software STATGRAPHICS Version XVI (Statgraphics Technologies, Inc., The Plains, VA, USA).

2.5.3. Determination of the Extraction Time

Once optimal extraction conditions were determined, a single-factor study was approached to determine the optimal extraction time. For that purpose, extractions were performed in triplicate at 2, 5, 10, 15, 20 and 25 min under optimized conditions.

2.5.4. Repeatability and Intermediate Precision

Once all factors were optimized, repeatability and intermediate precision were assessed. A total of 24 extractions under optimal conditions were performed (3 batches of 8 replicates each). Each batch of extractions was performed on a different day.

Repeatability was evaluated by determining the relative standard deviation (RSD) for each batch of extractions (intra-groupal RSD). Intermediate precision corresponds to the inter-groupal RSD, i.e., the RSD among different batches.

2.6. Determination of Naringenin

The amount of naringenin extracted was determined in the extracts obtained under optimal conditions. By doing that, the adequation of the method for obtaining great quantities of naringenin could be evaluated.

For determining naringenin, standard solutions were used as a reference. A retention time of 6.94 min corresponded to naringenin. Results were therefore expressed in terms of milligrams of naringenin per gram of lyophilized pulp.

3. Results and Discussion

3.1. Naringenin Identification

Naringenin was identified based on its chromatogram at 280 nm (Figure 2), considering the compounds detected at a retention time of 6.94 min, as this is the region corresponding to this compound when this chromatographic method is used.

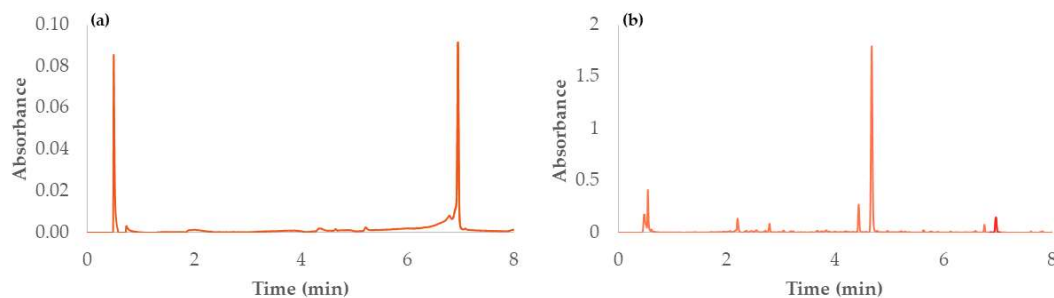


Figure 2. Chromatogram of naringenin standard at $\lambda = 280$ nm (a). Chromatogram of a pulp extract at $\lambda = 280$ nm (b).

The peak corresponding to naringenin is highlighted in red color in the chromatogram of pulp extract (Figure 2b).

3.2. Development of the Extraction Method of Naringenin

3.2.1. Plackett–Burman Experimental Design

Seven factors were considered for the development and optimization of the EE extraction method: time (X_1 , expressed in minutes), pH (X_2), temperature (X_3 , expressed in $^{\circ}\text{C}$), agitation (X_4 , expressed in revolutions per minute), solvent composition (X_5 , expressed in terms of ethanol percentage), sample-to-solvent ratio (X_6 , expressed as grams sample per 15 mL solvent) and enzyme-to-sample ratio (X_7 , expressed as units of enzyme per gram sample).

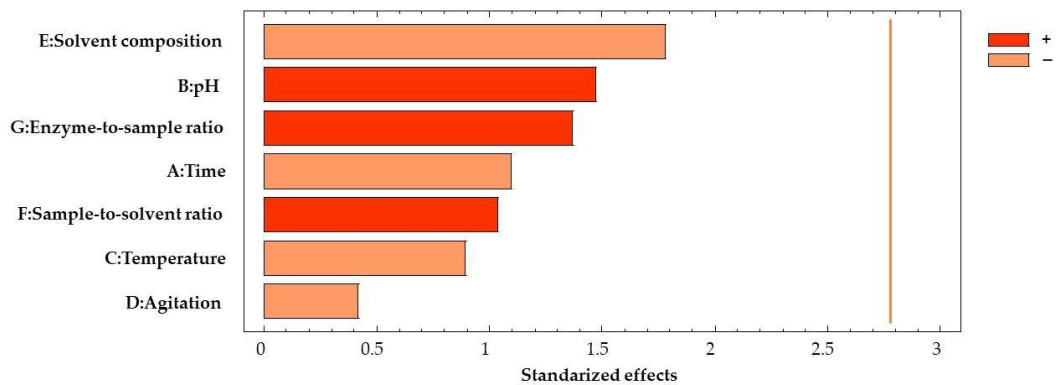
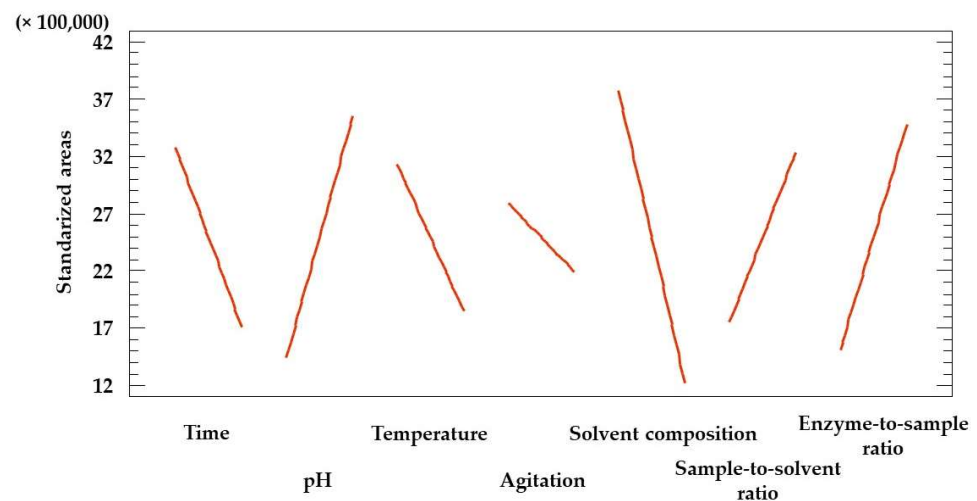
Two levels were considered for each factor in order to determine the effect of each factor over the response variable, expressed as standardized areas (units area per gram of dried pulp; UA/g) of the chromatographic peak of naringenin. The Plackett–Burman screening design therefore implied a total of 12 extractions.

An ANOVA was performed (Table 3) in order to determine the effect of each factor over the standardized areas of naringenin peaks. In the Pareto chart obtained from the statistical analysis (Figure 3), no factor was significant for the extraction yield. However, as only two levels were set for each factor, this design does not study the effect of each factor when intermediate values are considered. Thus, solvent composition (X_5), pH (X_2) and enzyme-to-sample ratio (X_7) were considered for the Box–Behnken design because these factors had the highest effects among all the factors studied. For the rest of the factors, the highest level studied was selected when they showed a positive effect (sample-to-solvent ratio (X_6)) and the lowest level was established when their effect was shown to be negative (time (X_1), temperature (X_3) and agitation (X_4)). All this information was extracted from the main effects plot (Figure 4). Therefore, 40 $^{\circ}\text{C}$, 50 rpm, 0.2 g/15 mL and 10 min were the values chosen for the Box–Behnken design extractions.

$$Y = -1.20964 \times 10^5 - 3.13936 \times 10^4 \times X_1 + 1.05270 \times 10^6 \times X_2 - 6.42033 \times 10^4 \times X_3 - 3.98142 \times 10^3 \times X_4 - 6.37572 \times 10^4 \times X_5 + 1.48935 \times 10^7 \times X_6 + 2.18122 \times 10^3 \times X_7 \quad (2)$$

Table 3. Analysis of variance (ANOVA) for the Plackett–Burman screening design.

Factor	Factor Code	Coefficients	Sum of Squares (10^{11})	Degrees of Freedom	Mean Square (10^{10})	F-Value	p-Value
Model		-1.20964×10^5					
A: Time	X ₁	-3.13936×10^4	7.39×10	1	7.39×10^2	1.20	0.3352
B: pH	X ₂	1.05270×10^6	1.32965×10^2	1	1.32965×10^3	2.16	0.2160
C: Temperature	X ₃	-6.42033×10^4	4.93256×10	1	4.93256×10^2	0.80	0.4218
D: Agitation	X ₄	-3.98142×10^3	1.06999×10	1	1.06999×10^2	0.17	0.6985
E: Solvent composition	X ₅	-6.37572×10^4	1.94922×10^2	1	1.94922×10^3	3.16	0.1501
F: Sample-to-solvent ratio	X ₆	1.48935×10^7	6.62147×10	1	6.62147×10^2	1.07	0.3587
G: Enzyme-to-sample ratio	X ₇	2.18122×10^3	1.15241×10^2	1	1.15241×10^3	1.87	0.2435
Total error			2.46782×10^2	4	6.16954×10^2		
Total correlation			8.86125×10^2	11			

**Figure 3.** Pareto chart of the Plackett–Burman screening design.**Figure 4.** Plot representing the main effects for the seven factors screened in the Plackett–Burman design.

The statistical analysis also generates a polynomial (Equation (2)), with a R^2 coefficient of 72.15%. This value means that the fit of the model is not high enough for the model to be considered as adequate to predict the standardized areas obtained from a determined combination of factors.

3.2.2. Box–Behnken Experimental Design

An ANOVA was performed to identify whether any of the simple, interaction and quadratic effects were significant (Table 4). Only pH had a significant effect over the extraction yield (Figure 5). The rest of the factors were not significant (p -values > 0.05), as their effects remained under the signification threshold (2.57).

Table 4. ANOVA for the Box–Behnken surface-response design.

Factor	Factor Code	Coefficients	Sum of Squares (10^{11})	Degrees of Freedom	Mean Square (10^{10})	F-Value	p-Value
Model		4.11482×10^6					
A: Solvent composition	X_5	-8.17423×10^3	4.60157×10^{-1}	1	4.60157	3.00	0.1440
B: pH	X_2	-1.53516×10^6	2.94607	1	2.94607×10	19.18	0.0072
C: Enzyme-to-sample ratio	X_7	-1.17820×10^3	2.20502×10^{-1}	1	2.20502	1.44	0.2845
AA	X_5^2	-3.31072×10	6.47534×10^{-3}	1	6.47534×10^{-2}	0.04	0.8454
AB	$X_5 X_2$	1.56784×10^3	3.93297×10^{-2}	1	3.93297×10^{-1}	0.26	0.6343
AC	$X_5 X_7$	−1.46991	7.00051×10^{-3}	1	7.00051×10^{-2}	0.05	0.8394
BB	X_2^2	155802×10^5	8.96281×10^{-1}	1	8.96281	5.84	0.0604
BC	$X_2 X_7$	1.93322×10^2	3.02724×10^{-1}	1	3.02724	1.97	0.2193
CC	X_7^2	3.51872×10^{-1}	1.87464×10^{-1}	1	1.87464	1.22	0.3195
Lack of fit			7.17897×10^{-1}	3	2.39299	9.58	0.0960
Pure error			4.99752×10^{-2}	2	2.49876×10^{-1}		
Total correlation			5.80282	14			

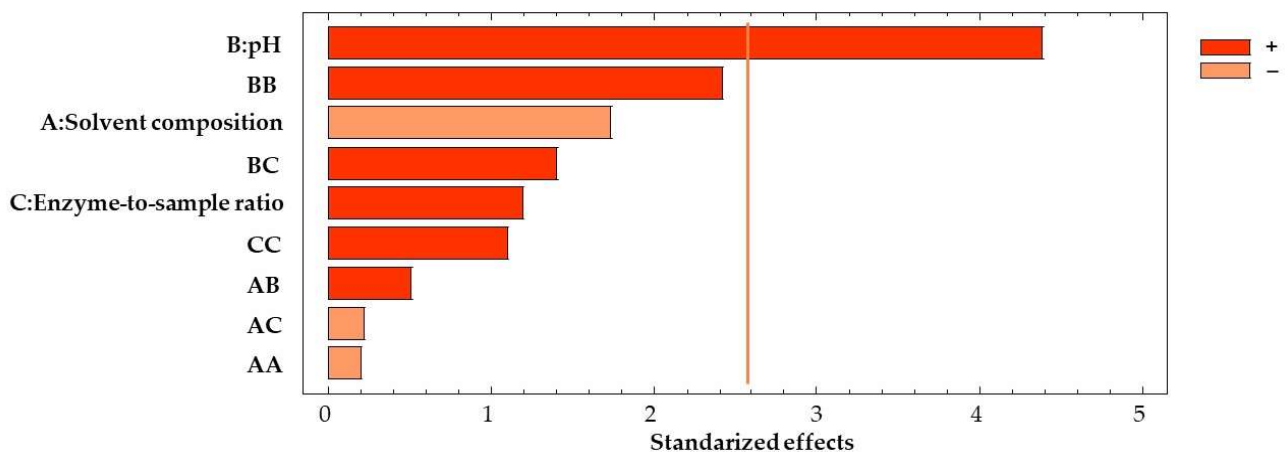


Figure 5. Pareto diagram of the Box–Behnken surface-response design.

In Figure 6, the optimal values for each of the variables optimized could be determined. Nevertheless, this graph only considers the individual effect for each factor. It does not include the interaction and the quadratic effects, so the real optimal values may differ from the values displayed by this graph. It can be observed that optimal solvent composition corresponds to the solvent with the minimum amount of ethanol (20%) employed for the experimental design, while pH and enzyme-to-sample ratio are optimized at their highest level (pH 6.0 and 1000 U/g).

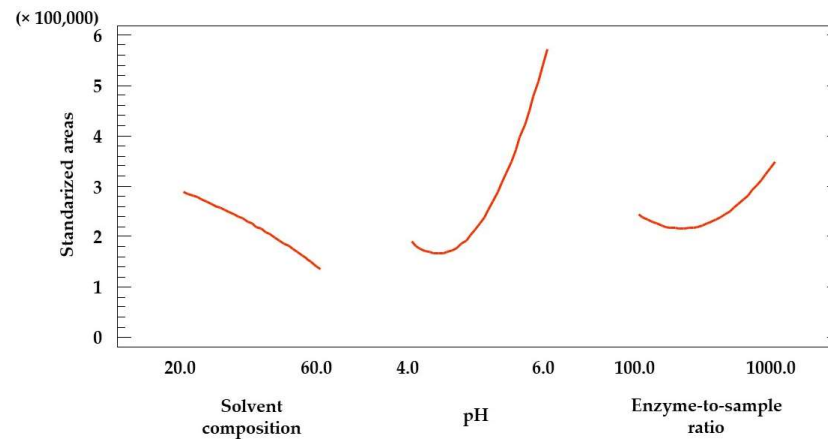


Figure 6. Plot representing the main effects of solvent composition, enzyme-to-sample ratio, pH and temperature.

For the remaining factors, the optimal values were assigned based on whether their effect was shown to be positive or negative for the extraction. For the former, it was determined that their optimal value corresponds to their highest level (1.0) and for the latter, the optimal value was set at their lowest level (−1.0). All these values are summarized in Table 5.

Table 5. Optimal values for enzymatic extraction.

Factor	Optimal Value
pH	6.0
Temperature (°C)	40
Agitation (rpm)	50
Solvent composition (%EtOH)	20
Sample-to-solvent ratio (g/15 mL)	0.2
Enzyme-to-sample ratio (U/g)	1000

As mentioned before, optimal values for agitation, temperature and sample-to-solvent ratio were set based on the main effects plot obtained from Plackett–Burman design (Figure 4).

Optimal pH (6.0) corresponds to the maximum value considered for this study. Despite the fact that extraction yield for pectinases decreases at pH values higher than 5.0, the combined effect of pH with other factors might alter this optimal. No higher values were considered for this study as the optimal value indicated by the technical bullet of the commercial pectinases indicates that the optimal pH should remain near 4.0 for this enzyme.

Optimal solvent composition (20% EtOH) matches with the minimum value studied. However, as it did not prove to be a significant factor, no further studies were performed.

Finally, optimal enzyme-to-sample ratio (1000 U/g) is the maximum value studied. Other studies suggest that high enzyme amounts lead to higher extraction yields until saturation is reached [32].

$$Y = 4.11482 \times 10^6 - 8.17423 \times 10^3 \times X_5 - 1.53516 \times 10^6 \times X_2 - 1.1782 \times 10^3 \times X_7 - 3.31072 \times 10 \times X_5^2 + 1.56784 \times 10^3 \times X_2 \times X_5 - 1.46991 \times X_5 \times X_7 + 1.55802 \times 10^5 \times X_2^2 + 1.93322 \times 10^2 \times X_2 \times X_7 + 3.51872 \times 10^{-1} \times X_7^2 \quad (3)$$

The polynomial (Equation (3)) obtained has an R^2 coefficient of 86.76%, which means that the predictability of this model is higher than the obtained for Equation (2). However, it is not high enough to consider the model as a good method to predict values for the response variable based on a determinate combination of values for each factor.

3.2.3. Determination of the Extraction Time

Once the method was optimized, a single-factor experiment was performed to determine extraction time. This experiment consisted of extractions in triplicate performed at 2, 5, 10, 15, 20 and 25 min. The results obtained are displayed in Figure 7.

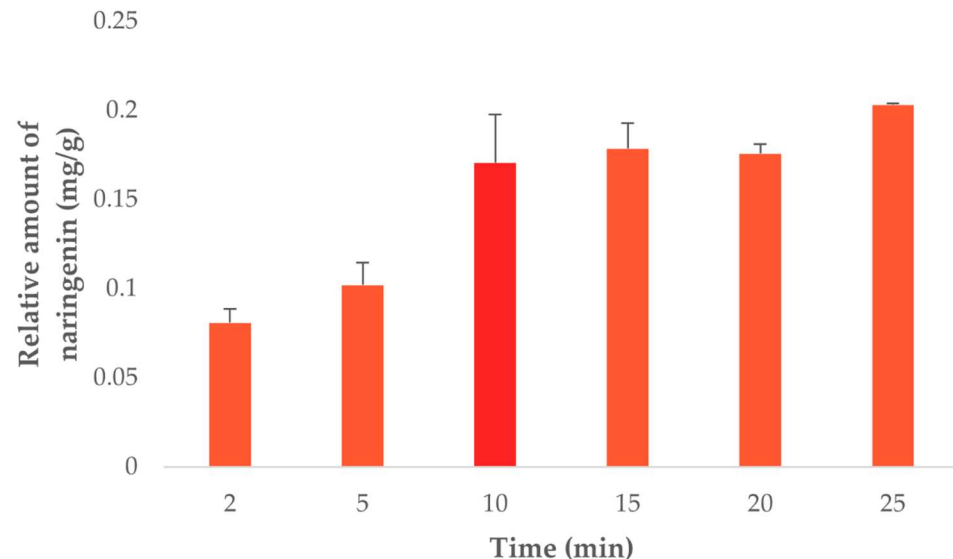


Figure 7. Single-factor study for the determination of the extraction time.

An analysis of variance (ANOVA) was applied to the obtained data in order to determine whether there were some significant differences between the yields obtained at the different times. As there were no significant differences between the extraction yields obtained at 10, 15, 20 and 25 min (p -values > 0.05), 10 min was accepted as the best extraction time as it implies lower energy and resources consumption.

3.2.4. Repeatability and Intermediate Precision

After determining optimal conditions, repeatability and intermediate precision were measured, resulting in 1.80% and 2.05%, respectively. As both values remain under 5%, the extraction method has good values for repeatability and intermediate precision [33].

3.3. Determination of Naringenin

As mentioned in Section 3.1, naringenin was determined according to its chromatogram at 280 nm (Figure 2). The amount of naringenin contained in the extracts was assessed by a chromatographic method, obtaining a mean value of 0.18 ± 0.02 mg naringenin per gram of dry sample.

Compared to other studies where comparable extraction yields of naringenin are obtained [34], the method described here is more eco-friendly as it employs 15 mL of an aqueous–organic mixture for the extraction instead of 60 mL of an organic solvent. It also consumes less energy than the extraction method described by Shin, K-C. et al. [35], where, despite obtaining higher relative amounts of naringenin, long extraction times (5 h) and higher temperatures (60 °C) are needed.

This extraction method is also faster than other methods previously developed as it needs a total processing time of 20 min per sample, compared to the 3 days employed in the method described by Takanaga, H. et al. [36]. Furthermore, even employing higher temperatures (100 °C) and longer extraction times (60 min), other extraction techniques such as Heat Reflux Extraction (HRE) report lower amounts of naringenin in grapefruit extracts [37].

Owing to all the above mentioned factors, this method results in a greener extraction method, as it employs lower amounts of organic solvents and consumes less energy, which

also makes it cheaper than other previously developed methods. It is also a rapid method with soft extraction conditions, which guarantees the preservation of the sample integrity.

Furthermore, this method is also applicable to other matrixes as polarity and degradability of naringenin rely on the extraction conditions, which would remain constant. It could be used, for example, to extract naringenin from other matrixes such as Mexican oregano (*Lippia graveolens*) [38].

4. Conclusions

The method described herein enables the obtention of grapefruit extracts with significant amounts of naringenin (0.18 ± 0.02 mg naringenin/g). This method also proves to be rapid, as it requires approximately 20 min per sample, and greener than previously described methods as it employs low amounts of organic solvents and low temperatures for the extraction. All the above mentioned factors make this a simpler and cheaper method for the acquisition of naringenin. Furthermore, the method proved to be repeatable (1.80%) and to have good intermediate precision values (2.05%).

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