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Improving Knowledge of Garlic Paste Greening through the Design of an Experimental Strategy

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The furthering of scientific knowledge depends in part upon the reproducibility of experimental results. When experimental conditions are not set with sufficient precision, the resulting background noise often leads to poorly reproduced and even faulty experiments. An example of the catastrophic consequences of this background noise can be found in the design of strategies for the development of solutions aimed at preventing garlic paste greening, where reported results are contradictory. To avoid such consequences, this paper presents a two-step strategy based on the concept of experimental design. In the first step, the critical factors inherent to the problem are identified, using a 2_{III}^{7-4} Plackett–Burman experimental design, from a list of seven apparent critical factors (ACF); subsequently, the critical factors thus identified are considered as the factors to be optimized (FO), and optimization is performed using a Box and Wilson experimental design to identify the stationary point of the system. Optimal conditions for preventing garlic greening are examined after analysis of the complex process of green-pigment development, which involves both chemical and enzymatic reactions and is strongly influenced by pH, with an overall pH optimum of 4.5. The critical step in the greening process is the synthesis of thiosulfinates (allicin) from cysteine sulfoxides (alliin). Cysteine inhibits the greening process at this critical stage; no greening precursors are formed in the presence of around 1% cysteine. However, the optimal conditions for greening prevention are very sensitive both to the type of garlic and to manufacturing conditions. This suggests that optimal solutions for garlic greening prevention should be sought on a case-by-case basis, using the strategy presented here.

KEYWORDS: Alliin; alliinase; cysteine; garlic paste; greening; optimization; thiosulfinate

INTRODUCTION

Color change during food processing is a major technological problem for the food industry. In particular, chopped garlic and garlic paste are adversely affected by browning and greening (*1*, *2*). Both of these are complex processes involving multiple enzyme and nonenzymatic reactions. Browning is a well-documented phenomenon affecting numerous food products, and the underlying chemical and biochemical processes involved are understood. Four major pathways lead to browning: (a) dicetone polymerization; (b) condensation and polymerization of dicetones with amino acids, where polyphenol oxidase plays a major role in transforming polyphenols into dicetones; (c) condensation and polymerization of amino acids with reducing sugars (Maillard reaction); and (d) ascorbic acid oxidation and polymerization.

Garlic discoloration, also known as "greening", is a major problem during the processing and storage of garlic products. The overall process is very complex and involves multiple compounds that undergo a number of enzyme and nonenzyme reactions. The greening process is known to be triggered by the action of the enzyme alliinase (EC 4.4.1.4) on (+)-*S*-2-propenyl-L-cysteine sulfoxide (alliin), to yield 2-propene-sulfenic acid. Two molecules of sulfenic acid then react nonenzymatically to yield 2-propene-thiosulfinate (allicin). Alliinase also acts on (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (isoalliin), which finally yields a thiosulfinate known as a color developer. This color developer reacts chemically with an amino acid to form a pigment precursor. Finally, the pigment precursor reacts nonenzymatically with allicin to form blue pigments (*2*–*8*).

A number of solutions have been proposed over the last 30 years, most involving the addition of substances that interfere with the chemistry and biochemistry of browning and greening. Reports show that several substances have been used to prevent or inhibit either browning or greening in processed garlic, including citric acid (*3*), ascorbic acid (*9*, *10*), cysteine (*4*, *11*, *12*), sodium metabisulfite (*13*–*15*), and EDTA (*10*). These substances interfere with discoloration processes at various levels. Citric

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acid is essentially an acidulant that prevents the proliferation of many microorganisms and also reduces the activity of most enzymes, whose pH optimum is around neutral. It is also a chelating agent that scavenges certain metal cations required as enzyme cofactors. Ascorbic acid is an antioxidant that prevents oxidative chemical processes taking place in processed food; it also has a specific action on polyphenol oxidase (PPO), being able to reduce dicetones back to polyphenol, thus reverting the reaction catalyzed by PPO. Sodium metabisulfite is also an antioxidant that inhibits the oxidation processes required for polymerization and browning (*16*). Cysteine is an amino acid with antioxidant properties, but most importantly it reacts with dicetones, thus blocking other condensation and polymerization reactions that lead to color development. EDTA is a versatile metal-chelating agent, useful to inactivate enzymes; it also prevents browning processes specifically related to ascorbic acid oxidation.

On the other hand, design of experiments (DOE) is a very powerful technique for the application of the scientific method, because it is a scientific approach which allows the experimenter to understand a process and to determine how the input variables (factors) affect output variables (responses). However, this potent tool is largely ignored by the scientific community. Plackett-Burman experimental design (PB-d), described on the basis of Hadamard matrices (*17*), and other two-level fractional factorial designs $2^{(k-p)}$ probably rank among the most powerful tools available for problem-solving based on the scientific method. Yet, despite these virtues, researchers appear to be largely unaware of PB-d, with the result that these tools are rarely used. Prvan and Street (*18*), for example, report that fractional factorial design was used in only 140 out of 6 million papers over a 5-year period (1997–2001), according to Science Citation Index (SCI) data. This report proves that researchers rarely use a statistical method such as DOE (*19*). Miller and Sitter (*20*) seek to explain this by suggesting that researchers find it very difficult or even impossible to extract information from PB-d. In view of this general situation, the aim of this paper is to present the full process to be followed in order to apply the scientific method to the solution of a given problem using the most adequate experimental design at each stage of the process.

According to earlier published data, prevention of garlic greening has been approached in a number of ways (**Table 1**). However, preliminary assays have shown that these approaches are not applicable to experimental conditions other than those in which they were developed. Since scientific knowledge continually grows on the basis of previously published data, this paper sought to apply an experimental design strategy to detect the key factors governing the garlic paste greening process and to optimize the modified process for the prevention of this discoloration.

MATERIALS AND METHODS

Samples and Process. Fresh garlic (*Allium sativum*, cultivar white basic) bulbs were supplied as peeled cloves by a company from Montalbán (Córdoba, Spain) and stored at 2–5 °C for a nine-month postharvest period until preparation of paste in the pilot plant. Thus, in all cases, dormancy-terminated bulbs were used, since greening is known to occur only once dormancy has finished (*21*); low-temperature storage was considered advisable to ensure that greening took place (*2*).

Aliquots of 500–600 g were weighed and ground in a Siemens 800 compact food processor (Siemens AG, Berlin, Germany) for 3 min to obtain garlic paste. Finally, aliquots of 300 ± 3 g of garlic paste and ingredients added—following the experimental design described below—at each experimental phase were blended for 5 min in a Stomacher 400 laboratory blender (Seward Ltd., U.K.), and the final mixture was bottled in crystal bottles with metal caps.

Table 1. Different Garlic Paste Greening Prevention Actions Previously Reported

action	source	level, range, or value
pH adjustment	(*2*)	4.0–4.3
	(*13*)	4.0
	(*32, 33, 51, 58*)	4.1
	(*57*)	4.9–5.9
citric acid	(*2*)	0.2 M
	(*3*)	1%
	(*13*)	1.000 ppm
	(*32*)	data not shown
	(*33*)	data not shown
	(*59*)	2.000 ppm
L-ascorbic acid	(*10*)	900 ppm
	(*59*)	500 ppm
cysteine	(*10*)	60 ppm
	(*22*)	data not shown
sodium disulfite	(*10*)	5.000 ppm
	(*13*)	3.000 ppm
EDTA	(*10*)	200 ppm
action on garlic bulbs	(*2*)	strorage for a month at 23 °C
	(*59*)	remove embryos or inner shoots
sanitation step (soaking)	(*13*)	NaHClO 200 ppm + citric acid 600 ppm
oil addition	(*22*)	data not shown
	(*59*)	0.1 mL/g of garlic paste

Table 2. Phase 1: Direct Optimization on the Basis of Published Data. Level of Key Additives Added to Garlic Paste as Percentages Added to Garlic Paste at the Box–Behnken Experimental Design

		symbol		level		
factor	unit	coded	uncoded	−1	0	+1
citric acid	%	X_1	Φ_1	0	1.50	3.00
ascorbic acid	%	X_2	Φ_2	0	0.50	1.00
cysteine	%	X_3	Φ_3	0	0.15	0.30
EDTA	%	X_4	Φ_4	0	0.10	0.20

Chemicals. All chemicals used were of analytical grade. Citric acid was purchased from Panreac Quimica SA (Barcelona, Spain); L-ascorbic acid, from Merck KgaA (Darmstadt, Germany); L-cysteine chlorhydrate, from Guinama (Valencia, Spain); and EDTA, from Merck KgaA (Darmstadt, Germany).

Experimental Design. *Phase 1. Direct Optimization on the Basis of Published Data.* Since the main effects of four factors have been described elsewhere (*3*, *9–15*, *22*), the previously established hypothesis regarding the additives preventing the greening process was taken as valid. Assuming that the most important factors (MIFs) governing the greening process were as identified by other authors, the aim was to optimize the solution directly in order to prevent greening. This was achieved using a particular type of $3^{(k-p)}$ experimental designs known as Box–Behnken (*23*) designs (BB-d), which are particularly useful when the necessary experimental runs are expensive to perform. Box and Behnken (*23*) derived a series of three-level second-order designs that has become very popular because, as in central composite designs (also called Box and Wilson experimental designs), BB-d are response surface designs that can be fitted to a full quadratic model. However, unlike most central composite designs, BB-d use just three levels per factor so that factor combinations lie at the midpoints of edges of the experimental space and at the center. **Table 2** shows the experimental design levels considered for each apparent key factor in preventing greening.

Because NaCl is able to stabilize the alliinase dimer (*24*) and since the garlic/water ratio may be a critical parameter in discoloration processes (*25*), both ingredients were kept constant: 5% (w/w) of NaCl and 5% (w/w) of tap water as common additives or ingredients.

Table 3. Phase 2: Key Factor Detection or Screening. Seven Apparent Critical Factors (ACF) Considered for the Two-Level Plackett–Burman Experimental Design at Resolution *III* [a], and Experimental Ranges Expressed in Coded and Actual Units

	symbol		levels	
ACF	code	actual	−1	+1
citric acid (%)	X_1	C_A	1	3
sodium disulfite (%)	X_2	S_2O_5	0	0.3
cysteine (%)	X_3	cys	0.6	1.2
sanitation treatment	X_4	S_T	no	yes
olive oil (5%)	X_5	oil	no	yes
hydroxylamine (%)	X_6	amine	0	0.07
benzoic acid (%)	X_7	B_A	0	0.1

[a] Confusion structure:

$X_1 = X_1 + X_2 \times X_4 + X_3 \times X_5 + X_6 \times X_7$
$X_2 = X_2 + X_1 \times X_4 + X_3 \times X_6 + X_5 \times X_7$
$X_3 = X_3 + X_1 \times X_5 + X_2 \times X_6 + X_4 \times X_7$
$X_4 = X_4 + X_1 \times X_2 + X_3 \times X_7 + X_5 \times X_6$
$X_5 = X_5 + X_1 \times X_3 + X_2 \times X_7 + X_4 \times X_6$
$X_6 = X_6 + X_1 \times X_7 + X_2 \times X_3 + X_4 \times X_5$
$X_7 = X_7 + X_1 \times X_6 + X_2 \times X_5 + X_3 \times X_4$

Table 4. Phase 3: Optimization under Specific Experimental Conditions. Two Key Factors To Be Optimized (FOs) in a Central Composite Experimental Design, with Experimental Ranges Expressed in Coded and Actual Units

	symbol		levels				
FO	code	actual	−1.41	−1	0	1	1.41
sodium disulfite (%)	X_1	S_2O_5	0.00	0.15	0.45	0.75	0.90
cysteine (%)	X_2	Cys	0.48	0.60	0.90	1.20	1.32

Phase 2. Detection of Key Factors (Screening). On the basis of published data (**Table 1**), seven apparent critical factors (ACFs) were selected with a view to preventing greening: citric acid (C_A), sodium disulphite (S_2O_5), cysteine (cys), sanitation treatment (S_T), olive oil addition (Oil), hydroxylamine (amine), and benzoic acid (B_A). **Table 3** shows the experimental design levels considered for each ACF. The aim was to determine whether a linear function (first-order model) of stepping factors (**Table 3**) would fit the kinetics of the greening process, in other words, to identify the key factors for greening prevention. For garlic paste manufacture, a 2^{7-4}_{III} Plackett–Burman experimental design was used as a screening design, which was highly fractionated (here, at resolution *III*, meaning that 4 of the 7 factors studied were generated by the interactions of a full 2^3 full factorial design). This experimental design allows the study of a large number of variables (up to 7) with a small number of trials, such as 8 experiments (*26*). Because the resolution of the fractionated experimental design was *III*, main or primary effects were confounded (overlapped) with some secondary effects, as shown by the confusion structure presented in **Table 3**.

Phase 3. Optimization for Specific Conditions. The simplicity of the screening phase lies in the assumption of a linear relationship between the settings of the factors and the greening process; however, the optimization phase requires a more complex experimental design including more than two points per factor, since a quadratic model must be obtained. Thus, once the key factors have been identified, other statistical techniques such as response surface methodology (RSM) may be used to optimize levels. After the screening process, as will be seen in the Results and Discussion sections, the number of key factors to be optimized (FOs) turned out to be just 2. For this reason, it was possible to use a central composite design (CCD) (*27*), which is a sum of a two-level factorial design and a star design, to obtain 5 levels per factor (*28*), as shown in **Table 4**.

For each MIF, ACF, and FO, a X_i coded independent variable was generated (where i = 1–4, 1–7, and 1–2, respectively), since it is advisable to transform natural variables into coded variables, and these coded variables are usually defined as dimensionless with mean zero and the same spread or standard deviation (*29*). Each MIF, ACF, and FO (independent variables) was coded according to the equation $X_i = (x_i - x_i^*)/\Delta x_i$, where X_i is the coded value of the ith independent variable, x_i is the uncoded value of the ith independent variable, x_i* is the uncoded value of the ith independent variable at the central point, and Δx_i is the step change value (*29*).

For optimization processes from both MIFs and FOs, each response variable was assumed to be influenced by four independent variables or MIFs at phase 1 (citric acid, ascorbic acid, cysteine, and EDTA) such as Φ_i (i = from 1 to 4), so that $\xi_m = f(\Phi_1,\Phi_2,\Phi_3,\Phi_4)$, where ξ is each response (m = from 1 to 5), Φ_1 is the proportion of citric acid formulated, Φ_2 is the proportion of ascorbic acid formulated, Φ_3 is the proportion of cysteine formulated, and Φ_4 is the proportion of EDTA formulated. Similarly, it was assumed that each response variable was influenced by two independent variables or FOs at phase 3 (sodium disulfite and cysteine) such as δ_i (i = from 1 to 2), so that $\xi_m = f(\delta_1,\delta_2)$, where ξ is each response (m = from 1 to 5), δ_1 is the proportion of sodium disulfite formulated and δ_2 is the proportion of cysteine.

The basic analysis, for a response surface experiment to obtain process optimization, consisted of fitting a quadratic model of the form $\xi = b_0 + \sum_{i=1}^{p} b_i X_i + \sum_{i=1}^{p} b_{ii} X_i^2 + \sum_{j=2}^{p} \sum_{i=1}^{j-1} b_{ij} X_i X_j + \epsilon$ where ξ is each response, X_i are factors or key ingredients (MIFs or FOs) considered for each garlic paste trial as coded independent variables, X_iX_j are the two factor interactions, b_0 is the intercept, b_i, b_{ii}, and b_{ij} are linear, quadratic, and cross-product regression terms, respectively, and ϵ is the error of model.

Finally, for all experimental designs, runs were performed in random order (trial order) because randomization allows the experimenter to avoid erroneous conclusions due to extraneous sources of variability (*30*, *31*).

Responses. Five responses were measured: color parameters such as L or lightness, and both a and b chromaticity coordinates (every three days); pH (at initial time (day 0), middle time (day 15), and final time (day 30)); and gas formation (at day 3). Both Statistica (StatSoft, Inc., Tulsa) and Design-Expert (Stat-Ease, Inc., Minneapolis) software were used to generate designs, fit the response surface model to the experimental data, and draw response surface figures. Differences were considered significant when $p < 0.05$.

pH Determinations. A mix of paste/water (1/10, w/w) was prepared, and pH was measured with a Crison GLP22 pH-meter.

Gas Production. A semiquantitative, rapid, and easy method of evaluating the amount of gas produced was used to measure paste evacuated when a bottle was opened at day 3. The amount of gas generated was estimated indirectly by quantifying the amount of paste evacuated when the bottle was opened. Gas formation was measured using a semiquantitative scale from 0 to 3, where 0 meant no gas generated (no paste expelled at bottle opening), 1 meant 1–20 g of garlic paste expelled, 2 meant 21–40 g of paste expelled, and 3 meant 41–60 g of paste expelled.

Color Measurements. Color was measured every 3 days over the 30-day experimental period. Although some authors have obtained paste color by placing an aliquot of paste on a Petri dish (*10*), a different procedure was used here. Color was measured through the bottom of the bottles to avoid bottle opening, because oxidation occurs rapidly on the paste surface after bottles are opened for sampling, and this would interfere with the effects of the various ingredients considered in experimental designs, thus yielding confusing results. The mean of three independent color measurements was considered as the final paste color. Color was determined using a Chroma meter CR-400 (Minolta, Japan) and by means of L, a, and b Hunter coordinates. Green-to-red and blue-to-yellow color dimensions corresponded to Hunter a and b parameters, and negative a values and positive b values were used to identify garlic paste greening.

RESULTS

Phase 1. Direct Optimization on the Basis of Published Data. Data on color modification, gas generation, and pH evolution under the experimental conditions tested are shown in **Table 5** and **Figures 1** and **2**. Most trials showed an inhibition of the greening process with respect to controls (**Table 2**).

Table 5. Phase 1: Direct Optimization on the Basis of Published Data[a]

run	trial	X_1	X_2	X_3	X_4	pH_0	gas_3	L_3	a_3	b_3
3/>	1	−1	1	0	0	5.34	1	39.58	−2.46	13.18
23	2	0	1	0	−1	4.12	1	45.22	−2.81	11.62
1	3	−1	−1	0	0	6.55	1	40.17	0.91	13.01
2	4	1	−1	0	0	4.45	0	40.17	−4.79	10.83
7	5	0	0	−1	1	4.24	1	43.26	−1.07	11.3
15	6	0	1	−1	0	3.97	0	44.13	−2.05	11.13
8	7	0	0	1	1	4.25	1	42.67	−1.41	10.96
17	8	0	1	1	0	4.23	0	44.87	−2.29	11.45
11	9	1	0	0	−1	3.75	0	44.37	−3.16	11.38
21	10	1	0	1	0	3.80	0	44.07	−2.82	10.79
14	11	0	−1	−1	0	4.45	0	40.48	−5.21	10.40
13	12	1	0	0	1	3.77	0	43.48	−2.09	10.98
20	13	−1	0	1	0	6.05	2	42.94	−0.67	13.68
22	14	0	−1	0	−1	4.45	0	39.22	−5.62	9.88
6	15	0	0	1	−1	4.34	0	45.22	−3.04	11.48
4	16	1	1	0	0	3.82	0	44.62	−2.62	10.90
10	17	−1	0	0	−1	6.16	3	42.26	−0.25	14.66
12	18	−1	0	0	1	6.15	3	42.21	−0.30	14.14
25	19	0	1	0	1	4.17	0	44.04	−1.36	10.74
9	20	0	0	0	0	4.28	0	43.66	−2.34	11.38
16	21	0	−1	1	0	4.55	0	40.57	−4.86	10.37
5	22	0	0	−1	−1	4.23	0	42.77	−2.87	11.37
18	23	−1	0	−1	0	6.01	3	38.17	−1.43	12.91
19	24	1	0	−1	0	3.75	0	43.71	−2.54	10.87
24	25	0	−1	0	1	4.50	0	40.48	−4.50	10.75

[a] Results obtained in garlic paste for pH at day 0, gas at day 3, and *L*, *a*, and *b* color parameters at day 3. The actual names of the X_i factors are as shown in **Table 2**.

Greening only appeared in trials 4, 11, 14, 21, and 25 (**Figure 1A**), in which parameter *a* returned to initial values (disappearance of green color) after a 30-day period, in agreement with previous results (*32*, *33*). However, taste and aroma deteriorated in these trials, due to the appearance of off-odors in garlic paste. Even in those trials where the greening process was inhibited, the solution of this problem prompted the appearance of a new one: all these trials displayed browning (**Figure 1A**), a highly complex phenomenon affecting many processed foods and involving a number of chemical and biochemical reactions (*3–5*).

In view of these results, it was concluded that, at least under the experimental conditions tested, garlic paste greening cannot be prevented with the solutions suggested by other authors (*1–3*, *6*, *9–15*, *22*). Nevertheless, the information obtained here may help to provide a better understanding of the nature of the problem and to find a real and effective solution.

Given that differences between the two sample groups (those displaying browning and those displaying greening) were evident as early as day 3 (**Figure 1A**), the effect of each factor on responses at day 3 was calculated. Under the testing conditions used (**Table 2**), the most important factor was citric acid (effect = 2.56, $p \leq 0.05$), which exerted significant effects on all the responses shown in the table. The second most important factor was ascorbic acid (effect = 2.36, $p \leq 0.05$), used at one-third the concentration of citric acid (**Table 2**), while cysteine and EDTA had no significant effects. Although it has been reported that metals do not have a significant influence on the formation of green pigments (*5*), some earlier authors suggested that EDTA prevented greening significantly (*10*), so EDTA was included in this phase. The present results do not support the conclusion obtained by Kim et al. (*10*), but they do tally with those obtained by Kubec et al. (*5*).

pH is mainly determined by factor X_1, particularly for values of $X_1 > 0$, while factor X_2 is relevant only when $X_1 < 0$. Citric acid had the strongest effect on pH at time zero (pH_0), being 4.5 times stronger than ascorbic acid. The value of pH at time zero (pH_0) critically determined gas generation in garlic paste

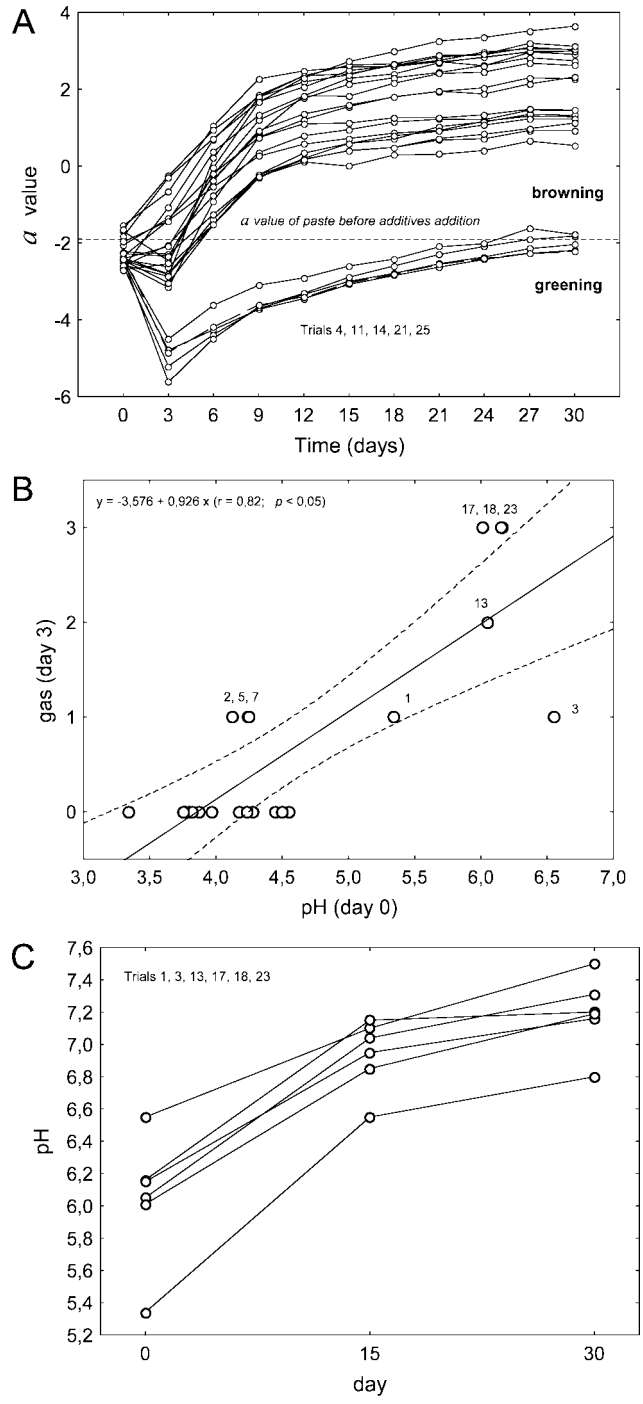

Figure 1. Phase 1: Direct optimization on the basis of published data. Results of Box–Behnken experimental design: (**A**) behavior of *a* values over the 30-day period; (**B**) gas production as a function of initial pH value (pH at day 0); (**C**) pH behavior over the 30-day period for selected trials (trials 1, 3, 13, 18, and 23, in the order shown in **Table 5**).

(**Figure 1B**), so that gas was always produced at pH values above 5.0 (trials 1, 3, 13, 17, 18, and 23, for $X_1 = -1$), while gas was seldom produced within the pH_0 interval between 4.0 and 5.0 (trials 2, 5, and 7, for $X_1 = 0$, and variable levels for remaining factors); only when $pH_0 > 5.0$ did garlic paste become more alkaline over the 30-day period of the experiment (**Figure 1C**). Clearly, gas formation in stored garlic paste is dependent on paste pH during manufacture.

The results obtained here suggest that the initial pH of garlic paste critically determined the behavior of the two sample groups. Group I included samples where $pH_0 > 5.0$ (**Figure**

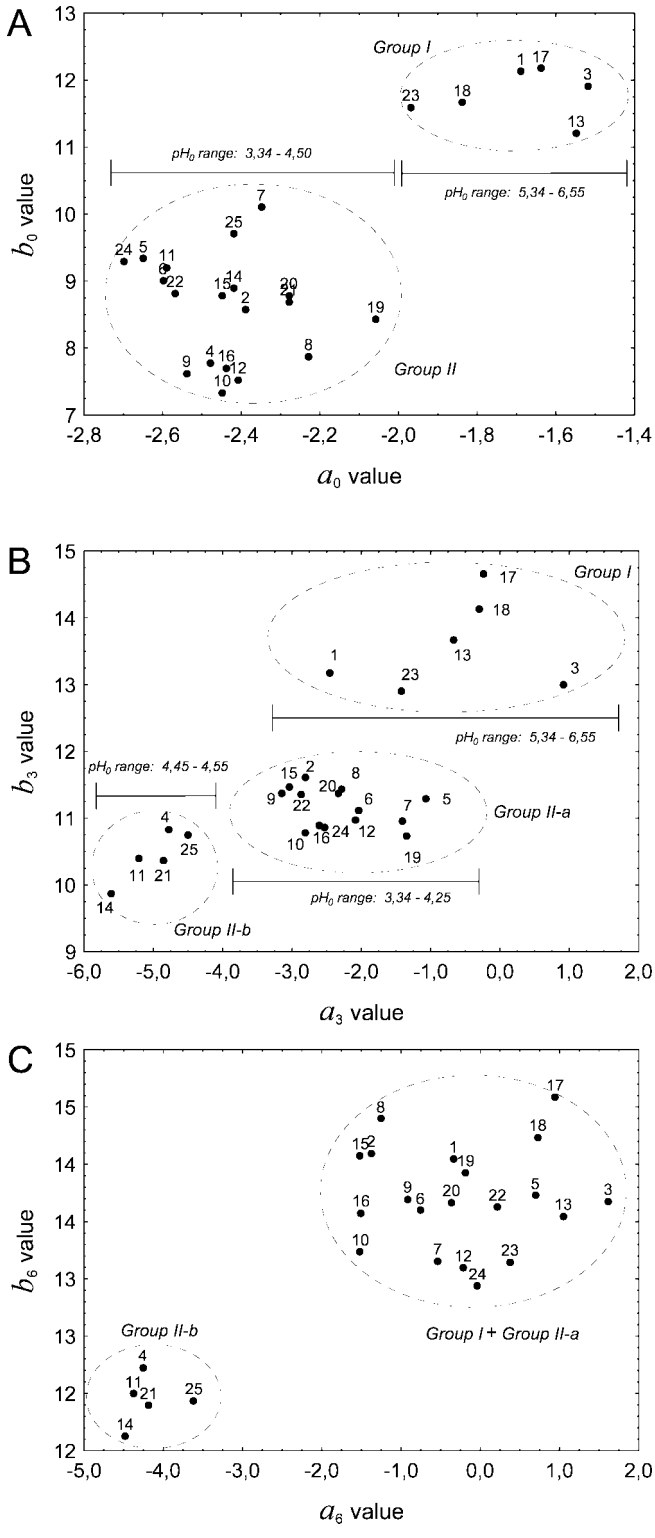

Figure 2. Phase 1: Direct optimization on the basis of published data. Evolution of garlic paste color at days 0, 3, and 6 (**A**, **B**, and **C**, respectively) as a function of Hunter coordinates.

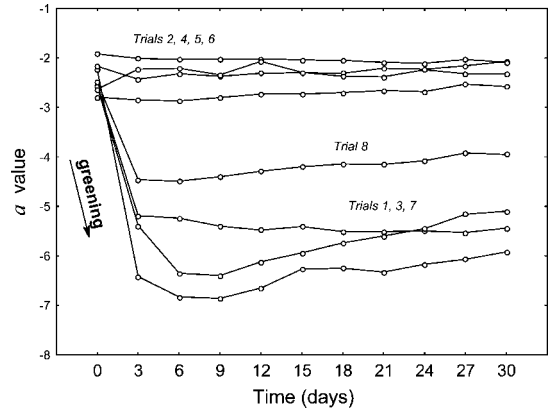

Figure 3. Phase 2: Key factor detection or screening. Behavior of the *a* value during the 30-day period using the 2_{III}^{7-4} Plackett–Burman experimental design results.

2A); these samples did not contain citric acid ($X_1 = -1$, **Table 5**), but they underwent alkalinization during storage (**Figure 1C**) and gas was generated (**Figure 1B**). Group II included those samples whose pH_0 was between 3.34 and 4.50; little or no gas was produced (**Figures 1B** and **2A**). With regard to alkalinization and gas production in the samples belonging to this group (1, 3, 13, 17, 18, and 23), although a slight acidification (not related to microbial growth) has been detected in pickled garlic (*34*, *35*), the alkalinization process observed here has not previously been reported. However, due to the antimicrobial effect of garlic (*36*, *37*), it may readily be assumed that alkalinization and gas production were not related to microbial growth and may be ascribed to the fact that thiosulfinate production via alliinase renders two NH_4^+ molecules (*38*), although at pH 6.5–6.7 no gas production has been detected in garlic cloves subjected to a prior sanitation process involving soaking in a sodium disulfite/citric acid solution (*13*). It is thus reasonable to assume a possible connection between gas production and microbial growth. Both hypotheses should be studied separately.

Color evolution at day 3 enabled discrimination between two subgroups of samples within group II. Group II-b included samples that displayed greening (**Figures 1A** and **2B**); pH_0 was 4.5 ± 0.5 in this group. Group II-a included samples that underwent browning (**Figures 1A** and **2B**). Finally, at day 6 two groups were clearly formed (**Figure 2C**).

All in all, the results suggest that the presence of ascorbic acid as an additive favors garlic paste browning, while greening requires a pH of 4.5 ± 0.5. In the absence of citric acid, pH_0 is above 5.0, which in turns prompts alkalinization and gas production during garlic paste storage.

Phase 2. Key Factor Detection (Screening). Results obtained at this phase are shown in **Table 6**. In accordance with Miller and Sitter's criteria (*20*), results in this phase will be discussed on the basis of three empirical principles, i.e. the sparsity principle (only a small number of the candidate factors (ACFs) will be critical), the hierarchy principle (primary effects are more likely to be critical than secondary effects from two-factor interactions, which in turn are more likely to be critical than three-factor interactions, and so forth), and the heredity principle (it is unusual for an interaction to be critical unless at least one of the factors involved has an active primary critical effect).

Since garlic paste greening was evident 6 days after paste manufacture (**Figure 3**), effects of each ACF on a_6 were calculated at this time, including 0.13, 1.10, 3.37, 0.16, −0.49, −0.63, and 0.03 for X_1, X_2, X_3, X_4, X_5, X_6, and X_7, respectively. Because the resolution of the experimental design was *III*, the main effects were mixed with some interactions (**Table 3**). Any true interactions could be readily detected using a simple graphical method described previously (*39*); however, no active interactions were detected at this phase of the present experiment.

For an adequate interpretation of effects, it was borne in mind that a negative value for the *a* coordinate indicated a predisposition to greening, while a positive value indicated greening prevention. This meant that both oil and amine addition could

Table 6. Phase 2: Key Factor Detection or Screening. Experimental Design 2^{7-4}_{III} and Response Values[a]

run	trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	R_1	R_2
5	1	1	−1	−1	−1	−1	1	1	−6.35	3.70
6	2	1	−1	1	−1	1	−1	−1	−2.87	3.77
1	3	−1	−1	−1	1	1	1	−1	−6.83	4.45
8	4	1	1	1	1	1	1	1	−2.21	3.70
2	5	−1	−1	1	1	−1	−1	1	−2.32	4.50
4	6	−1	1	1	−1	−1	1	−1	−2.03	4.57
3	7	−1	1	−1	−1	1	−1	1	−5.24	4.57
7	8	1	1	−1	1	−1	−1	−1	−4.49	3.70

[a] R_1 = *a* Hunter coordinate at day 6 postmanufacture; R_2 = pH of garlic paste at manufacture day. The actual names of X_i factors are as shown in **Table 3**.

Table 7. Phase 3: Optimization under Specific Experimental Conditions. Central Composite Experimental Design and Results Obtained for the pH of Garlic Paste (R_1) and *a* Hunter Coordinates at Day 6 (R_2) and at Day 30 (R_3)[a]

run	trial	X_1	X_2	R_1	R_2	R_3
5	1	−1.41	0	3.52	−2.36	−1.98
2	2	−1	1	3.36	−2.01	−2.06
9	3	0	0	3.54	−2.17	−2.15
6	4	1.41	0	3.58	−2.08	−2.12
1	5	0	−1.41	3.55	−4.89	−3.95
7	6	−1	−1	3.64	−3.10	−2.64
10	7	0	0	3.50	−1.99	−2.03
3	8	1	−1	3.50	−2.92	−2.60
8	9	0	1.41	3.50	−2.17	−2.15
4	10	1	1	3.44	−1.88	−1.93

[a] The actual names of the X_i factors are as shown in **Table 4**.

be discarded, because their presence in the paste causes a reduction of the *a* value and a concomitant increase in greening.

With regard to the effect of olive oil, the stability of allyl thiosulfinates is known to be influenced by the presence of oils (*22*). On the other hand, the effect of hydroxylamine may be explained by the fact that hydroxylamine can reduce alliinase activity only by roughly 50% (*40*), and irreversibly (*41*). Because alliinase degrades *S*-alkyl-L-cysteine sulfoxides, if alliinase activity is partially inactivated, less thiosulfinates such as allicin will be produced, which in turns enhances the greening process.

By contrast, effects with a positive sign increased the *a* value and, therefore, reduced or prevented the greening process; thus, since the effect of S_T was positive (0.16) and this factor was a qualitative variable, S_T was included in the next optimization step as a constant step in the process. With regard to the factor C_A, since a pH of around 4.5 was obtained with a 1% level and greening was observed (see results at phase 1), the 3% level (mean pH = 3.7) was taken as a constant step in the process when planning the optimization phase. Another positive effect was detected for B_A (0.03), but because it was very slight, this candidate factor was discarded in the optimization phase.

Thus, on the basis of the initial ACF set established in accordance with the scientific literature (**Table 3**) and in view of the results obtained during the screening phase, two active factors were kept constant in the optimization process: citric acid (3% w/w) and a sanitation treatment as described by Carbonell et al. (*13*). Therefore, two quantitative and active effects were considered as FOs: Cys (effect 3.37) and S_2O_5 (effect 1.10), which are, incidentally, two sulfur compounds.

Phase 3. Optimization under Specific Conditions. Using the central composite design shown in **Table 4**, the results obtained for the most relevant responses (pH, a_6, and a_{30}) are shown in **Table 7**, and the effect obtained for each term of the quadratic model is given in **Table 8**. The fitness of the model

Table 8. Phase 3: Optimization under Specific Experimental Conditions. Effects in Terms of Coded Factors on the pH of Garlic Paste (R_1) and *a* Hunter Coordinates at Day 6 (R_2) and at Day 30 (R_3)[a]

	responses		
factor	R_1	R_2	R_3
X_1-lineal	0.01	0.18	−0.04
X_1-quadratic	0.00	0.06	0.19
X_2-lineal	−0.11	1.49[b]	0.94[b]
X_2-quadratic	−0.02	−1.25[b]	−0.85[b]
X_1X_2	0.11	−0.02	0.04
R^2	0.65	0.91	0.89
R^2_{adj}	0.22	0.79	0.77

[a] The actual names of the X_i factors are as shown in **Table 4**. [b] Significant effects ($p \leq 0.05$).

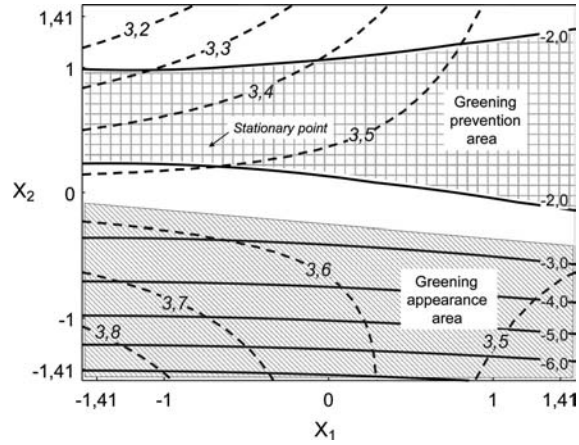

Figure 4. Phase 3: Optimization under specific experimental conditions. Evaluation of greening appearance and greening prevention areas on the basis of *a* Hunter coordinates (continuous lines) and garlic paste pH (discontinuous lines) as a function of both sodium disulfite (X_1) and cysteine (X_2).

was checked by the determination coefficient (R^2), and because $R^2 \times 100$ explains the percentage of variability of the results that can be predicted by the model, it is clear that none of the FOs were critical factors in modifying the pH of garlic paste (**Table 8**). In fact, according to the results obtained in phase 1, citric acid had the strongest influence on the pH of garlic paste and this factor was kept constant (at 3% level) in the optimization phase, so the pH range was narrow (from 3.36 to 3.61). However, both linear and quadratic significant effects were detected for the Cys FO (**Table 8**). Comparison of the effects on a_6 and on a_{30} showed that the intensity of both effects decreased with time (**Table 8**). Because the fitness of the model for predicting greening was good (see R^2 in **Table 8**), it was possible to obtain the stationary point of the system (saddle point), identified for S_2O_5 at 0.3% and for Cys at 1.09% or 1090 mg/100 g (**Figure 4**), with −1.91 being the predicted value in solution for a_6. Most of this cysteine must be consumed quickly in chemical reactions, since the concentration of free cysteine determined immediately after garlic paste manufacture was as low as 0.05 mg/100 g (data not shown).

In addition to identifying a solution for the prevention of garlic paste greening, the most striking finding in the optimization phase was that greening also appeared at pH values ≠ 4.5 (**Figure 3**). Results obtained in phase 1 showed that this pH value was critical for greening development. This correlation between greening and pH supports the view that both chemical and enzyme reactions underlie the greening process: the various chemical and enzyme reactions have specific optimum pH values, resulting in a global pH optimum of 4.5 for the entire

process (*42*). In fact Lawson et al. (*4*) showed that all dipropenyl thiosulfinates, precursors of green pigments, were formed at an optimum pH of 4.5–5.0. Moreover, the stability of thiosulfinates is greatest at pH 4.5–5.5 (*43*). Isoalliin has been identified as a necessary cysteine sulfoxide for developing a dark blue color (*5*), and Ichikawa et al. (*44*) report a very low first order rate constant (k) for the degradation of isoalliin (0.027 day^{-1} at pH 4.6 and 25 °C), while at pH 6.5 the k value is increased by a factor of 5.5; so, a high level of greening precursors at pH 4.5 would account for earlier (phase 1) results relating greening with a pH of 4.5.

DISCUSSION

Garlic alliinase has an optimum pH of 7.0 (*45*), whereas the pH of garlic paste affected by greening is very close to 4.5; therefore, alliinase activity would not seem to be necessary for the greening process (*6*), even though this enzyme is released in an active form after garlic crushing (*46*).

Lukes (*2*) has reported that *S*-(1-propenyl)cysteine sulfoxide is one of the major participants in the chemical reactions responsible for garlic greening, while Kubec et al. (*5*) have shown that isoalliin and alliin are involved in blue color formation *in vitro* in the presence of alliinase. This points to the involvement of allyl thiosulfinates, derived from alliin and isoalliin either enzymatically or nonenzymatically, in the greening process.

The nonenzymatic derivation appears to be more relevant, due to the difference in optimum pH between garlic allinase and garlic paste affected by greening. Moreover, other authors have shown that, to optimize the manufacture of blue pigment by allicin and alliinase reactions, pH must be adjusted to 4.0, and under these conditions, these authors believe that the enzyme is isoelectrically precipitated (*48*). However, the isoelectric point has been reported at 6.35, close to the optimum alliinase pH (*47*). Therefore, the observations reported by Sawada et al. (*48*) are more likely to be due to the lack of cofactor than to the isoelectric precipitation of the enzyme cofactor (*38*). In fact, at pH 4, this cofactor is almost 30 times less soluble than that at pH 7, and substrate and free enzyme compete for soluble pyridoxal phosphate (*49*). A lack of cofactor would yield an inactive enzyme, although Sawada et al. (*48*) justify this inactivation by enzyme isoelectric precipitation. In addition, the titration curve of allicin shows an isoelectric point of 5.6 (*50*). Given all these assumptions, and the fact that a pH of 4.5 ± 0.5 is required for the greening process to occur, it seems very unlikely that thiosulfinates are produced enzymatically during garlic paste storage (**Figures 1A** and **2C**). Nonenzymatic production of thiosulfinates may thus have some influence on greening, but it is unlikely to be the only factor involved.

Reports on strategies to avoid loss of quality in manufactured garlic are contradictory. For example, although citric acid has been proposed as a browning suppressor (*3*), Kim et al. (*10*) found that it had no effect on greening suppression. Similar contradictory reports involve the effect of cysteine as an efficient (*22*) or inefficient greening suppressor (*10*). In addition, L-ascorbic acid has been presented as a suppressor of both greening and browning. Kim et al. (*10*) reported that 0.06‰ cysteine or 0.09% L-ascorbic acid could prevent both browning and greening. Here, however, over the experimental range 0–0.3% for cysteine and 0–1.0% for L-ascorbic acid (**Table 2**), no preventive effects were noted. Similar conclusions are to be reached from a comparison of the present results with those obtained by Ahmed and Shivhare (*51*), Carbonell et al. (*13*), Lawson and Gardner (*22*), and Kim et al. (*10*). These results suggest that other undetermined factors (either factors intrinsically related to garlic composition or factors related to garlic processing) have a major influence on garlic paste greening.

Allium sp. contains a high concentration of sulfur compounds, most of them nonprotein sulfur amino acids (between 1 and 5% on a dry weight basis). The most abundant sulfur compounds in garlic bulbs are three γ-glutamyl peptides: γ-L-glutamyl-*S*-(2-propenyl)-L-cysteine (GSAC), γ-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine (GSPC), and γ-L-glutamyl-*S*-methyl-L-cysteine (GSMC). These γ-glutamyl peptides have their corresponding cysteine sulfoxide derivatives (flavor precursors): (+)-*S*-allyl-L-cysteine sulfoxide (alliin or 2-PeCSO), (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (isoalliin or 1-PeCSO), and (+)-*S*-methyl-L-cysteine sulfoxide (methiin), respectively (*44*, *52*). Upon garlic bulb crushing, allyl-sulfoxides (alliin and isoalliin) are rapidly converted to a number of sulfur-containing compounds. The process begins with the action of alliinase on allyl-sulfoxides. Alliinase catalyzes a β-elimination–deamination reaction, involving an aminoacyl intermediate bound to a pyridoxal-5′-phosphate cofactor, to yield allyl-sulfenic acid. Two molecules of allyl-sulfenic acid then react nonenzymatically to yield allyl-thiosulfinate (*7*), and the most abundant allyl-thiosulfinate (69–73%) is allicin (2-propene-thiosulfinate) (*22*).

Alliinase also catalyzes the conversion of isoalliin into another thiosulfinate known as a color developer. The color developer reacts chemically with an amino acid to form a blue-pigment precursor, which finally reacts nonenzymatically with allicin to form the blue-green pigments (*8*, *42*). Therefore, any discussion of the effect of cysteine on garlic greening prevention should take into account the interference of this amino acid at different points in the greening process.

Thiosulfinates react significantly with cysteine. When cysteine is present at a high concentration, allicin rapidly reacts with two molecules of cysteine to form two molecules of *S*-allyl-L-cysteine, so that allicin levels rapidly decrease (*53*). In addition, a high concentration of cysteine would also inhibit the synthesis of new thiosulfinates (*41*). This inhibitory effect is related to *S*-allyl-L-mercaptocysteine, a competitive inhibitor of alliinase; through the reaction between allicin and cysteine and a series of disulfide/sulfide interconversion reactions, multiple cysteine *S*-conjugates are formed such as *S*-allyl-L-cysteine (SAC), *S*-allylmercapto-L-cysteine (SAMC), *S*-methylmercapto-L-cysteine, *S*-propylmercapto-L-cysteine, *S*-penta-1,3-dienylmercapto-L-cysteine, and ajocysteine (*7*). The different amounts of cysteine added to garlic paste in the optimization phase (phase 3) using the Box and Wilson experimental design would lead to different proportions of these compounds, so that the relative abundance of *S*-allyl-L-mercaptocysteine, and the overall inhibitory effect of added cysteine, would be the result of a complex equilibrium. The inhibitory effect of cysteine on the greening process may also be related to the fact that alliin reacts with compounds containing a free -SH group such as thioacetic acid, hydrogen sulfide, and cysteine (*54*), so that added cysteine causes a reduction of the level of alliin and a reduction in the greening process.

Although a recent study of 11 garlic varieties showed no significant differences in total free amino acid content among these varieties, free cysteine did vary, ranging from 52.8 to 397.9 mg/100 g (*55*). The cysteine added to garlic paste according to the Box and Wilson experimental design (**Table 3**) meant an increase in natural cysteine levels by 48, 60, 90, 120, and 132 mg/100 g for each level of FO (**Table 3**), and the optimum value (stationary point) for this FO resulted in an increase of

the background level by 1090 mg/100 g. Interestingly, higher levels of cysteine had a negative effect on greening prevention, because this FO showed a significant negative quadratic level (**Table 8**). This negative effect at higher concentrations of cysteine could be due to an enhancement of alliin and isoalliin synthesis, as previously shown by Prince et al. (*56*).

All these arguments support the idea that not only the absolute concentration but also the relative abundance of cysteine in garlic paste, with respect to the precursors of the blue-green pigments, is critical for the prevention of the greening process. Under the present experimental conditions, 1.09% cysteine prevented garlic paste greening. However, the optimal concentration of added cysteine for greening prevention could vary depending upon garlic variety, culture conditions, garlic bulb storage conditions, and the actual conditions of garlic processing for the manufacture of garlic paste, since the relative concentrations of all the precursors and enzymes involved in the production of blue pigments depend on all the variable parameters discussed above (*2*, *24*, *57*).

A similar caveat must be made when considering the effect of garlic paste pH on greening prevention. At phase 1, a pH of 4.5 was found to be essential for greening development (see results for phase 1), as suggested by other authors (*6*). However, in the present paper, greening was also seen to occur at lower pH, within the range 3.6–3.8 (**Figure 4**). Thus, the complexity of the problem may give rise to different pH optima for garlic paste greening prevention depending on garlic variety, culture conditions, garlic bulb storage conditions, and the actual protocol of garlic processing. Nevertheless, a critical factor governing greening development is the presence of an active alliiase in the garlic paste at least for a sufficient time to transform alliin and isoalliin into thiosulfinates. An irreversible inactivation of alliinase at very low pH (i.e., pH < 3.0) before or during paste manufacture should completely inhibit garlic paste greening. This could be achieved by grinding garlic cloves in the presence of an acidulant, so that the substrate is never exposed to an active alliinase.

As an overall conclusion, the results obtained regarding the effects of cysteine and pH on greening development by application of the scientific method suggest two alternative strategies to prevent greening: (1) inactivate or inhibit aliinase activity or (2) divert the precursors of the blue pigments through other chemical pathways. However, these theoretical solutions cannot be automatically implemented in every case. Specific optimal solutions must be identified for each case. An optimization strategy such as that described here may be of great value in finding an optimal solution.

The authors intend to apply this optimization strategy to other cases, with a view to identifying critical factors other than the use of additives; these will include garlic variety, culture and storage conditions, and garlic processing.

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