CHEMICAL MARKERS OR SIGNALS FOR DETERMINATION OF STORABILITY OF CHROMATOGRAPHIC SEPARATOR EXTRACT

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Introduction:

American Crystal Sugar Company has been storing extract from chromatographic separators for approximately fifteen years. For almost all of this time, storage has generally been successful with minor loss of sucrose. There have been a couple of instances where the storage was not considered successful with the result that the extract had to be processed earlier than originally planned or quality of the extract deteriorated and processing of the extract was more difficult. In one case, the extract deterioration was due to addition of low solids content juice to a tank. In the other case, discussed below, the deterioration of the extract is thought to have been due to enzymatic contamination. We have reacted to high fastidious microbial counts by disrupting beet slicing campaign in order process the extract in order to avoid sugar loss. Generally, changing campaign processing plans at the expense of beet slice is a costly choice. The question that was raised was: Are microbial counts are a good indicator of extract storage or are there better indicators?

Because of these instances of problematic extract storage, we have set strict controls on temperature, pH, and RDS of extract sent to storage and we have worked to ensured that storage conditions are sufficient for successful storage. We have implemented comprehensive chemical and microbiological testing of extract sent to storage and extract under storage. Total extract in storage increases across the campaign and may total 1-1.5 million dry CWT equivalent of sugar at each of the two plants with chromatographic separators.

One of the questions that have been raised internally is whether the microbial and chemical testing provide complementary information and if one set of tests could provide sufficient information to determine whether the extract is or is not storing well. The results of this testing indicate that the chemical tests provide sufficient information on extract storage and that the extensive sampling and microbial testing that we had been performing can be substantially reduced. This testing does confirm that microbiological testing is important to ensure the extract sent to storage is not contaminated with microbiological organisms, that the tank is clean, and that the surface of the extract is not a growth area for yeasts and molds. One may not see changes in microbial counts under recommended storage conditions (68+ RDS., pH =9.0, and temperature of <20 °C, but chemical changes may still occur and those appear to be driven by either microbial contamination or enzymatic action. This work indicates that tight controls of RDS of extract to storage, pH, and temperature are essential control parameters to consider for prevention of spoilage during storage. In addition, ensuring that the juice has a low microbial load as it enters storage will help to reduce deterioration of the juice during storage.

Several studies on thick juice storage have been completed; some of the results of those studies will be briefly discussed in this paper. In addition to thick juice storage, Fiddlers (*1*) discussed storage of raw thick juice in 1993 and Groom presented a paper on extract storage at the 2003 ASSBT (*2*). In all cases, the conditions for successful storage of concentrated juice are similar: RDS of 67% or higher or super saturation of 1.05, pH of approximately 9 or greater, temperature of storage 25 ºC or lower, and control of infections at the surface of the juice through the application of 25% sodium hydroxide or other approved chemicals that have shown efficacy in this application.

Pollach, Hein, and Rösner discuss method for improving the storability of juice for long periods of time. In particular, they developed the technique of adding a layer of sodium hydroxide to the surface of the tank in order to control the growth of yeasts and molds. They also discuss the use of ß-hop acids for controlling infections and found that these acids also delayed the start of pH drop in a tank that was undergoing microbial degradation. Marin discussed the presence of ethanol and acetic acid in the headspace of the tank as an indication that deterioration had occurred (*3*). Hein also indicated that ethanol levels in the headspace could be used as an indicator of spoilage due to yeast activity and proposed the use of a NIR for continuous monitoring of the headspace (*4*).

Sargent (*5*) presented a paper in 1997 that laid out the conditions necessary for long term storage of thick juice and provided graphs that could be used to predict how long properly prepared thick juice could be stored at different temperatures. They noted that long term storage is feasible if the supersaturation of the juice is at 1.05, pH = 9.0 ± 0.2 units, and the temperature is maintained at 10 ºC. In one tank that they were monitoring, they noted that pH drop did not occur for 160 days and then dropped from 8.9 to 7.1 in forty days. Yeasts and molds were present initially, but then disappeared during storage so that none were found at the point of the pH drop. Mesophile counts also dropped during storage, peaked at the time of the pH drop, followed by a decrease as the pH dropped. Sargent et al. indicated the mesophile count increases were preceded by an increase in temperature.

Groom found that high microbial counts were not present in juice that was degrading. The cause of the decline in juice quality in this case was thought to have been due to the presence of invertase in the extract from contamination during a low temperature evaporation operation. Microbial counts made on extract sent to storage and extract in storage showed that the extract was cleaner than extract from our East Grand Forks (EGF) facility from a microbial contamination sense, but during storage, juice color increased, pH dropped, invert concentration increased, and purity decreased whereas the extract from EGF remained stable during storage. Addition of formaldehyde or sulfur dioxide had no affect on the continued spoiling of the juice, an observation that was consistent with a note from Pollach (*6*). Only an increase in temperature sufficient to deactivate the enzyme stopped the spoilage of the extract in the laboratory. It was not possible to do anything with the extract in tank storage other than process the material earlier than had been planned as a means of forestalling any additional deterioration. Due to lower purity and higher color, the extract was more difficult costly to process.

A more recent paper by Schrevel et al. (*7*) discussed the spoilage of beet thick juice. They note that decline in pH is caused by lactic acid fermentation and that this drop was correlated the high fastidious bacteria levels and an increase in reducing sugar

on the surface. *Tetragnoccus halophilus* was found to be the culprit in storage problems. This organism does not survive under the temperatures and RDS levels in factory evaporation, so the source of contamination has to occur after evaporation. The authors determined that the source of contamination was from the air that was drawn into the tank during loading and unloading of the storage tank.

When American Crystal Sugar Company stores extract, we generally do not have problems with varying tank levels and the influx of air as a tank is drawn down and then refilled. The tanks are filled during the beet campaign and then emptied during the intercampaign period. Influx of air resulting in surface contamination of the extract is not as generally a problem. Even so, attention has to be paid to the surface condition of the juice in order to prevent airborne contamination from causing subsequent juice spoilage. The method of application of sodium hydroxide outlined by Pollach has helped prevent those contamination issues. In addition, air entering the tank should be conditioned by filtration and temperature and humidity control in order to prevent condensation on the surface of the extract or the sidewalls of the tank.

Determination of the specific organism at done by Schrevel would not be feasible in our laboratories, nor have we had acceptable results when measuring fastidious organisms. The high degree of variability between analysts caused us to discontinue using that measurement as a means of assessing the risk of juice spoiling. The work of Sargent suggests that measurement of yeasts, molds, and mesophiles may not yield results that will be indicative of spoilage of thick juice or in our case extract, although they did see a spike in mesophile count prior to the drop in pH. Microbial counts have been shown to decrease after the pH drop occurred; it is conceivable that one could miss the point of the infection if the interval between samples is too long. In addition, we have found that the presence of microbial invertase can result in juice degradation without any increase in microbial counts (*2*).

Proper conditions for long term storage of thick juice have been established through the work of authors mentioned above. One question that remained for us was: what is the best set or combination of testing methods to use for accurately predicting how well the extract is storing? Ideally, the testing could be completed in the factory lab with supplemental testing at our laboratory at the American Crystal Sugar Company Technical Services Center. In addition, the tests would not give false positive or negative indications of storage problems that would lead to either large losses of sucrose or premature (and expensive) processing of the extract.

Experimental Methods and Materials:

Inoculum for these tests was prepared using the methods described by Samaraweera et al. in 2008 (*8*). Briefly, the technique involved culturing a mesophilic population by diluting the storage extract to ~ 20 RDS followed by incubation at 35 ºC for 48 hours. After 48 hours of incubation, a 30 ml aliquot of the mixture was transferred to a new bottle containing 20 RDS extract and incubated at 35 ºC for 24 hours. This cycle continued through four cycles with the final transfer made into three bottles that were subsequently mixed together after 24 hours of incubation. After multiple transfers, the inoculum reached a count of 4.8 E+07 in the study started on April 28, 2008 compared to an initial count of zero mesophilic counts in the control. Samples of extract

that had been concentrated to ~74 RDS were mixed with inoculum to make a mix of 69 RDS mixture. In a similar manner, the same volume of inoculum was mixed with 69 RDS extract to produce a 64 RDS mixture. The inoculated extract was stored at 20 and 30 ºC for the duration of the trial. In addition, 68 RDS uninoculated samples were stored as controls at the same temperatures. Duplicates of all inoculated samples were run. The experimental design for the simple trial is a replicated two by two factorial without center points. This simple design allows for determination of main effects and interactions in the design. Initial mesophilic count data are shown in the Table 1.

The laboratory tests that we carried out were designed to determine the stability of extract under conditions of high microbial loads at RDS levels of 64 and 69 and temperatures of 20 ºC and 30 ºC. Three sets of trials were run over a three year period with the first set of trials discussed by Samaraweera (*9*) The second set of trials followed mesophilic counts on a monthly basis until the last sample was analyzed and the entire range of analytical testing was completed. The third set of trials was run in a manner similar to the first, but chemical and microbiological testing was completed on all samples on an approximately monthly schedule. Graphical data from the last two sets of trials are labeled II and III accordingly.

Extract used for all tests was from our East Grand Forks facility. Extract was placed in sterilized 1-liter plastic bottles and stored in either a 20 ºC or 30 ºC incubator after the addition of cultured inoculum. Samples were removed aseptically approximately monthly intervals through the duration of the tests. Laboratory analyses included microbiological analysis for mesophilic bacteria in the first study. Tests for RDS, purity by polarimetry and ion chromatography, glucose and fructose by ion chromatography, lactic acid and volatile fatty acid analyses by HPLC, color, and pH were incorporated into the third set of tests. Results from the two sets of trials are discussed below. In addition, an uninoculated sample at 69 RDS was stored at each temperature as controls.

It should be noted that initial attempts were made to prepare an inoculum with extract from the Hillsboro factory; however, we were not able to achieve the required level of growth of mesophilic bacteria in the Hillsboro extract. It was possible to achieve high counts in inoculum prepared from a 50:50 mixture of Hillsboro and EGF extract. The reason for the failure of growth in the Hillsboro extract is not precisely known, but it was interesting to note that microbial growth did not readily occur in the Hillsboro extract; the stability of this extract was in sharp contrast to our earlier experience with deterioration of this extract during long term storage.

Results and Discussion:

During the fall of 2006, a trial was initiated to study the effect of a high load of bacterial loading on extract storage under variable conditions of RDS and temperature. The test protocol called for samples of extract to be inoculated with high counts of bacteria that had been cultured in diluted extract. Control or non-inoculated samples had a mesophilic log count of 1; the inoculated samples had log counts ranging from 3.46 to 4.01 one day after inoculation. Samples were stored at 20 or 30 ºC for an extended period of time. Periodically samples were analyzed for microbial counts and pH, but not for VFAs, invert, color, or IC purity. The final samples were taken on August 9, 2007 and the analyses shown in Table II completed. Analytical test results as well as the

results of statistical analysis of the data are shown in Table II and in the response surface graphs that follow.

Microbial counts for trial II are shown in Table III. They did not show great deal of variation over the period of tests. Inoculated samples maintained or decreased counts throughout the period of the testing, with a couple of samples increasing by 0.25 log units and one sample decreasing by 0.41 log units. These changes were not found to be significant at the 95% CI. The control samples (69 RDS) which were not inoculated and stored at 20 and 30 ºC showed a decrease in microbial counts, decreasing from log count of 1 to 0.

Table 1—Initial mesophilic count on samples tested.

Sample ID	Mesophiles/g 12-04-06 Trial II	RDS 12-04-06	Mesophiles/g 4-28-08 Trial III	RDS 4-28-08
Starting Inoculum	9.80E+07		4.80E+07	
Control	2.00E+01	68.45	0.00E+00	68.05
64 RDS Samples	6.00E+03	64.45	1.00E+01	63.74
69 RDS Samples	6.30E+03	68.93	3.30E+03	69.41

Table II—Final Analytical Test Data from Trial II

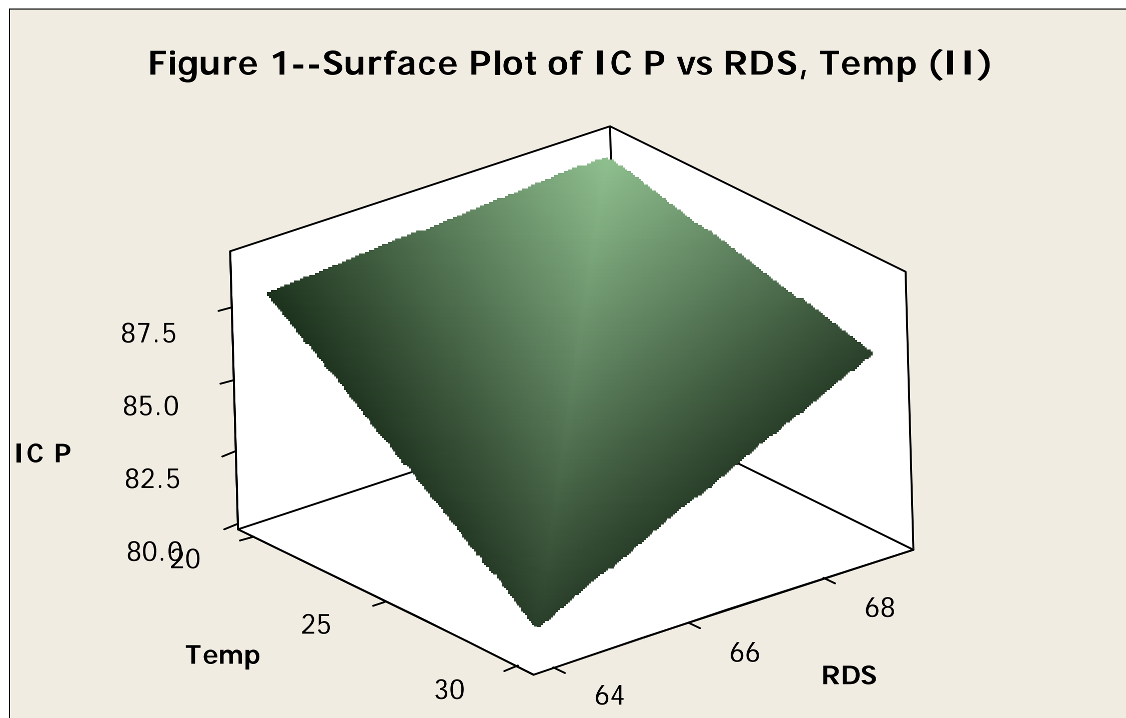
RDS	Temp	AP	pH	Color	IC P	Invert	Lactic	Formic	Acetic
64	20	88.91	8.61	10760	87.92	0.507	2967	146	536
69	20	89.70	9.18	12380	88.93	0.278	0	137	571
64	30	79.97	7.26	18420	80.95	4.573	4172	270	1356
69	30	87.53	7.60	24980	86.46	0.816	2754	359	1364
64	20	88.97	8.70	11040	88.17	0.474	2251	104	577
69	20	89.50	9.18	12310	88.91	0.296	0	71	467
64	30	79.80	7.22	18010	81.04	4.626	4310	96	703
69	30	87.96	8.18	25810	86.76	0.653	670	106	697
Control (69 RDS)	20	91.11	9.33	7490	89.69	0.041	ND	62	151
Control (69 RDS)	30	91.08	9.3	9020	90.27	0.031	ND	---	195

Table III—Mesophile counts by day in trial II

RDS	Temp	D2	D3	D8	D16	D46	D81	D163	D248	Change
69	20	3.81	3.85	3.79	3.85	3.95	3.88	3.75	3.81	0.00
69	20	4.01	3.79	3.77	3.79	3.80	3.76	3.70	3.84	-0.17
69	30	3.80	3.76	3.88	3.86	3.90	3.82	3.64	3.84	0.04
69	30	3.76	3.91	3.69	3.70	3.81	3.86	3.79	3.80	0.04
64	20	3.45	3.57	3.37	3.40	3.45	3.38	3.54	3.70	0.25
64	20	3.46	3.58	3.57	3.48	3.52	3.36	3.51	3.61	0.15
64	30	3.61	3.53	3.49	3.48	3.45	3.38	3.49	3.10	-0.51
64	30	3.46	3.60	3.36	3.43	3.43	3.53	3.43	3.05	-0.41

Change referred to in the last column refers to the change in mesophile counts from day 2 to day 248, with the change reported in log units.

Responses were analyzed using Minitab version 15, with the results shown below in the form of response surface graphs. The data analysis shows that RDS, temperature, and the interaction of these terms have a significant effect on extract purity during storage. That effect is also illustrated in the Figure 1 below, which shows the changes in IC purity at the different RDS and temperatures**.** Low RDS and high temperatures have a negative effect on purity of extract. This is not surprising, but the noted effect in the absence of significant changes in mesophilic bacterial populations was surprising.



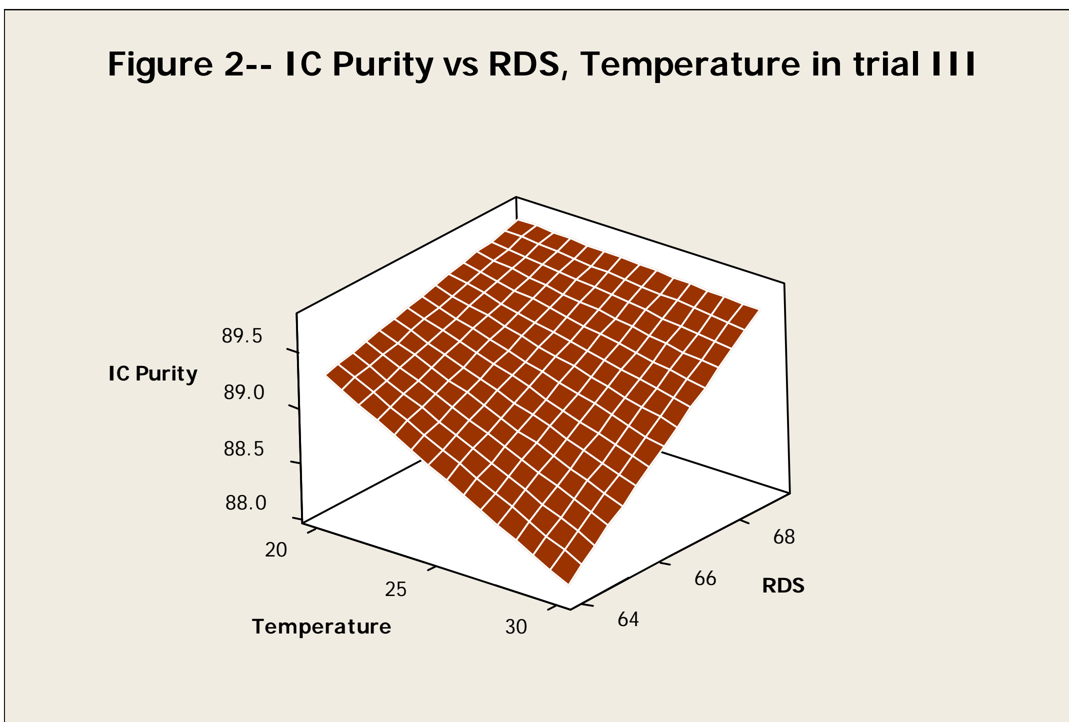
As expected, the effects of temperature and RDS on apparent purity were similar to the effects noted above. Low RDS and high temperatures resulted in a decrease in IC purity of ~8 points more than the control and ~10 points in apparent purity for trial II.

Trial III did not show the same absolute value of changes in purity as Trial II with a decrease in apparent purity of ~3.5 points and an IC purity change of ~3.6 points. However, the Trial III did show the same directional changes as Trial II. The trials were run for 248 and 280 days for trial II and III respectively. Extract and inoculum used for the two trials were different and that may explain the differences in the magnitude of changes in purity. The response surface graph for IC purity changes in Trial III is shown in Figure 2.

Figure 3 and the data in Table II show that the highest color was generated in samples stored at 30 C and 69 RDS. Invert in these samples was also lower than samples stored at 30 C and 64 RDS. Decreases in pH and increases in lactic acid were greatest for samples stored at higher temperature and lower RDS.

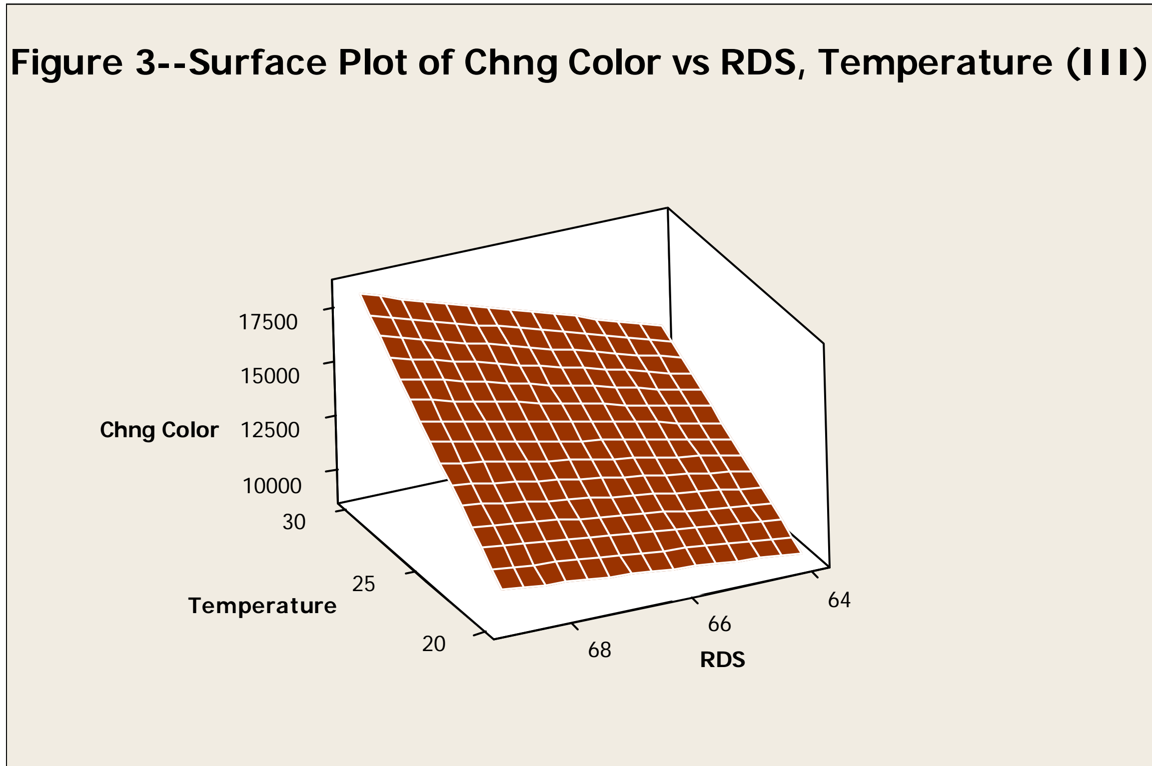
Differences of pH and temperature are known to have an effect on the reaction of the invert and on reactions that consume sucrose. The higher RDS samples generally maintained a pH above 9 even when stored at 30 °C. Given that there was low acid production under these conditions, but the greatest generation of color, it may be that alkaline degradation mechanism of both sucrose and invert are causing the color increase and the color changes are not primarily due to microbiological or enzymatic activity.

Production of invert under the different storage conditions could be due alkaline degradation of sucrose. Such degradation is possible at pH greater than 8.3 at the moderate storage temperatures. Clarke et al, Kelly and Brown and others have discussed mechanisms for both acid and alkaline degradation of sucrose and invert (*10, 11*). de Bruijn has discussed alkaline degradation of monosaccharides and the production of color in numerous articles (*12-14*). Discussion of mechanisms for color and acid formation are beyond the scope of this paper and the readers are directed to the literature cited for additional discussion of mechanisms. Sucrose lost either due to alkaline degradation or through microbial/enzymatic activity could be converted to color or organic acids with the conversion route dependent on the pH of the solution. Under conditions of pH greater than 8.5, formation of color through combination of invert with amino acids present in the extract is possible and is discussed in the literature. We did not test for any intermediate products that would allow us to determine mechanisms of generation or reaction of invert.



Vaccari noted that storage at lower RDS resulted in a drop in pH and an increase in invert, a conclusion that is similar to the results that we obtained (*15*). Figure 4 shows the change in total invert (approximated as 2x glucose) over the course of Trial III.

The initial increase in invert between the controls and the inoculated samples may be due to the increase in invert in the inoculum due to microbial growth. We did not analyze those samples for invert etc. after the three or four days of microbial growth. The controls were relatively stable with respect to changes in invert compared to the other samples.



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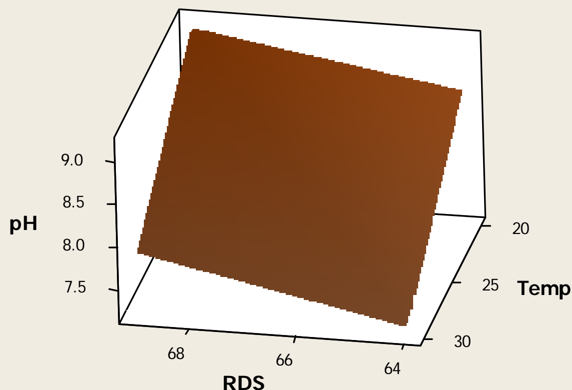
Final pH of the extract is negatively affected by high temperature and low RDS storage conditions. Figure 5 provides an indication of the magnitude of the effect of the changes during storage. The greatest pH drop occurred in samples stored at lowest RDS and the highest temperature. Microbial, enzymatic, and chemical degradation of sucrose would be expected to be greater at the lower pH values. Formation of acids from the degradation of invert or from microbial action would be expected to further lower the pH.

Figure 5--Surface Plot of pH vs Temp, RDS



Both acetic acid and lactic acid concentrations show increases that differ depending on storage conditions. Acetic acid concentration shows the greatest increase at 68 RDS and 30 C whereas lactic acid has the greatest increase at 64 RDS and 30 C. These differences may point to different mechanism of degradation of the extract. High RDS samples may be undergoing chemical changes; the lower RDS samples may be showing the effects of changes due to microbial growth with the generation of lactic acid that would decrease the pH of the extract. Colonna and Marin showed that small amounts of acid can decrease the pH of stored extract or thick juice from 9.6 down to 7 or less. The amount of acid required is comparable to the amount that would be generated from the destruction of relatively small amount of sucrose through microbial action (*3, 16*). Figures 6 and 7 show the changes in acetic and lactic acid concentrations under the conditions of Trial III.

Figure 6--Surface Plot of Acetic Acid vs RDS, Temperature (III)

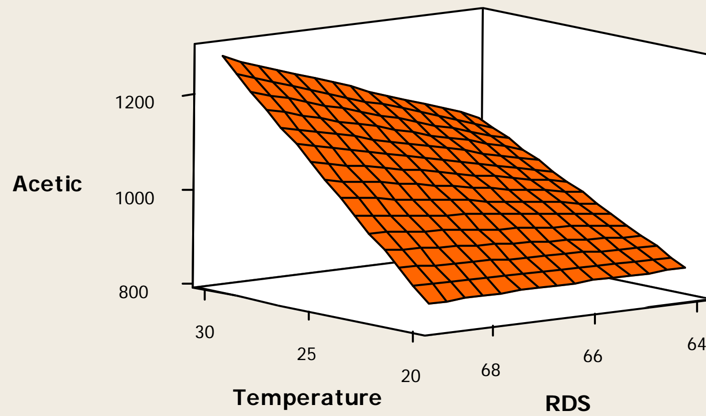
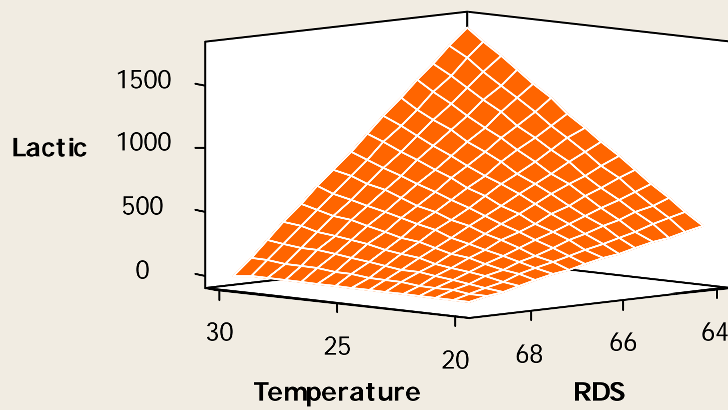


Figure 7--Plot of Lactic vs RDS, Temperature (III)



Invert levels showed the greatest increase when the extract was stored at 64 RDS and 30C. The combination of low RDS and high temperature resulted in a nearly ten point decreases in purity, with about half of that sugar showing up as invert in the stored sample.

Table IV shows the magnitude of changes between the control samples and the inoculated samples stored under comparable conditions. Several differences are listed below for samples stored at 20 ºC and 69 RDS.

- pH drop in the 69 RDS inoculated sample stored at 20 C was 0.31 units greater
- Apparent purity drop was 0.83 greater and IC purity 1.67 units higher
- Color increased by an additional 7325 RBU in the inoculated sample
- Invert was lower in the control than in the inoculated sample
- Acetic acid increased in the inoculated sample

Data for the 69 RDS and 30 ºC samples show the following with the changes being Control-inoculated sample

- Apparent purity decreases 1.19 units and IC purity by 1.47 units
- pH deceases by 0.6 units
- color increases by 13555 RBU
- Invert increases by -0.09 units
- Acetic acid increases by 704 PPM

In all cases, the inoculated samples showed worst storage capabilities than the non-inoculated samples.

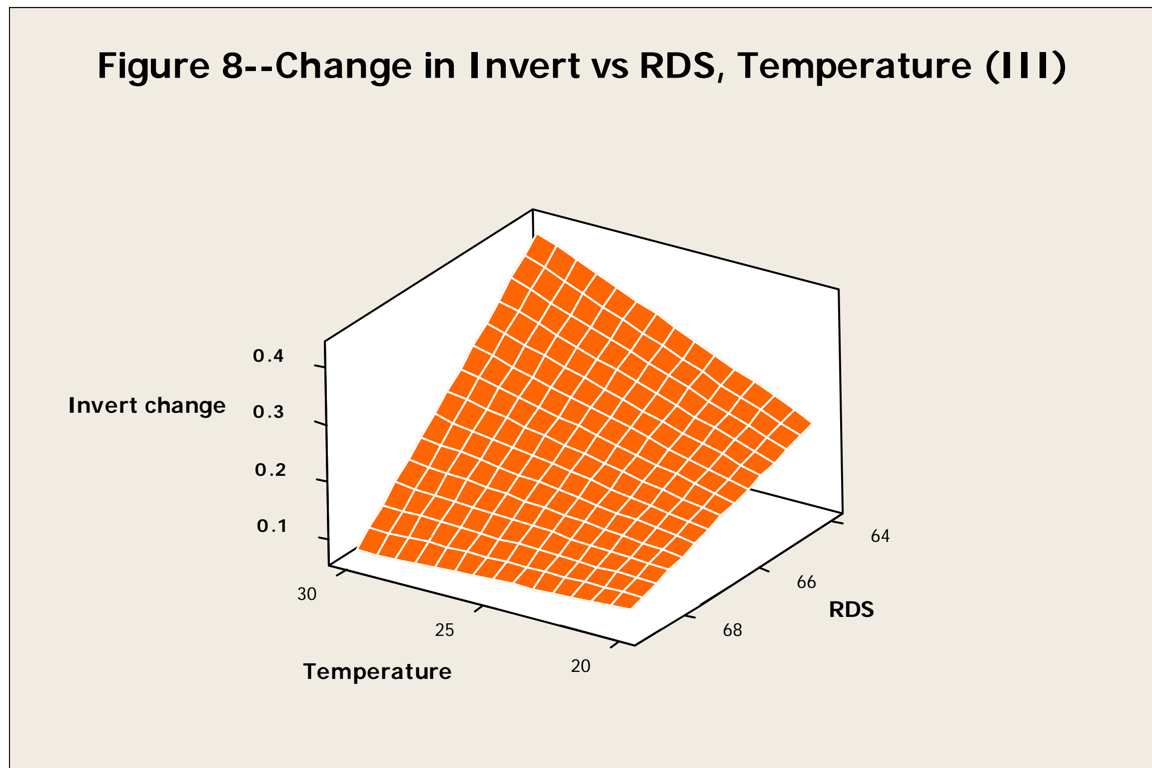


Table IV—Differences between Control samples and inoculated samples

Sample	AP	pH	Color	IC	Invert	2X Glucose	Lactic	Formic	Acetic
Control 20C-69/20 inoc	.83	.31	-7325	1.67	-.1	-.17	0	-94	-398
Control 20C-64 Inoc	1.73	.72	-6110	2.01	-.23	-.4	-380	-103	-436
Control 30-69/30c inoc	1.19	.6	-13555	1.47	-.09	-.15	86	-193	-704
Control 30-64/30 inoc	2.69	1.55	-9245	2.88	-.44	-.68	-1647	-120	-540

Conclusions:

The data presented above shows that the best extract storage conditions in this study are RDS of 68-69 or SS of >1.0 and temperature of 20 C; these are not surprising results given all the results of all the studies that have been completed over the last 20 or more years. We were surprised that we did not see any appreciable change in the bacterial populations over the course of both trials even while the extract was undergoing degradation. It may be that the organisms were active, but not actively reproducing which would explain the why the counts did not change. Whether the microbes are active or if the effects are from invertase in the solution or from alkaline degradation of sucrose in the higher RDS and temperature samples is not clear; the investigation of the mechanisms is beyond the scope of this study. We did see changes due to these mechanisms as increases in invert, organic acids, color, and decreases in purity or sucrose content and decreases in pH. Clearly, all changes were exacerbated by increasing the microbial load present in the extract sent to storage. These results point to the need to ensure that the extract is as microbiologically as clean as possible when it goes to storage.

Extract should go to storage with minimal microbial contamination and should be stored at 20C or lower and 69 RDS or a concentration that results in minimal crystallization of sucrose. Monitoring of extract storage through microbial examination does not appear to be a viable means of detecting degradation. A combination of chemical measurements may provide a more sensitive indicator of extract degradation. The measurement of pH with analyses for invert, color, and organic acids including acetic may provide the most sensitive means of detecting any changes in the quality of stored extract. Lactic acid monitoring of properly stored extract does not appear to provide useful information on success of storage of extract of proper RDS and temperature. If lactic acid is produced, it would appear that a low RDS extract had been placed into storage and microbial attack resulted in the production of lactic acid.

Recommendations:

Drop most microbial testing of extract in storage except for analysis of samples sent to storage and those from the top surface of the tank. Surveys of the stored extract may be completed on a much lower frequency. Chemical analysis of stored extract at the factory labs should be completed on a bi-weekly or more frequent schedule. Analyses completed should include AP, pH, lactic acid, and color.

Microbial testing of extract in storage does not appear to be a viable means of detecting spoilage and can be replaced by chemical testing for routine testing. Microbial activity can be continued on a much more limited schedule. This recommendation applies to extract and not to thick juice. A reading of the literature suggests that thick juice storage may be more problematical that extract storage.

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