Interactions of Sulfur Dioxide, Lactic Acid, and Temperature During Simulated Corn Wet Milling[1]

D. L. SHANDERA,[2] A. M. PARKHURST,[3] and D. S. JACKSON[2,4]

Cereal Chem. 72(4):371-378

ABSTRACT

Response surface methodology was used to investigate the interactions of lactic acid, sulfur dioxide (SO_2), and steeping temperature and their effects on corn wet milling yields. A regular dent corn hybrid and a more vitreous dent corn hybrid were laboratory batch steeped. Kernel absorption of SO_2 was higher for the more vitreous dent corn hybrid. Absorption also increased with lactic acid use and at lower steeping temperatures. Lactic acid concentrations in steepwater remained constant over time, but kernels absorbed more steepwater at higher temperatures. When wet milled on a laboratory scale, vitreous corn was more resistant to grinding and less millable. Significant first-order response surface models of starch, germ, fines, gluten, and steep solids yields were predicted as functions with nonlinear influences of lactic acid and SO_2. Higher starch yields were obtained when steeping at 43°C than at 57°C; higher yields were predicted with moderate lactic acid and high SO_2 concentrations. Steeping temperature interactions with lactic acid and SO_2 during the steeping period limited its integration as a second-order modeling factor for starch, germ, and fines fractions. For all treatments, gluten recovery and steepwater solubles responses were predicted by lactic acid concentrations. Lactic acid, which influenced all significant fraction yield models, affected SO_2 absorption.

Steeping corn (*Zea mays,* L.) for starch production softens and degrades kernel structure, thus aiding kernel component separation during the physical stages of the wet milling process. Sulfur dioxide (SO_2) and elevated steeping temperatures (45–55°C) are used to control the growth of putrefactive microorganisms within the steeps, as well as to aid in kernel degradation. Lactic acid is usually formed by bacterial fermentation in commercial steeps and is often added to steepwater used in laboratory batch steeping.

Cox et al (1944) identified SO_2 as an important steeping agent that peptidizes protein matrices enveloping endosperm starch granules. The degree of protein peptization in whole kernels increased over the 24-hr steeping period with increasing SO_2 concentrations (up to 0.4% tested) and higher steeping temperatures (up to 55°C tested). When steeping horny endosperm sections (10 μm thick, unlimited steepwater diffusion), Watson and Sanders (1961) observed increased starch granule release from the surrounding protein matrix with increased SO_2 concentrations. In commercial steeping, kernel degradation for starch release does not occur until kernels are exposed to SO_2 (Wagoner 1948). Bisulfite ions, a form of aqueous SO_2, reduce and peptidize native kernel proteins and form sulfo-protein complexes (Boundy et al 1967). Steepwater pH affects bisulfite ion formation (King et al 1981). Eckhoff and Okos (1990) showed that gaseous SO_2 penetrates corn kernels 100 times faster than the steepwater diffusion rate calculated by Fan et al (1965). Also, Eckhoff and Okos (1990) observed a higher net absorption of gaseous SO_2 at temperatures lower than those typically used for steeping (3°C). Steeping times have been decreased and starch yields have been increased by mechanically (Hassanean et al 1986, Roushdi et al 1979) and enzymatically (Caransa et al 1988, Du Ling and Jackson 1991, Steinke and Johnson 1991) increasing steepwater and SO_2 penetration.

Cox et al (1944) reported that lactic acid softened the kernel and increased the effectiveness of SO_2, but acetic and hydrochloric acids did not have softening or degrading effects. Watson and Sanders (1961) reported that lactic acid alone did not influence granule release, but Watson (1967) reported that lactic acid has a softening action on kernel structure. Rhousdi et al (1979) reported that high levels of lactic acid in commercial steeping systems reduced yield and quality of starch. Eckhoff and Tso (1991) increased the titratable (water soluble) SO_2 content within kernels and also increased starch yields by adding lactic acid to laboratory batch steeps containing 0.1% SO_2.

The interactions and the importance of lactic acid concentration and temperature on wet milling fraction yields has never been fully established. Thus, our objective was to better understand the interactions of lactic acid, SO_2, and temperature that promote separation of kernel constituents during the corn wet milling steeping process.

MATERIALS AND METHODS

Maize Samples

Golden Harvest 2572 (GH2572), typical of yellow dent hybrid types used by the wet milling industry, and Asgrow 404Y (A404Y), a harder kernel yellow dent hybrid more typically used by the alkaline processing industry, were field dried (12.5 ± 1% moisture), mechanically harvested, and stored at −10°C. Samples were hand-sieved on a 5.6-mm (U.S. 3.5) standard sieve and equilibrated overnight to ambient temperature before steeping.

Steeping Procedure

The batch steeping procedure described by Watson et al (1955) and modified by Krochta et al (1981) and Steinke and Johnson (1991) was used. Corn (300 g) was steeped in 1,000-ml flasks containing 600 ml of steep solution prepared with distilled water, sodium bisulfite (67F-0469, Sigma Chemical Co., St. Louis, MO) as a SO_2 source (Rausch et al 1993), and synthetically derived 85% DL-lactic acid syrup (91H0693, Sigma). Free lactic acid was obtained from synthetic syrup by diluting to a 10% stock solution (v/v) and heating at 95°C for 24 hr (Shandera and Jackson 1993). Levels of steeping temperature, SO_2 concentration, and lactic acid concentration were assigned using a uniform precision central composite design to sequentially fit first- and second-order response surfaces (Montgomery 1991) (Table I). Steeping flasks were preheated for 20 min before adding corn. Flasks were submerged and heated in a 2,400 W water bath (Blue M, Blue Island, IL). Steepwater chemical concentrations, which change with kernel absorption, were equilibrated by circulating steepwater from the top of the steep to below the steeping kernels at 150 ml/min during the first hour, and for 15 min/hr intervals thereafter. Concentrations of SO_2 and lactic acid were monitored and quantified

[1]Journal series 10935, Agricultural Research Division, Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln.
[2]Research assistant and associate professor, respectively, Cereal/Oilseed Science and Technology Laboratory, Dept. of Food Science and Technology, University of Nebraska-Lincoln.
[3]Professor, Dept. of Biometry, University of Nebraska-Lincoln.
[4]Author to whom all correspondence should be addressed.

over the 40-hr steep time by high performance liquid chromatography (HPLC) (Shandera and Jackson 1993). Steep solution absorption rate into kernels, as affected by steeping temperature, lactic acid, and SO_2 concentration, was monitored by removing kernels at 0.5-hr intervals, blotting, and drying (AACC 1983). All SO_2 and lactic acid measurement treatments were performed in duplicate (high performance size exclusion chromatography test measurements were also replicated for each sample treatment); kernel moisture measurement treatments were performed in triplicate. Because of limited milling capacities, steeps were given a latent period of refrigeration (at least 24 hr, but no more than one week) at 4°C before milling. Du Ling and Jackson (1991) found that refrigeration of steeped corn at 4°C for 0–168 hr did not change starch yields.

Milling

Milling procedures were based on Watson et al (1951, 1955), Anderson (1963), Krochta et al (1981), and Steinke and Johnson (1991). Drained samples were degerminated in 500 ml of distilled water with a 1-L Waring commercial laboratory blender (model 7010H, Dynamics Corp., New Hartford, CT), fitted with a 53- × 7- × 3-mm blunt blade, for 2 min at 90 V and a low-speed setting. Germs were recovered by adding 250 ml of distilled water to settle bran and endosperm fragments and then suspending the starch and gluten-protein with a 4-cm diameter perforated plastic disk fitted to the blender operating at 20 V. Floating germs were skimmed with a screen strainer of ~850 μm (U.S. 20) mesh and washed on a 118-μm (U.S. 16) standard sieve by spraying with 2 L of distilled water. Spent germ washwater was reused as fiber washwater during sieving.

Degerminated slurry and germ washwater settlings were reground, 500 ml at a time (1/2 blender jar), with the Waring blender (original blades reversed) for 1 min at high speed and 120 V. Bran was recovered over a 425-μm (U.S. 40) standard sieve, washed with used germ washwater, and spray-washed with 1,500 ml of distilled water in 500-ml aliquots. Fines, composed of finely ground pieces of bran, germ, and endosperm (inseparables), were collected on a 63-μm (U.S. 230) standard sieve placed directly under the 425-μm (U.S. 40) standard sieve and spray-washed with three 750-ml aliquots of distilled water. Fiber and fines were hand-mixed with a 7.5-cm wide, round-edged plastic spatula. Water was removed from the fiber by hand-squeezing with the spatula. Throughs, containing mostly starch and gluten-protein, were settled in a 4-L plastic container overnight at 4°C.

The mill starch slurry was decanted, adjusted to 8° baume (Weller et al 1988), and immediately pumped (150 ml/min) onto the upper end of a 0.15- × 3.05-m (6 in. × 10 ft) flat-bottomed aluminum trough inclined at a 0.75% slope and prewetted with distilled water. Collected starch was washed with the previously decanted supernatant (~3 L total volume) at 1,000 ml/min. A second rinse was made with 1 L of distilled water. Starch was air-dried on the table for 1 hr before collecting for further analysis. Overflow water was considered to contain the gluten (protein) fraction.

Fraction Analysis

All milled fractions were predried in an air oven at 55°C to prevent gelatinization of starch. Moistures for corn, starch, bran, and fines fractions were determined in triplicate (AACC 1983). Moisture content of germs and solids content of steepwater and gluten were determined in triplicate (AACC 1983). Fraction yields were calculated on a percentage (db) of original unsteeped corn. Protein contents of starch and gluten fractions were determined (N × 6.25) with a Kjeltech automated system (Tecator Inc., Herdon, VA) (AACC 1983). Table overflow (gluten) was predried at 103°C overnight before protein determination. Kernel ash content and lipid content were determined (AACC 1983).

Experimental Design

A two-phase study using response surface methodology was performed to determine the effects of steeping temperature, lactic acid concentrations, and SO_2 concentrations on corn wet milling yields. During the first phase, the objective was to determine whether a linear function (first-order model) of the steeping factors would fit the corn wet milling yields. Each corn hybrid was tested at two steeping temperature extremes (43 and 57°C) in duplicate, using a 2^2 factorial design augmented with five outer points. A fifth treatment, a surface midpoint, was included as a quadratic check of the surface function (lactic acid) and for estimation of the stationary point. The treatment combinations of SO_2 and lactic acid are shown in Table I. First-order models tested linear and possible quadratic steeping effects of SO_2 and lactic acid on starch, germ, bran (425-μm sieve overs), fines (63-μm sieve overs), gluten-protein, and steepwater solids fraction yields. Yield data was analyzed for model fit using the RSREG procedure in the Statistical Analysis System (SAS 1992). In addition, fraction yields were compared by hybrid and by steeping temperature using the method of Fisher's protected least significant differences in the GLM procedure.

After analysis of the first phase experiments, second phase experiments were conducted to test the fit of first-order and then second-order models that integrated steeping temperature with lactic acid and SO_2 effects on the GH2572 corn hybrid (A404Y was not tested further because it was deemed of limited importance to the wet milling industry). A uniform precision, rotatable central composite design (2^3 factorial) was developed sequentially from the first-phase, first-order treatment design. The 2^3 factorial was augmented by six replicates of the center point (Table I), and the data were used to fit a first-order model. Then data from the six axial points were collected and combined with the previous data to fit a second-order model for the yields of each fraction. Second-order mathematical models for predicting wet milling fraction yields (y) were created as functions (f) of the three steeping factors (X), including experimental error (ϵ):

$$y = f(X_{\text{sulfur dioxide}}, X_{\text{lactic acid}}, X_{\text{steeping temperature}}) + \epsilon$$

TABLE I
Steeping Treatment Levels Used for Response Surface Methodology Modeling

Steeping Temperature (°C)	Lactic Acid Concentration (%, v/v)	Sulfur Dioxide Concentration (%, v/v)
Experimental Phase 1		
57	1.5	0.3
57	1.5	0.05
57	0.2	0.3
57	0.2	0.05
57	0.65	0.125
43	1.5	0.3
43	1.5	0.05
43	0.2	0.3
43	0.2	0.05
43	0.65	0.125
Experimental Phase 2		
Center point		
50.5	0.65	0.20
Factorial points		
46.0	0.30	0.10
46.0	0.30	0.30
46.0	1.00	0.10
46.0	1.00	0.30
55.0	0.30	0.10
55.0	0.30	0.30
55.0	1.00	0.10
55.0	1.00	0.30
Axial points		
50.5	0.65	0.032
50.5	0.65	0.369
50.5	0.061	0.20
50.5	1.239	0.20
42.9	0.65	0.20
58.07	0.65	0.20

RESULTS AND DISCUSSION

Hybrid Effects

Comparisons of composition of the GH2572 and A404Y hybrids showed that GH2572 kernels initially contained significantly ($P < 0.05$) more starch (79.3 vs. 73.6%), less lipid (3.7 vs. 4.8%), and less protein (8.5 vs. 9.2%) than those of A404Y. After wet milling the two hybrids of the first-phase modeling, samples of GH2572 (averaged over chemical treatments and temperatures) had better milling properties with significantly higher starch yields, similar germ yields, and lower by-product fraction yields than those of A404Y (Table II). Although GH2572 had a higher initial starch content, that does not necessarily equate to higher starch yields. Morphology, kernel structural density, protein distribution, and other factors also affect the millability of a hybrid (Weller et al 1988, Fox et al 1992). Higher fines yields and lower starch recovery of A404Y (77.2 vs. 79.9% for GH2572, expressed as the percentage of starch obtained vs. total starch content) were reflective of its harder structure. A404Y was more resistant to grinding (torquing of the blender motor) and had less endosperm fragmentation during the first grind. A404Y absorbed more SO_2 (probably because of its higher protein content) from the steepwater (Fig. 1). A404Y also released more steepwater solids (including protein) when compared to GH2572 (Table II). Steepwater concentrations of lactic acid did not differ between hybrids nor for any treatment during the 40-hr steep period. Lactic acid was absorbed in proportion to the steepwater. Because kernel structure affects the amount of steepwater absorbed (Fan et al 1965), the higher vitreousness of A404Y slowed aqueous and lactic acid penetration within the kernels and resulted in reduced overall millability. Although A404Y had a higher SO_2 absorption (independent of steepwater absorption), its higher protein content and vitreousness resulted in less kernel degradation during steeping.

TABLE II
Effects of Hybrid and Steeping Temperature on Product Yields[a]

	Corn Hybrid[b]		Steeping Temperature[c]	
Fraction	GH2572	A404Y	43°C	57°C
Starch	63.39 a	56.85 b	61.67 a	58.92 b
Germ	7.35 a	7.49 a	7.56 a	7.24 b
Bran	7.41 b	7.86 a	7.38 b	7.90 a
Fines	3.54 b	5.26 a	3.58 b	5.21 a
Gluten	13.02 b	17.22 a	15.01 a	14.83 a
Steep solids	5.17 b	6.13 a	5.63 a	5.58 a

[a] Means for hybrids or temperatures followed by the same letter are not statistically different ($P < 0.05$).
[b] Averaged over steeping temperatures.
[c] Averaged over hybrids.

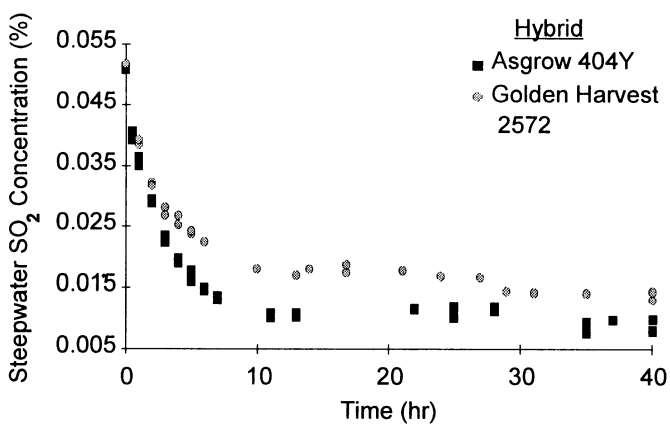

Fig. 1. Differentiation between corn hybrids by sulfur dioxide (SO_2) disappearance (kernel absorption) using a 0.05% SO_2 and 1.5% lactic acid steep at 43°C. Similar trends were observed using different steeping conditions.

Steeping Temperature Effects

Fraction yields (averaged over hybrid and chemical treatments) were significantly affected by steeping temperature (Table II). Steeping at the lower temperature (43° C) resulted in higher starch and germ yields, lower bran and fine yields, and similar gluten and steep solids yields. Kernels absorbed somewhat more SO_2 at lower steeping temperatures, but at a slower rate (Fig. 2). GH2572 steeped at 43° C had a significantly lower moisture (steepwater) uptake than it did at 57°C (Fig. 3); absorption was not significantly affected by SO_2 and lactic acid concentrations. The slower SO_2 absorption rate at lower steeping temperatures (43°C) is linked to the amount of time the kernel moisture remains below 30% (Figs. 2 and 3). Absorption of gaseous SO_2 is not limited when kernel moisture is above 30% (wb), and it increases at lower temperatures (Eckhoff and Okos 1990). Sulfo-compounds were leached from inside the kernels (observed as a rise or recovery in steepwater SO_2 concentrations) ~15 hr later at the lower steeping temperatures (Fig. 2). Lower steeping temperatures resulted in kernels absorbing more SO_2, with reduced moisture content; sulfo-species formation remained within the kernel longer over the 40-hr steep. The higher overall SO_2 concentration within the kernels allowed for increased kernel degradation, but with slower kenetics due to a lower temperature. While Cox et al (1944) reported that kernel degradation increased with temperature during a 24-hr steep, Figure 2 shows that at 24 hr, sulfo-compounds are only beginning to leach at 43°C. The 40-hr steep in this study gave sufficient time for kernel degradation to negate the short-term kenetic benefits (higher initial SO_2 concentrations within the kernel) of steeping at higher temperatures. Delayed

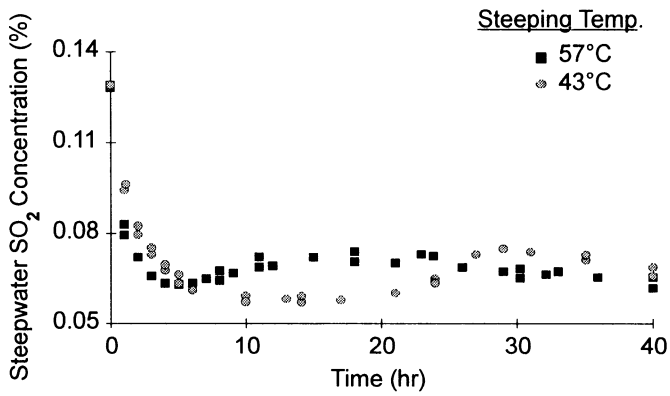

Fig. 2. Steeping temperature effect on rate and amount of sulfur dioxide (SO_2) absorbed from steepwater by GH2572 corn using a 0.125% SO_2 and 0.65% lactic acid steep. Similar trends were observed with different steeping conditions and hybrids.

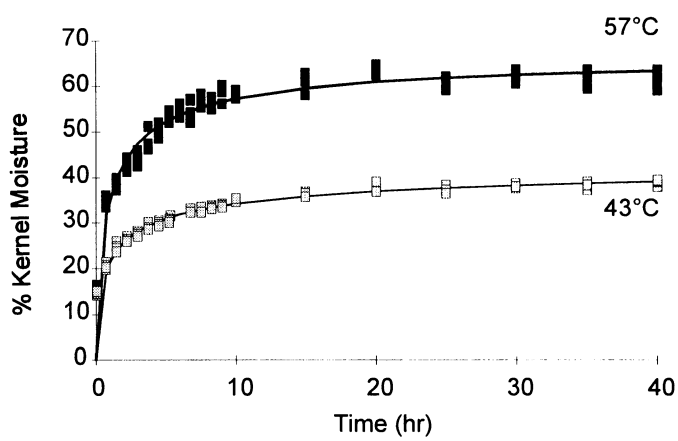

Fig. 3. Steeping temperature effect on kernel moisture uptake of GH2572 corn. Significant ($P < 0.05$) difference in rate and total uptake was observed between steeping temperatures, but moisture uptake was not affected by sulfur dioxide or lactic acid concentrations.

TABLE III
P-values for Significance of First-Phase Yield Models Parameters[a]

Fraction	Hybrid[b]	Batch Steeping Temperature (°C)	(Prob > T) for Testing H_0: Parameter = 0					Regression[c] ($P > F$)				Stationary Point[d]
			β_0	β_1 (%LA)	β_2 (%SO_2)	β_3 (%LA^2)	β_4 (%LA ×%SO_2)	Total	Linear	Quadr	Cross-Product	
Starch	GH2572	43	0.000	0.499	0.833	0.588	0.201	0.262	0.186	0.588	0.201	Saddle
	GH2572	57	0.000	0.016	0.038	0.016	0.119	0.046	0.137	0.017	0.119	Saddle
	A404Y	43	0.000	0.007	0.267	0.010	0.289	0.047	0.202	0.011	0.289	Saddle
	A404Y	57	0.000	0.288	0.015	0.387	0.059	0.084	0.077	0.451	0.059	Saddle
Germ	GH2572	43	0.000	0.630	0.199	0.673	0.087	0.153	0.144	0.673	0.087	Saddle
	GH2572	57	0.000	0.714	0.930	0.941	0.340	0.574	0.419	0.984	0.340	Saddle
	A404Y	43	0.000	0.018	0.860	0.018	0.514	0.104	0.513	0.019	0.514	Saddle
	A404Y	57	0.000	0.856	0.950	0.657	0.228	0.356	0.274	0.608	0.228	Saddle
Bran	GH2572	43	0.000	0.159	0.835	0.175	0.885	0.582	0.679	0.175	0.885	Saddle
	GH2572	57	0.000	0.568	0.290	0.488	0.185	0.396	0.436	0.444	0.185	Saddle
	A404Y	43	0.000	0.214	0.010	0.664	0.005	0.040	0.915	0.846	0.005	Saddle
	A404Y	57	0.000	0.008	0.003	0.009	0.091	0.009	0.009	0.010	0.091	Saddle
Fines	GH2572	43	0.000	0.001	0.000	0.005	0.002	0.001	0.003	0.005	0.002	Saddle
	GH2572	57	0.003	0.200	0.202	0.225	0.991	0.229	0.134	0.224	0.991	Saddle
	A404Y	43	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Saddle
	A404Y	57	0.000	0.000	0.000	0.000	0.073	0.000	0.000	0.000	0.073	Saddle
Gluten	GH2572	43	0.001	0.595	0.088	0.948	0.415	0.041	0.012	0.948	0.415	Saddle
	GH2572	57	0.001	0.871	0.752	0.752	0.194	0.016	0.006	0.809	0.194	Saddle
	A404Y	43	0.004	0.657	0.051	0.814	0.124	0.110	0.067	0.742	0.124	Saddle
	A404Y	57	0.001	0.002	0.119	0.003	0.745	0.007	0.012	0.003	0.745	Saddle
Steep solids	GH2572	43	0.036	0.166	0.043	0.569	0.024	0.015	0.012	0.568	0.024	Saddle
	GH2572	57	0.000	0.602	0.567	0.152	0.935	0.005	0.002	0.153	0.944	Saddle
	A404Y	43	0.001	0.018	0.022	0.102	0.029	0.006	0.003	0.126	0.029	Saddle
	A404Y	57	0.000	0.076	0.783	0.262	0.833	0.006	0.002	0.257	0.833	Saddle

[a] Yield = $\beta_0 + \beta_1$ (%LA) + β_2 (%SO_2) + β_3 (%LA^2) + β_4 (%LA×%SO_2). LA = lactic acid, SO_2 = sulfur dioxide.
[b] Dent corn hybrids tested: Golden Harvest 2572 (normal, yellow); Asgrow 404Y (harder type, yellow).
[c] Fraction model's regression significance (*p*-value) for *F*-test.
[d] Stationary point of surface is a minimum, maximum, or saddle point.

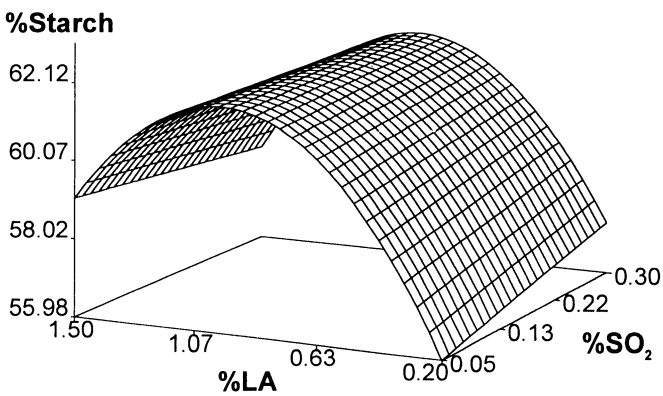

Fig. 4. First-phase, first-order response surface model for starch on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration for GH2572 corn steeped at 57°C.

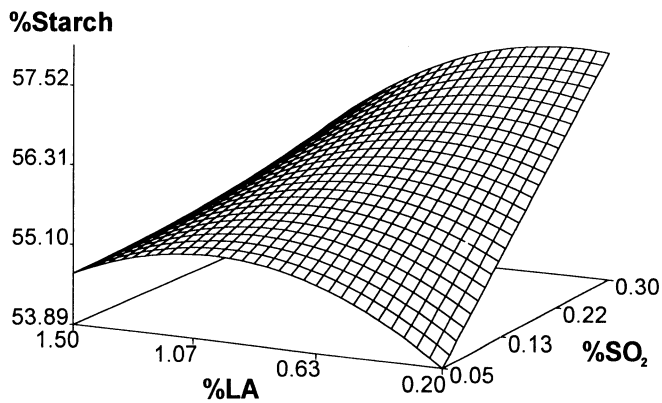

Fig. 5. First-phase, first-order response surface model for starch on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration for A404Y corn steeped at 43°C.

SO_2 absorption and leaching trends, as shown by HPLC, indicate that results would differ with reduced steeping times or if kernels were exposed to differing concentrations of SO_2 and lactic acid over time.

First-Phase Modeling

Product yield data from the first-phase experiments were analyzed for lactic acid and SO_2 influences in predicting wet-milling fraction yields for each hybrid at 43 and 57°C. Response surface models with a significance of $P < 0.10$ were found for starch, bran, fines, gluten, and steep solids for both hybrids (Table III). These first-order models estimated the linear influences of lactic acid and SO_2, with inclusion of a quadratic check for surface curvature due to lactic acid, and estimation of the stationary point. The generated surfaces are not intended to precisely predict fraction yields but to exhibit the effects of lactic acid, SO_2, and their interaction on the milling yields of each fraction. Significance levels associate the relative importance of surface models for further study and help characterize the influencing factors.

Starch yield models with a significance of $P < 0.05$ were obtained for GH2572 steeped at 57°C (Fig. 4) and A404Y steeped at 43°C (Fig. 5). Both models were influenced quadratically by lactic acid concentrations during steeping. The 57°C model for GH2572 was also somewhat influenced by SO_2 ($P = 0.083$) linearly and interactively with lactic acid. Highest starch yields were predicted when steeping with moderate concentrations of lactic acid (~1.0%) and high concentrations of SO_2. The 57°C model for A404Y ($P = 0.084$) was influenced linearly by SO_2 and by a lactic acid and SO_2 interaction (Fig. 6). Thus, the effect of SO_2 on starch yields depended on the level of lactic acid in the steeps. The combination of lactic acid and SO_2 that approximated a maximum or minimum yield response for each response surface is the "stationary point". When lactic acid and SO_2 had opposing quadratic influences on yields, there was no true surface optimum. Thus, the stationary point existed as a "saddle point" on the surface. The saddle point is a combination of factor levels where

the quadratic response of one factor is minimized and the response curvature of the other factor is simultaneously maximized. The stationary point for the A404Y 43°C starch yield model is located outside of the tested parameter ranges at 1.13% lactic acid and <0% SO_2, and predicts 61.6% starch yield. The predicted stationary point is a saddle point. Because of its relative location on the surface, higher concentrations of SO_2, combined with moderate lactic acid concentrations, resulted in higher starch yields within the tested parameters. The stationary point (saddle point) of the GH2572 57°C model predicts 60.8% yield at 1.60% lactic acid and <0% SO_2. The stationary point of the A404Y 57°C model predicts 54.5% yield at 1.58% lactic acid and <0% SO_2. Starch recovery for all treatments (both hybrids at both temperatures) had an average coefficient of variation (CV) of 1.61% for the first phase. The collected starch fractions were reasonably pure with all protein contents at <0.5% (average 0.33%, CV = 1.61%).

Germ models for each hybrid were somewhat significant at 43°C steeping (Table III, surfaces not shown). The A404Y germ model ($P = 0.104$) was predicted quadratically by lactic acid. The GH2572 germ model ($P = 0.153$) was predicted by a lactic acid and SO_2 cross-product interaction ($P = 0.087$). Lactic acid and SO_2 concentrations did not predict germ yields during steeping at 57°C.

Bran yield first-phase models were significant for A404Y steeped at 43 and 57°C (Table III, surfaces not shown). A significant lactic acid and SO_2 cross-product interaction was observed for the 43°C model. The response surface is characterized by a valley that extends from high lactic acid and low SO_2 combinations, through the surface center, to low lactic acid and high SO_2 concentrations. The surface also has ascending curvatures toward high and low combinations of lactic acid and SO_2 at the opposing corners. The A404Y 57°C bran model is aligned quadratically to lactic acid concentrations, with some interaction by SO_2. Lower bran yields are predicted at moderate levels of lactic acid. High SO_2 concentrations lowered yields at low lactic acid concentrations, but increased yields at high lactic acid concentrations. Steeping chemical concentrations did not significantly predict GH2572 bran yields.

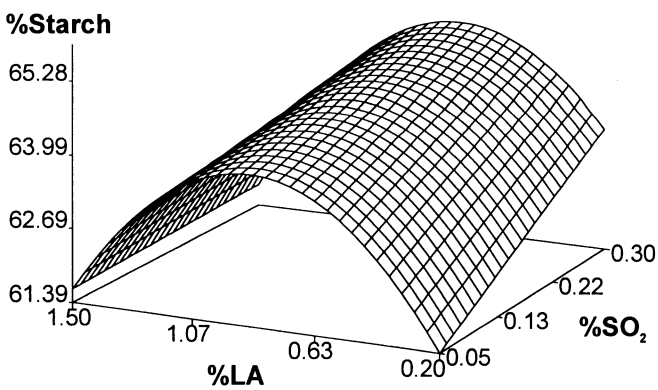

Fig. 6. First-phase, first-order response surface model for starch on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration for A404Y corn steeped at 57°C.

Three significant first-phase models were obtained for fines yields: GH2572 at 43°C and A404Y at 43 and 57°C (Table III, surfaces not shown). All significant models were fitted both linearly by lactic acid and SO_2 concentrations, and quadratically by lactic acid concentrations. For both hybrids, 43°C models had cross-product interactions of lactic acid with SO_2. A404Y yield data fitted the 43 and 57°C models with correlation coefficients (R^2) of 0.93 and 0.98, respectively. Lowest fines yields (best milling) for all three models were predicted under moderate lactic acid concentrations (1.0–1.2%), and somewhat by high SO_2 concentrations. Cross-product interactions caused high SO_2 concentrations to decrease fines yields at low lactic acid concentrations and to increase yields at high lactic acid concentrations.

The gluten fraction first-phase models were linearly significant for GH2572 steeped at 43 and 57°C. Moreover, the A404Y 57°C model was also quadratically significant (Table III, surfaces not shown). The hill curvature of the A404Y 57°C model was quadratically aligned to lactic acid. Highest gluten yields were predicted at moderate lactic acid concentrations (0.9–1.2%). A404Y 43°C gluten ($P = 0.110$) was linearly predicted by SO_2. Higher SO_2 concentrations increased yields for both A404Y models. Lactic acid and SO_2 were approximately equivalent in linearly predicting GH2572 43°C gluten; highest yields were obtained with highest concentration combinations. GH2572 43°C gluten was aligned positively with increasing lactic acid concentration; highest yields were obtained with high lactic acid and SO_2 combinations. The gluten fractions had an average protein (N × 6.25) content of 32% (CV = 14%). Protein contents were generally higher for lower steeping temperatures.

First-phase steep solids yields were linearly predicted by lactic acid for both hybrids at both temperatures and with some SO_2 interactions at 43°C (Table III, surfaces not shown). Highest steep solids yields were obtained with high concentrations of lactic acid and lowest SO_2 concentrations. Except for the GH2572 model at 57°C, SO_2 increased steep solids yields under low levels of lactic acid and decreased yields with high levels of lactic acid. Due to difficulty in determining the net amounts in the dried fraction, lactic acid and SO_2 levels used during steeping contribute to the mass weight of the steep solids.

Results of the first-phase experiments indicated that laboratory wet milling yields could be mathematically modeled using steep chemical concentrations. All first-order model stationary points were saddle points. The model curvatures indicated the need for development of second-order models to adequately explain the variability in the data caused by the steeping treatments. The objective of comparing millability between hybrids was met with the first-phase models. In comparing first-order plots of A404Y to GH2572 fractions, overall trends in chemical effects were generally similar, with a shift in the A404Y response surface curvatures towards a larger chemical requirement, especially SO_2, to attain equivalent milling efficiency. Higher SO_2 concentration require-

TABLE IV
***P*-Value for Significance of Second-Phase, First-Order Yield Model Parameters[a]**

	(Prob > T) for Testing H_0: Parameter = 0								Regression[b] (Prob > *F*)				
Fraction	β_0	β_1 (°C)	β_2 (%LA)	β_3 (%SO_2)	β_4 (°C ×%LA)	β_5 (%LA^2)	β_6 (°C ×%SO_2)	β_7 (%LA ×%SO_2)	Total	Linear	Quadr	Cross-Product	Stationary[c] Point
Starch	0.000	0.019	0.069	0.127	0.655	0.050	0.063	0.063	0.015	0.021	0.064	0.064	Saddle
Germ	0.000	0.463	0.437	0.131	0.089	0.781	0.166	0.059	0.068	0.075	0.784	0.072	Saddle
Bran	0.000	0.052	0.802	0.493	0.269	0.157	0.663	0.233	0.127	0.076	0.153	0.402	Saddle
Fines	0.240	0.001	0.065	0.665	0.557	0.013	0.195	0.135	0.000	0.000	0.015	0.198	Saddle
Gluten	0.011	0.393	0.791	0.285	0.319	0.947	0.442	0.947	0.002	0.000	0.967	0.679	Saddle
Steep solids	0.658	0.329	0.266	0.398	0.664	0.929	0.654	0.037	0.006	0.001	0.963	0.181	Saddle

[a]Yield = $\beta_0 + \beta_1$ (°C) + β_2 (%LA) + β_3 (%SO_2) + β_4 (°C×%LA) + β_5 (%LA^2) + β_6 (°C×%SO_2) + β_7 (%LA×%SO_2). LA = lactic acid, SO_2 = sulfur dioxide.
[b]Fraction model regression significance (*p*-value) for *F*-test.
[c]Stationary point of surface is a minimum, maximum, or saddle point.

ments and longer steeping periods were needed to adequately penetrate and degrade the higher protein content and more vitreous endosperm structure of the A404Y hybrid.

Second-Phase Modeling

The second phase focused on the typical dent corn hybrid (GH2572) and included steeping temperature as an additional steeping factor in the response surface modeling. The initial experiments of this phase were focused on expanding the GH2572 first-phase modeling into first-order models that integrated steeping temperature as a modeling factor (Table I). A quadratic test of lactic acid was made in these first-order models to determine the need for development of second-order models, but quadratic influences of temperature and SO_2 were not modeled.

The second-phase, first-order model of starch was influenced by lactic acid concentrations, steeping temperature, and SO_2 interactions with lactic acid and temperature (Table IV). The stationary point of the model (saddle point) predicts 64.8% starch; just beyond our tested parameter levels at 47.8°C, 0.63% lactic acid, and 0.35% SO_2. Lactic acid had a significant quadratic fit to the model and predicts higher starch yields at moderate concentrations. This hill curvature of the surface runs parallel to SO_2 concentrations, which is similar to the first-phase models (Fig. 7). The SO_2 effect on yield was dependent on lactic acid concentrations, but higher yields were predicted by using low SO_2 concentrations with moderate lactic concentrations. Because it is a stationary point, there is no effect of temperature at 0.35% SO_2, but lower steeping temperatures generally resulted in higher starch yields. A nonsignificant lack-of-fit was obtained for the model, with 62% of the data variability explained by the model and a 1.86% CV in starch yields.

The germ fraction first-order model was primarily predicted by lactic acid and SO_2 and lactic acid and temperature cross-product interactions, with some linear influence of SO_2 (Table IV, surface not shown). The stationary point (saddle point) is contained within the tested parameter limits at 49.6°C, 0.42% lactic acid, and 0.23% SO_2, and predicts 7.28% yield. The downward curvatures of the saddle extend diagonally across the surface to low and high concentrations of lactic acid and SO_2. The opposing upward curvatures extend diagonally with high and low chemical concentration combinations. Highest germ yields are predicted by high lactic acid, high SO_2, and low temperature levels.

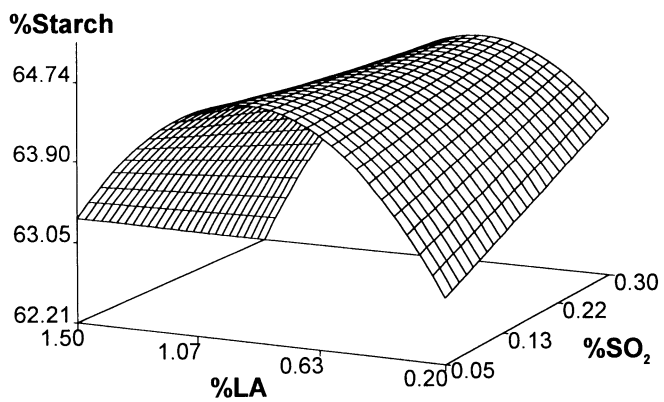

Fig. 7. Second-phase, first-order response surface model for starch on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration for GH2572 corn steeped at 50°C. Quadratic effects of LA were tested.

The bran first-order model was only somewhat significant ($P = 0.127$), but temperature linearly increased yields for the developed model (Table IV, surface not shown). The plotted surface was also characterized by a valley at moderate lactic acid concentrations that extend parallel to that of SO_2. The stationary point (saddle point) predicted a 7.14% yield beyond the tested parameter range at 0.57% SO_2, 1.23% lactic acid, and 43.6°C.

A very significant first-order model ($P < 0.001$) was linearly ($P < 0.001$) and quadratically ($P = 0.015$) fit to the fines fraction (Table IV, surface not shown). The stationary point (saddle point) predicted −0.211% yield outside of the tested range at 23.4°C, 0.54% lactic acid, and 0.76% SO_2. The valley shape of the surface showed that lactic acid quadratically influences fines yields. Moderate (~0.8%) lactic acid concentrations predicted best milling (lowest yields) in combination with high SO_2 concentrations. Lower steeping temperatures decreased yields linearly.

The first-order gluten model is linearly influenced by lactic acid (Table IV, surface not shown). The stationary point of this model (saddle point) at −48.4% yield, is beyond the tested parameter range at 96.9°C, −27.24% lactic acid, and −7.12% SO_2. Due to the extreme location of the stationary point, the surface within the parameter limits is somewhat flat and well defined. Higher lactic acid concentrations, alone and in combination with SO_2, increased gluten yields. Higher temperatures decreased yields.

The steep solids first-order model stationary point (saddle point) predicted 5.25% yield at 58.5°C, 0.72% lactic acid, and 0.41% SO_2 (Table IV, surface not shown). Steep solids yields increased linearly with lactic acid concentrations. A cross-product effect of SO_2 resulted in increased yields at low lactic acid concentrations and decreased yields at high concentrations. Temperature had only a minor influence on yields.

The reduction in error was significant for starch, germ, fines, gluten, and steep solids first-order models, as significant differences in yields existed between treatments. Quadratic (hill) curvature of the starch fraction first-order models indicated that the optimum combination of parameters was near, or included within, the range of tested chemical concentrations. This check also indicated that the models could be improved by a second-order model. In developing second-order models for GH2572, the central composite design was expanded to integrate additional treatments with the previous models. A rotatable, second-order model was constructed and tested for the three steeping factors (lactic acid concentration, SO_2 concentration, and steeping temperature).

The gluten fraction second-phase, second-order model was significant at the $\alpha = 0.10$ level (Table V). Because of the nonsignificant lack-of-fit ($P = 0.846$), it was not likely that the model would be improved by fitting more tested points. Approximately

TABLE V
***P*-Values for Significance of Second-Phase, Second-Order Yield Model Parameters[a]**

	(Prob > T) for Testing H_0: Parameter = 0								Regression[b] (Prob > *F*)						
Fraction	β_0	β_1 (°C)	β_2 (%LA)	β_3 (%SO_2)	β_4 (°C^2)	β_5 (°C ×%LA)	β_6 (%LA^2)	β_7 (°C ×%SO_2)	β_8 (%LA ×%SO_2)	β_9 (%SO_2^2)	Total	Linear	Quadr	Cross-Product	Lack-of-fit
Starch	0.594	0.555	0.578	0.863	0.507	0.976	0.099	0.956	0.706	0.348	0.559	0.482	0.218	0.984	0.300[c]
Germ	0.956	0.347	0.365	0.797	0.377	0.176	0.091	0.794	0.431	0.925	0.140	0.054	0.255	0.450	0.004
Bran	0.034	0.185	0.436	0.635	0.174	0.206	0.108	0.523	0.706	0.613	0.257	0.220	0.171	0.519	0.080
Fines	0.575	0.720	0.495	0.462	0.690	0.853	0.132	0.777	0.647	0.166	0.423	0.216	0.274	0.950	0.000
Gluten	0.107	0.123	0.365	0.850	0.107	0.998	0.034	0.961	0.221	0.245	0.095	0.104	0.043	0.648	0.846[c]
Steep solids	0.707	0.412	0.382	0.489	0.499	0.717	0.791	0.616	0.239	0.744	0.010	0.001	0.871	0.596	0.328[c]

[a]Yield = $\beta_0 + \beta_1$ (°C) + β_2 (%LA) + β_3 (%SO_2) + β_4 (°C^2) + β_5 (°C×%LA) + β_6 (%LA^2) + β_7 (°C×%SO_2) + β_8 (%LA×%SO_2) + β_9 (%SO_2^2). LA = lactic acid, SO_2 = sulfur dioxide.
[b]Fraction model regression significance (*p*-value) for *F*-test.
[c]Stationary point of surface is a saddle point.

68% of the variability found in the data was explained by the model. Lactic acid with four degrees of freedom was the significant factor ($P = 0.058$) in the model. The estimated response surface of the gluten model at 50°C does not have a unique optimum because of the saddle point (Fig. 8). The predicted stationary point (saddle point) is contained within the tested parameter ranges. It predicts 14.64% gluten yield at 48.05°C, 0.75% lactic acid, and 0.24% SO_2. The hill orientation, which is aligned quadratically to lactic acid, has more curvature than the valley, which is aligned with steeping temperature or SO_2. Ridge analysis indicates that maximum gluten yields are associated with moderate levels of lactic acid, moderate temperature, and low SO_2 levels.

The steep solids second-order model adequately approximated the true response surface ($P = 0.010$, $R^2 = 0.817$) (Table V). Because of a nonsignificant lack-of-fit ($P = 0.328$), the model would not be improved with more fitted points. The stationary point (saddle point) predicts 4.99% yield at 50.91°C, −0.004% lactic acid, and 0.52% SO_2. A negative concentration for lactic acid makes the stationary point unfeasible. The estimated surface does not have a unique optimum and is linearly fitted to lactic acid ($P = 0.001$) at 50°C (Fig. 9). The surface hill orientation, which is more aligned with SO_2, is slightly more curved than the valley, which is aligned with lactic acid or temperature. Ridge analysis indicates that maximum steep solids yields will result from steeping with relatively high concentrations of lactic acid, high temperatures, and low concentrations of SO_2.

The experimental results of the second phase did not show significant second-order models for GH2572 starch, germ, bran, and fines fractions. The multiple correlation coefficient (R^2) for quadratic fitting of starch yields decreased from 0.62 for the first order to 0.45 for the second order when more yield data was collected over a temperature range. Although the CV (2.73%) of the second-order starch model was greater than that of the first phase at 57°C (1.24%) or the second-phase, first-order model (1.86%), the value is not experimentally excessive. The best fitting of a starch model was quadratic ($P = 0.218$) with a nonsignificant lack-of-fit ($P = 0.300$). The second-order model would not have been improved by fitting more points. The data for the germ and bran second-order models had a lower CV than those of the first-order models. The significant lack-of-fit statistics for fitting second-order models to these fractions may indicate a need for higher degree polynomials (cubic or quartic) to model the interactions of temperature, lactic acid, and SO_2. Germ, bran, and fines lack-of-fit P values were 0.004, 0.079, and <0.001, respectively. The loss in significance between the first- and second-order modeling was not due to an increase in experimental error. The testing of more treatments and inclusion of temperature and SO_2 quadratic terms in the second-order model detected a steeping phenomenon that was not modeled at the quadratic level.

Results of the second-phase experiments, as well as differences found between shapes of 43 and 57°C response surfaces in the first-order model, may indicate that more complex, interactive influences of lactic acid and SO_2 occur over the tested temperature ranges, which were not adequately explained using a second-degree polynomial. Higher steeping temperatures increased kernel moisture uptake and the proportional (net) absorption of lactic acid. The rate of SO_2 absorption increased initially with temperature, but the total amount absorbed during 40 hr decreased (Fig. 2). The combination of higher internal kernel moisture and reduced net uptake of SO_2 also reduced internal SO_2 concentration of the kernels. To further complicate the model, it is likely that the reaction rates were changing, as was the timing of sulfo-species leached from the kernel. In addition, higher starting concentrations of SO_2 resulted in a higher absorption rate (Figs. 1 and 2). The individual effects of lactic acid, SO_2, or temperature were also dependent on steeping length, as these factors change and interact. A 40-hr period may nullify individual effects on most milling fraction yields. These temperature interactions with steepwater chemicals and kernel structure over time would be extremely difficult to meaningfully model.

Lactic acid had an effect on all significant fraction models (as measured by separate parameter testing). The significant lactic acid interaction terms (quadratic) for starch, germ, bran, and fines in the second-order models suggest at least some influence of lactic acid in wet milling. Because lactic acid was proportionally absorbed with the steepwater, and steepwater concentrations did not change over time, lactic acid probably does not react covalently with kernel constituents (as evident with SO_2). Nor is the impact of lactic acid on fraction yields due to a direct acid hydrolysis of kernel structure (Cox et al 1944, Watson et al 1951). Steeping with only lactic acid (1.5% lactic acid, 0.0% SO_2, 50°C, 40 hr) resulted in incomplete disruption of endosperm structure for starch release, low starch yields, high fines collection, and poor overall milling properties; however, steeping without lactic acid is feasible. The dissociated base of lactic acid may interact with the structural cell membranes, causing a solvating effect that results in the softening action reported by Watson (1967). We speculate that the softening action of lactic acid probably increases porosity of cellular membranes or softens the protein matrix in vitreous regions of the kernel, which promotes absorption of SO_2 over the first 10 hr of steeping (Fig. 10). The increased cellular concentration of SO_2 ions increases endosperm starch release (Eckhoff and Tso 1991). Although very high concentrations of lactic acid enhance SO_2 absorption within the kernel, these concentrations result in a lowering of internal pH. This shifts the aqueous sulfite equilibrium state from HSO_3^- ions to formation of a dissolved SO_2 gas species that is not effective in reducing disulfide bonds within the kernel (King et al 1981). Lowering the pH (below ~2.5) nullifies the beneficial effects of lactic acid. The result of using excessive lactic acid during steeping is a quadratic decrease in the millability of the steeped kernels.

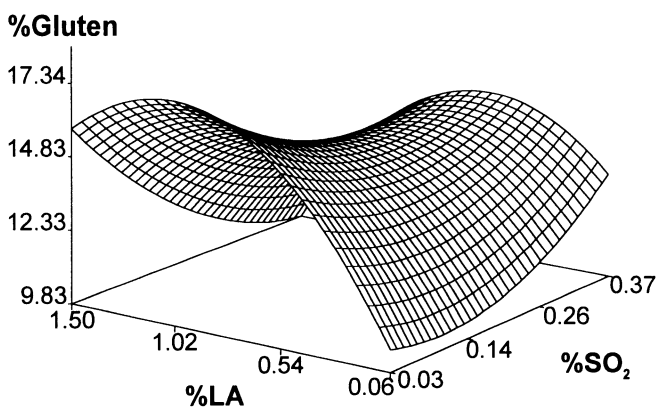

Fig. 8. Second-order model for gluten-protein on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration drawn at 50°C for GH2572 corn.

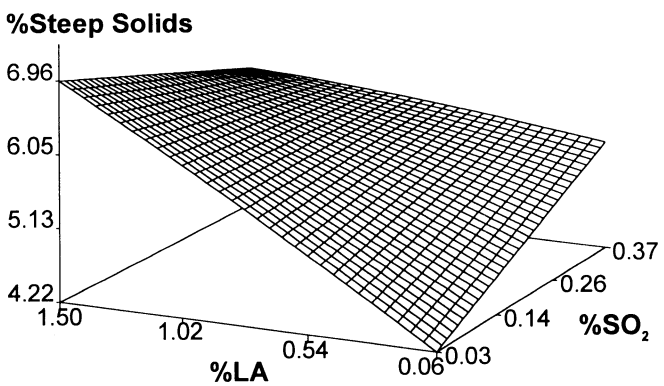

Fig. 9. Second-order model for steep solids on the effects of lactic acid (LA) and sulfur dioxide (SO_2) concentration drawn at 50°C for GH2572 corn. Steep solids yields not corrected for LA or SO_2 contents.

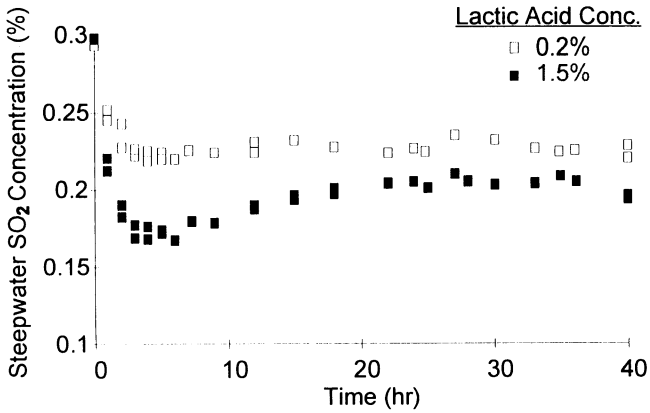

Fig. 10. Effect of lactic acid concentration on rate and absorption of sulfur dioxide (SO_2) from steepwater by GH2572 corn using a concentration of 0.3% SO_2 at 57°C steep. Similar trends were observed under different steeping conditions and for A404Y.

CONCLUSIONS

The steepwater factors of lactic acid, SO_2, and temperature were not equally important to both corn hybrids, but there were clearly similarities within fractions. Corn type (hardness) had a greater effect on most fraction yields than did steep chemical concentrations or temperature. Lactic acid concentrations significantly predicted gluten and steep solids yields of GH2572 in the second-order model. Because of the complex interactions involving steepwater uptake and SO_2 absorption with temperature, the second-order mathematical models for high value fractions (starch, germ) yields of GH2572 were not significant. When temperature was held constant (first-order models), significant models for starch and germ were obtained. Steeping temperature affects kernel moisture uptake and the proportional lactic acid absorption. HPLC data suggest that lactic acid concentration, steeping temperature, and kernel morphology influence SO_2 absorption. SO_2 is necessary for efficient wet milling and its concentration within the kernel affects millability. Moreover, very low SO_2 concentrations seem feasible when moderate (~1%) lactic acid concentrations are also used. HPLC data also suggest that shorter steeping periods would result in different yield results. A 40-hr steeping period allowed SO_2 concentration fluctuations to stabilize, and there was sufficient time for reactions to occur within the kernel, thereby reducing the effects of varying the initial SO_2 concentration. Commercial steeping operations and alternative laboratory methods, which introduce steep chemicals at different times in the wet milling process, will have different SO_2 absorption trends and will produce different yield models. On the basis of previous research and our observations in this study, lactic acid has two major effects during steeping: 1) it softens kernel components and promotes SO_2 absorption, and 2) it interacts with SO_2 to determine ionic species, which may or may not have sulfo-reducing properties. It is unlikely that lactic acid directly degrades starch-protein complexes within the endosperm.

ACKNOWLEDGMENTS

We wish to acknowledge H. Gu for his assistance with the moisture absorption studies. This research was partially funded by the Nebraska Corn Development, Utilization and Marketing Board and the Corn Refiners Association.

LITERATURE CITED

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1983. Approved Methods of the AACC, 8th ed. Method 08-03, approved April 1961, revised October 1994; Method 30-25, approved April 1961, revised October 1976, October 1981, October 1991; Method 44-15A, approved October 1975, revised October 1981; Method 44-40, approved April 1961, revised October 1994; Method 46-12, approved October 1976, revised October 1986. The Association: St. Paul, MN.

ANDERSON, R. A. 1963. Wet milling properties of grains: Bench scale study. Cereal Sci. Today 8:190-195, 221.

BOUNDY, J. A., TURNER, J. E., WALL, J. S., and DIMLER, R. J. 1967. Influence of commercial processing on composition and properties of corn zein. Cereal Chem. 44:281-287.

CARANSA, A., SIMELL, M., LEHMUSSAARE, A., VAARA, M., and VAARA, T. 1988. A novel enzyme application for corn wet milling. Starch/Staerke 40:409-411.

COX, M. J., MacMASTERS, M. M., and HILBERT, G. E. 1944. Effects of the sulfurous acid steep in corn wet milling. Cereal Chem. 21:447-465.

DU LING, and JACKSON, D. S. 1991. Corn wet milling with a commercial enzyme preparation. Cereal Chem. 68:205-206.

ECKHOFF, S. R., and OKOS, M. R. 1990. Sorption Kinetics of Sulfur Dioxide on Yellow Dent Corn. ASAE Trans. 33:855-861.

ECKHOFF, S. R., and TSO, C. C. 1991. Wet milling of corn using gaseous sulfur dioxide addition before steeping and the addition of lactic acid on steeping. Cereal Chem. 68:248-251.

FAN, L. T., CHEN, H. C., SHELLENBERGER, J. A., and CHUNG, D. S. 1965. Comparision of the rates of absorption of water by corn kernels with and without dissolved sulfur dioxide. Cereal Chem. 42:385-396.

FOX, S. R., JOHNSON, L. A., HURBURGH, C. R., JR., DORSEY-REDDING, C., and BAILEY, T. B. 1992. Relations of grain proximate composition and physical properties to wet milling characteristics of maize. Cereal Chem. 69:191-197.

HASSANEAN, A., and ABDEL-WAHED, A. 1986. A new method to short the steeping period of corn grains. Starch/Staerke 38:417-419.

KING, A. D., PONTING, J. D., SANSHUCK, D. W., JACKSON, R., and MEHARA, K. 1981. Factors affecting death of yeast by sulfur dioxide. J. Food Prot. 44:92-97.

KROCHTA, J. M., LOOK, K. T., and WONG, L. G. 1981. Modification of corn wet milling steeping conditions to reduce energy consumption. J. Food Process. Preserv. 5:39-46.

MONTGOMERY, D. C. 1991. Design and Analysis of Experiments. John Wiley & Sons: New York.

RAUSCH, K. D., SINGH, S. K., and ECKHOFF, S. R. 1993. Magnesium bisulfite and sodium bisulfite as alternative steeping agents for wet milling. Cereal Chem. 70:489-490.

ROUSHDI, M., GHALI, Y., and HASSANEAN, A. 1979. Factors improving the steeping process of corn grains. Starch/Staerke 31:7-9.

SAS. 1992. SAS Proprietary Software, Release Version 6.08. SAS Institute: Cary, NC.

SHANDERA, D. L., JR., and JACKSON, D. S. 1993. A simplified method for measurement of lactic acid and sulfur dioxide in corn wet milling steep water. Starch/Staerke 45:351-354.

STEINKE, J., and JOHNSON, L. 1991. Steeping maize in the presence of multiple enzymes. Cereal Chem. 68:7-17.

WAGONER, J. A. 1948. Microscopic observations and procedures used in a study of industrial corn steeping. Cereal Chem. 25:354-361.

WATSON, S. A. 1967. Manufacture of corn and milo starches. Page 48 in: Starch: Chemistry and Technology, Vol. II. Academic Press: New York.

WATSON, S. A., HIRATA, Y., and WILLIAMS, C. B. 1955. A study of the lactic acid fermentation in commercial corn steeping. Cereal Chem. 32:382-394.

WATSON, S. A., and SANDERS, E. H. 1961. Steeping studies with corn endosperm sections. Cereal Chem. 38:22-33.

WATSON, S. A., WILLIAMS, C. B., and WAKELY, R. D. 1951. Laboratory steeping procedures used in a wet milling research program. Cereal Chem. 28:105-118.

WELLER, C. L., PAULSEN, M. R., and STEINBERG, M. P. 1988. Correlation of starch recovery with assorted quality factors of four corn hybrids. Cereal Chem. 65:392-397.

[Received November 4, 1994. Accepted April 13, 1995]