ABSTRACT: Washing of fresh-cut spinach with aqueous chlorine solutions eliminates foreign matter, removes cellular fluids produced by cutting and reduces the initial microbial contamination. Response Surface Methodology was used to evaluate the effect of initial chlorine concentration (25 to 125 ppm), water-to-produce ratio (10 to 26 L/kg) and washing time (2 to 8 min) on the total and available chlorine depletion and the total microbial count reduction after washing. All variables affect the chlorine depletion but the microbial count reduction was only influenced by initial chlorine concentration and washing time. Different combinations of the variables were determined by the predictive models to reduce microbial contamination assuring residual levels of chlorine in the washing solution.

Keywords: washing, chlorine, fresh-cut spinach

Introduction

Producing fresh-cut vegetables usually involves cleaning, trimming, coring, slicing, shredding, washing, centrifugal drying, and packaging (Yildiz 1994).

Shelf life and safety are both important aspects of the microbiological quality of fresh-cut vegetables (Brackett 1992, Nguyen-the and Carlin 1994). Bolin and others (1977) reported that the initial microbial load of shredded lettuce influenced the storage life of the product. Besides eliminating soil residues, plant debris, and nutrient rich cellular fluids, washing permits the reduction of the initial microbial load. The efficacy of this operation is often improved by the inclusion of antimicrobials in the wash water. Many antimicrobial wash solutions have been reported but probably the most widely used for vegetables is a hypochlorite solution containing 50 to 100 ppm available chlorine (Adams and others 1989, Zhuang and others 1995, Mermelstein 1998, Izumi 1999).

Mazollier (1988) reported a reduction of the mesophilic aerobic microflora of 1.6 to 2.4 log cycles for chlorine concentrations between 50 and 200 ppm and washing times between 5 and 20 min during washing of green salad leaves. Garg and others (1990) reported a reduction of approximately 2.7 log cycles when dipping lettuce in ice water containing 300 ppm available chlorine in commercial fresh-cut vegetable processing lines. Adams and others (1989) achieved about 1.7 log cycle reduction in mesophilic aerobic bacteria counts when washing lettuce leaves in 100 ppm available chlorine solution (pH 9).

Generally, a certain initial concentration of chlorine is recommended, without specifying whether the chlorine is total or free. The water-to-produce ratio and time of contact that will permit an effective microbial reduction may also be unspecified.

Sometimes, fresh-cut processors measure the total chlorine instead of available chlorine. So, it is useful to know how important the difference is. This difference depends on the amount of organic matter, which reacts with free chlorine (resulting in combined chlorine) during washing.

Our objectives were: (a) to study the effects of operational variables (initial chlorine concentration, water-to-produce ratio and washing time) on the available and total chlorine depletion and total microbial reduction on washing of fresh-cut spinach and (b) to find conditions to improve this operation.

Materials and Methods

Spinach processing

Spinach (Hybrid 424 cv.) was obtained from a local farm near Santa Fe (Argentina), transported without refrigeration and immediately processed in the laboratory (no more than 10 h after being harvested). Outer and damaged leaves were removed and the rest were chopped by hand to 2 cm wide by 2 to 3 cm long, with a sharp stainless steel knife. Samples of cut spinach were taken to determine the viable bacteria counts before washing. Washing tests were performed using a 2 L glass beaker placed on an agitated ice-water bath to maintain a constant temperature of 4 ± 0.5 °C in the washing solutions. Samples (50 g) were successively placed in a stainless steel basket in such a way that all the produce was submerged in the washing water. The contents were then continuously stirred moving the basket up-and-down at a rate of 60-times/min. Spinach was washed with selected initial chlorine concentrations, times and ratios, according to the experimental design, allowed to drain during 5 min, placed in a plastic container and submitted to microbiological analysis.

The washing solutions were prepared by adding appropriate amount of 60 g/L commercial solution of sodium hypochlorite to 0.1 mol/L potassium phosphate buffer (pH 7.0). Samples of washing solutions were taken at the beginning and at the end of each experiment to evaluate free and total chlorine concentrations.

Experimental design

Response surface methodology (RSM) was employed to investigate washing of fresh-cut spinach. RSM is a statistical method that uses quantitative experimental data from appropriate experimental design to determine and solve multivariate equations. Other researchers have used RSM and second order polynomial equations to evaluate microbial viabi-
Ranges of variables were selected based on practical concerns. Table 1 shows the coded and uncoded variables and their levels. The selected responses were: available (free) chlorine concentration after washing (AC), total chlorine concentration after washing (TC), and total microbial count reduction (MR). Total microbial count reduction (MR) is expressed as (-log 10 Nt/No), where Nt is the viable bacteria count after washing (CFU/g) and No is the viable bacteria count before washing (CFU/g).

A mathematical function f_k (k = 1,2,3) was generated for each response variable Y_k (AC, TC and MR) in terms of three independent processing variables:

\[ Y_k = b_{0k} + b_{1k}X_1 + b_{2k}X_2 + b_{3k}X_3 + b_{11k}X_1^2 + b_{12k}X_1X_2 + b_{13k}X_1X_3 + b_{22k}X_2^2 + b_{33k}X_3^2 \]

where b_{0k}, b_{1k}, b_{2k}, b_{3k}, b_{11k}, b_{12k}, b_{13k}, b_{22k}, and b_{33k} are regression coefficients and X_i the coded independent variables, linearly related to C, t and r. Table 1 shows the coded and uncoded variables and their levels. Ranges of variables were selected based on practical concerns.

Chlorine analysis

Total and free (available) chlorine concentrations were determined by the N, N-diethyl-p-phenylenediamine (DPD) colorimetric method (APHA 1998) using a Spectronic Genesys 5 spectrophotometer (Rochester, N.Y., U.S.A.). The standard curve was generated from a series of potassium permanganate solutions covering the chlorine equivalent range from 0.05 to 4 ppm. The experimental samples were appropriately diluted with chlorine demand-free water to contain less than 4 ppm.

Microbiological analysis

Shredded spinach (10 g) was mixed with 90 mL 0.1% peptone water and homogenized with a Stomacher Lab-Blender 400 (London, UK) for 2 min. Further decimal dilutions, as required, were prepared with the same diluent and plated, in duplicate, on PCA and incubated during 48 h at 30 °C. Viable counts were reported as colony forming units per gram (CFU/g).

Verification

For the model verification (AC, TC, and MR) an experiment on pilot plant, using predetermined conditions, was done. The responses were compared to values predicted by the models.

Statistical analysis

Statgraphics Plus 7.1 (Manugistics, Inc., Rockville, M.D., U.S.A.) was used to perform analysis of variance, to fit the second-order polynomial equations to experimental data and to generate response surface and contour plots. Tests to verify that the residuals satisfied the assumptions of normality, independence, and randomness were also evaluated. Differences between experimental pilot plant results and predicted values were determined by a t-test analysis.

Results and Discussion

TABLE 2 SHOWS EXPERIMENTAL RESULTS OBTAINED FOR THE available chlorine (AC), total chlorine (TC), and total microbial count reduction (MR) under different treatment conditions (coded, refer to Table 1 for coding) of initial chlorine concentration, water-to-produce ratio and washing time of fresh-cut spinach.

First, the assumptions underlying the ANOVA test were investigated. Normal probability plot of residuals, plot of residuals against estimated values for the responses, and plot of residuals against random order of runs revealed that the residuals satisfied the assumptions of normality, independence, and randomness. Therefore, the ANOVA (Table 3) was performed on the full models for each response (AC, TC, and MR) and the residual (total error) was separated into lack of fit and pure error to have an indication of whether the model appears to fit the data or not.

None of the predicted models were shown to have significant lack of fit (P > 0.05) and the coefficients of determination (R^2) were satisfactory (0.998 for AC, 0.997 for TC, and
Based on all these tests, the proposed models were accepted. The regression coefficients \( b_{ki} \) are presented in Table 4.

### Available and total chlorine

The linear terms of initial chlorine concentration \( X_1 \), water-to-produce ratio \( X_2 \), and time \( X_3 \) had a significant effect \( (P \leq 0.01) \) on the available chlorine concentration model \( (AC) \). The concentration-ratio \( X_1 \cdot X_2 \) and ratio-time \( X_2 \cdot X_3 \) interaction terms had also a significant effect \( (P \leq 0.05 \text{ and } P \leq 0.10, \text{ respectively}) \) but none of the other terms were significant for the AC model (Table 3).

If a simpler model is preferred, the nonsignificant terms could be removed from the full model. In this case, the reduced model for available chlorine concentration has an \( R^2 = 0.992 \), which is still an excellent coefficient of determination.

Figure 1 shows response contour plots for AC obtained using full predicted model. Contours represent lines of equal available chlorine after washing as a function of time and water-to-produce ratio. As shown in Figure 1a (C = 25 ppm), when water-to-produce ratio is low and washing times are long (that is \( r = 10 \text{ L/kg} \) and \( t = 8 \text{ min} \)), the reduction of available chlorine could be critical (available chlorine concentration as low as 2.6 ppm). However, when the initial concentration is high (about 125 ppm) for the same washing ratio, the available chlorine decreased slightly (Figure 1b) remaining about 100 ppm even with washing times as long as 8 min.

For the total chlorine model (TC) only the linear terms were significant (Table 3). The generated response surfaces (not shown) were similar to the available chlorine model surfaces (Table 4). In this experiment, the difference between available chlorine and total chlorine concentration was small, probably due to the minimum amount of organic cellular compounds released as a consequence of the previous cutting operation.

### Total microbial count reduction

The mean count on spinach samples before washing (No) was \( 24 \times 10^8 \text{ CFU/g} \) and the counts after each experimental washing test (Nt) are shown in Table 2.

The initial chlorine concentration \( X_1 \) \( (P \leq 0.01) \) and time \( X_3 \) \( (P \leq 0.10) \) linear terms and the interaction term \( X_1 \cdot X_3 \) of both variables \( (P \leq 0.10) \) were significant on the microbial reduction model (Table 3). All the other terms were not significant \( (P > 0.10) \), which means that water-to-produce ratio did not affect the reduction of microbial counts within the experimental region.

Figure 2 shows the predicted response surfaces for total microbial count reduction \(-\log N_t/No\) as a function of initial chlorine concentration and water-to-produce ratio for 2 min and 8 min washing time. For long washing times...
the predicted population reduction ranged from 2.8 to 3.5 log CFU/g, regardless of the water-to-produce ratio and initial chlorine concentration (Figure 2b). However, when short times are used, the initial chlorine concentration markedly affects the achieved microbial count reduction (Figure 2a).

Model verification at pilot plant
Adequacy of the model equations was tested in pilot plant with 8 kg of spinach using an initial concentration of 89 ppm, a water-to-produce ratio of 18 L/kg and a washing time of 7.5 min.

The predicted values for AC, TC, and MR were 69.1 ppm, 79.1 ppm, and 3.3 log cycles, respectively. The mean experimental values were AC = 71.0 ppm, TC = 73.3 ppm, and MR = 2.6 log cycles. There were no differences (P > 0.05) between the predicted and experimental results.

Application of the models
The developed models (AC and MR) could be used as an approach to find the conditions to improve the washing of fresh-cut spinach. Generally, the aims of industry are to achieve an adequate microbial count reduction for extending shelf life of produce using non-abusive chlorine concentrations and assuring residual levels of available chlorine in the washing solution during the operation.

As an example or alternative, the following criteria were selected: AC $\geq$ 80 ppm and MR $\geq$ 3 log cycles. Contour plots of available chlorine (AC), microbial count reduction (MR) and the superimposed plot of them for a specific water-to-produce ratio (18 L/kg) are presented in Figure 3. The shaded areas in Figure 3a and 3b indicate the conditions that satisfy each selected criteria. The superimposed contour plot of AC over MR (Figure 3c) delimits the area which satisfies both conditions simultaneously. It can be seen that there are many different combinations of working conditions (times and initial concentrations) which can be selected from this area.

Conclusions
The derived models used to predict available and total chlorine concentration in wash water and total microbial count reduction on fresh-cut spinach appeared to be adequate, showing no significant lack of fit.

The reduction in the available chlorine concentration during washing in a single stage operation (without recycling) was similar to the total chlorine concentration reduction. So, for this type of fresh-cut vegetable, one can measure either the total chlorine reduction or the available chlorine reduc-

Figure 2—Response surface plots of microbial count reduction (MR) as related to water-to-produce ratio and initial chlorine concentration at t=2 min (a) and t=8 min (b).

Figure 3—Contour plots at r = 18 L/kg for (a) available chlorine concentration (AC). The shaded area indicates AC $\geq$ 80 ppm. (b) microbial count reduction (MR). The shaded area indicates MR $\geq$ 3 log cycles. (c) Superimposed plots of (a) and (b).
tion during washing.

Although chlorine did reduce total microbial populations, it was only 2-3 log cycles, which is far from being a total reduction in microbial numbers. However, it could be convenient to leave some nonpathogenic microorganisms to compete with likely pathogens that could be present in the produce.

Processors can use the models obtained herein as an approach to find the conditions to improve the washing of fresh-cut spinach or other similar leafy vegetables.

References

This study was partly supported by CAI+D of Universidad Nacional del Litoral (Santa Fe - Argentina). We gratefully acknowledge the help of Lic. MS Salai, Bioq. MC Tiburzi and Tec. MA Mogalevsky (microbiological analysis) and Lic. NA Gordo (English version correction).

Authors are affiliated with the Instituto de Tecnología de Alimentos, Universidad Nacional del Litoral- C.C. 266-3000, Santa Fe, Argentina. Direct correspondence to author Pirovani at mpirovani@fiqus.unl.edu.ar