 doi 10.1515/ijfe-2012-0156 — International Journal of Food Engineering 2013; 9(1): 67–74

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Effect of Storage Conditions on Microbiological and Physicochemical Parameters of Cloudy Apple Juice Concentrate

Abstract

The effects on microbiological parameters of minimally pasteurized cloudy apple juice concentrate obtained in a pilot scale from two apple varieties, and their changes with storage time and temperature were investigated. The juice obtained from Red Delicious apples showed an adequate biological stability for import and export market for storage at −8.5 and −20°C during a period of three months. The preservation of samples at −20 and −8.5°C limited to acceptable values the growth of molds and yeasts during the first two months in the case of juice obtained from Granny Smith apples. Neither lactic acid nor thermo acidophilic bacteria were isolated from the samples in all storage conditions. No noticeable differences in pH and soluble solids content were observed among storage times and temperatures. Turbidity increased slightly with storage time. Viscosity decreased substantially during storage at 2°C. Color was stable for all storage conditions evaluated.

Keywords: cloudy apple juice, storage, microbiology, color, turbidity

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1 Introduction

Cloudy or unclarified apple juice is a complex multicomponent system, opalescent or turbid due to the presence of insoluble solids in suspension [1]. It can be considered a minimally processed product that is increasingly preferred by the consumers over the world [2].

Cloudy apple juice contains significant quantities of suspended pulp and is perceived as a natural food product that supplies dietary fiber and important nutrients [3]. Although it can be an important source of bioactive compounds, techniques used for its processing and subsequent storage can cause alteration to its contents and consequently it might not provide the benefits expected by the consumer. All these considerations led the food industry to develop alternative processing technologies in order to produce foods with a minimum of nutritional, physicochemical, or organoleptic changes induced by technologies themselves. Attention has also been focused on the evaluation of the microbiological or toxicological risks that can be involved in applying these processes, and their effect on food safety, in order to obtain safe products that do not present health risks [4].

Processing is the step of the food chain that mainly affects the physical and bio-chemical characteristics of the juice and determines the safety and shelf life of the product. Nagel [5] describes a cloudy (opalescent) apple juice as a light, whitish-yellow juice, clearly showing cloudiness, which presents no sedimentation, full-bodied and juicy, and not astringent nor bitter. Peleg and Noble [6] found that a low value of pH in beverages is associated with astringency. It is also known the influence of pH on juice flavor and solubility of carbohydrates and proteins, which are the main insoluble solids in suspension in cloudy apple juices. It has been reported [7] that cloudy apple juice concentration up to 45–50 °Brix (i.e., increasing the concentration of total soluble and insoluble solids) does not affect the colloidal stability of the system.

However, processing is not the only factor that affects the quality of these products. It is also necessary to consider the conditions of refrigerated storage, mainly how temperature and time affect the microbiological, physicochemical, and organoleptic characteristics of the beverage.

It is desirable that microbiological deterioration and changes in physicochemical properties are reduced to a minimum during storage, so it is also necessary to optimize the conditions for refrigerated storage. An important number of deterioration reactions should be avoided, such us cloud loss, microbial spoilage, development of off-flavor, changes in pH, soluble solids concentration, color, texture, and appearance, in order to preserve product quality.

Soluble solids of apple juice consist mainly of sugars, pectin, salts, and organic acids. The evaluation of changes on their concentration during storage has importance due to the variations of their solubility with temperature and their relationship with the browning rate.

Another factor that affects the consumer's perception of quality is the color of the cloudy apple juice. The European Union [8] and the USA [9] have implemented this parameter as part of the quality control in the food industries. In cloudy apple juice, accumulation of brown color during storage is mainly attributed to five causes: enzymatic browning of the phenols, ascorbic acid oxidation, caramelization, formation of brown-red polymers by oxidized lipids and Maillard reaction [10, 11]. This also may be associated to losses in nutritional value of the product.

Besides, one of the main problems with cloudy apple juice storage is the assurance of cloud stability: even after prolonged storage only a very small part of the cloud particles should precipitate [12]. The determination of turbidity and viscosity changes during storage can be considered as a possible index of juice stability.

Another important factor that limits the shelf life of a food is the microbial growth. The presence of atypical flavor and appearance, as a result of it, diminishes the quality of the product and can, therefore, lead to the rejection of the food by the potential buyers and consumers [13].

Under the conditions prevailing in concentrated apple juice (pH 3.0 to 4.0 with high sugar content), lactic acid bacteria (LAB), molds, yeasts and *Alicyclobacillus* species comprise the typical microbiota that should be expected to be found. Lactic acid bacteria are the primary spoilage bacteria in fruit beverages; however, their number is greatly reduced after pasteurization, concentration, and refrigeration. On the other side, molds and yeasts tolerate high-osmotic and low-pH conditions. They grow even at refrigeration temperatures and can, therefore, cause spoilage in the processed product. These microorganisms that use fruit as substrate will generate flavors and odors, discoloration of the product, and fermentation.

Species of *Alicyclobacillus* are acid-tolerant and heat-resistant bacteria (thermo-acidophilic bacteria, TAB) that cause spoilage of fruit juices stored at room temperature. Spores of *Alicyclobacillus* species are known to survive heat pasteurization processes applied to fruit, vegetable, and fruit/vegetable-based beverages, fruit concentrates and purees, sugar, sugar syrups, tea, isotonic drinks (sports drinks), and other low pH products [14]. Some strains of the bacterium can grow at pH 2.0 and at temperatures as high as 70°C [15–17]. Germination of spores, followed by vegetative cell growth can occur within a few days or be delayed well into the expected shelf life of products. Medicinal or disinfectant off odors attributable to guaiacol and halogenated phenols produced by *Alicyclobacillus* [18, 19] can be detected at concentrations as low as 2 ng/mL of beverage [20, 21]. Reviews [22–26] provide extensive discussions of taxonomy, physiological characteristics, and conditions affecting survival and growth of *Alicyclobacillus*.

Good hygiene alone is not sufficient to control the occurrence of *Alicyclobacillus* in fruit juices. Soil is considered to be the main repository for spores to TAB and it is believed that fruit in contact with the soil during harvesting become contaminated. Fruit juice contamination results from unwashed or poorly washed raw fruit that is processed, as well as contaminated water used during the production of fruit juices. The only viable count control measure at present is the thorough washing of the raw material before it is processed. Heat treatment alone has been shown to be inefficient to eliminate *Alicyclobacillus* from fruit juices without altering the organoleptic qualities or vitamin content of fruit juices [23, 25].

Argentine is the fifth world producer and exporter, offering 4% of the world trade, and leads the production of concentrated apple juice in the southern hemisphere. In average, 95% of Argentine production of concentrated apple juice is exported, mainly to the USA and to a lesser extent, to Europe and Japan. In recent years, the increase of the world offer implies higher quality demands to Argentine and the need for new alternatives to reduce costs. To export, cloudy apple juice concentrated to 45 ° Brix produced in Argentina is currently transported and stored at −20°C [27], but important savings in energy could be achieved if the temperature were increased without affecting the quality and microbiological safety of the product. This study was undertaken to determine the impact of three storage conditions (−20°C, −8.5°C and 2°C) during a period of four months on some physicochemical properties (pH, soluble solids concentration, turbidity, viscosity, and color) and microbiological parameters (LAB, TAB, molds, and yeasts) of cloudy apple juice concentrate obtained from two varieties of apple (Granny Smith and Red Delicious) in pilot scale.

2 Materials and methods

2.1 Juice samples preparation

The general manufacturing process for cloudy apple juice concentrate has been conducted as follows: apples (Granny Smith and Red Delicious) were obtained from commercial sources. After suitable washing and hygienization of the fruits, they were crushed in a mill, pressed in a hydraulic press, and heated by means of a stream of steam to a temperature of 65–70°C for 15–20 seconds to inactivate native polyphenol oxidase (PPO). Cloudy apple juice was concentrated to 46–47 °Brix at 45°C in a rotoevaporator of 5 L capacity (Rotavapor R-151, Büchi, Switzerland) and rapidly cooled to a temperature of 2°C.

For microbiological and physicochemical analysis, samples by triplicate were packed in 200 mL sterile flasks and were used as control group. Other twelve samples (three for each storage temperature and time) were stored at −20, −8.5 and 2°C during one, two, three and four months. In all experiences, each sample was analyzed by triplicate.

2.2 Physicochemical properties analysis

2.2.1 pH measurement

Apple juice pH was recorded at 20°C using a pH electrode Altronix TPX-1 model (SAEN S.R.L.) calibrated with Anedra (Argentine) standard buffers (pH 4.0) before and after each pH determination.

2.2.2 Total soluble solids (TSS)

Total soluble solids were determined by measurement of the refractive index (°Brix) on juice samples using a digital refractometer (Abbe Mark II, Reichert, USA) equipped with a temperature compensation sensor at 20°C $\pm$ 0.1°C.

2.2.3 Viscosity

A Brookfield model DV-II Viscometer (Brookfield Engineering Labs. Inc. Stoughton, MA 02072 U.S.A) was used to measure shear stress versus shear rate in order to determine the viscosity. A concentric cylinder geometry was employed. The tests were conducted at 20°C.

2.2.4 Turbidity

Turbidity was determined at room temperature in a 15 mL standard vial with a PC Compact 4206020 turbidimeter (Aqualitic, Germany) and expressed in Nephelos Turbidity Units (NTU).

2.2.5 Color

Color was determined using a Hunterlab UltraScan XE spectrophotometer (Hunter Assoc. Laboratory, Reston, VA, USA) in the reflectance mode and calibrated with black and white standard tiles. Samples were placed in 5 cm path sample cells and filled near the top, and color was recorded using the CIE Lab uniform color at room temperature. Commission International de l'Eclairage (CIE) classifies color in three dimensions; L* (brightness), a* (green to red color), and b* (blue to yellow). A shift toward direction of decreasing L*, and increasing a* and b* would indicate a browning trend. The results were expressed in accordance with the CIE Lab system with reference to illuminant D65 and with a visual angle of 10°.

2.3 Microbiological analysis

2.3.1 Isolation and identification of molds and yeasts

One milliliter of apple juice samples and decimal dilutions in 0.1% w/v peptone water (Merck KGaA, Darmstadt, Germany) were cultured on Yeast Extract-Glucose-Chloramphenicol Agar (YGC) (Merck KGaA) [28–30]. The plates were incubated at 25°C for five days. Each sample was analyzed by triplicate. The mold colonies were identified by their macro and micro morphology.

2.3.2 Isolation and identification of LAB

One milliliter of apple juice samples and decimal dilutions in 0.1% w/v peptone water (Merck KGaA) were cultured on M17 agar (Biokar, France) and MRS Agar (Britania, Argentine). The MRS plates were incubated at 25°C for 48 h in 6 % CO_2, and M17 plates were incubated at 25°C for 48 h in aerobic conditions. Each sample was analyzed by triplicate. The different colonies isolated on MRS and M17 agar were characterized by Gram coloration and catalase test.

2.3.3 Isolation and identification of TAB

Three different protocols were evaluated for ability to support growth of TAB cells:

Protocol 1: Ten grams of each sample were diluted in 90 mL of YSG broth: 2 g/L yeast extract (Merck KGaA), 1 g/L glucose (Merck KGaA), 2 g/L soluble starch (Merck KGaA). The dilution was heat shocked at 70°C for 20 min and cooled immediately. Heat shock is applied in order to obtain a uniform germination of *Alicyclobacillus* spores and to eliminate other microbial contaminants, such as yeasts and lactic acid bacteria. When a sample is tested after a long storage period under refrigerated conditions, synchronized germination is obtained after heat shock treatment [25]. The pH was adjusted to 3.7 ± 0.1 with 1N hydrochloric acid and the dilution was pre-incubated at 45°C for three to five days (enrichment). Then, 0.3 mL were cultured at 45°C for three to five days by spread plate technique on YSG agar: 2 g/L yeast extract (Merck KGaA), 1 mL glucose (Merck KGaA), 2 g/L soluble starch (Merck KGaA) and 14 g/L bacteriological agar (Biokar). Sterile molten agar (50°C) was adjusted to pH 3.7 ± 0.1 with 1 N hydrochloric acid.

Protocol 2: Ten grams of each sample were diluted in 90 mL of K broth: 2.5 g/L yeast extract (Merck KGaA), 5 g/L peptone (Merck KGaA), 1 g/L glucose (Merck KGaA) and 1 g/L Tween 80 (Merck KGaA). The dilution was heat shocked at 70°C for 20 min and cooled immediately. The pH was adjusted to 3.7 ± 0.1 with 25 % filter-sterilized malic acid and the dilution was pre-incubated at 43 ± 1°C for three days (enrichment). Then, 0.3 mL were cultured at 43 ± 1°C for three days by spread plate technique on K agar: 2.5 g/L yeast extract (Merck KGaA), 5 g/L peptone (Merck KGaA), 1 g/L glucose (Merck KGaA), 1 g/L Tween 80 (Merck KGaA), and 14 g/L bacteriological agar (Biokar). Sterile molten agar (50°C) was adjusted to pH 3.7 ± 0.1 with 25 % filter-sterilized malic acid.

Protocol 3: Ten grams of each sample were diluted to 12 °Brix with sterile distilled water. This dilution was heat shocked at 70°C for 20 min and cooled immediately. The pH was adjusted to 3.5 ± 0.1 with sterile 10% tartaric acid and the dilution was pre-incubated at 46°C for two days (enrichment). Then, 0.3 mL were cultured at 46°C for two days by spread plate technique on PDA agar (Merck KGaA). Sterile molten agar (50°C) was adjusted to pH 3.5 ± 0.1 with sterile 10% tartaric acid.

The resulting data were analyzed using Two-way ANOVA to perform multiple analyses of the interactions between all factors. When appropriate, Bonferroni post-test was used for comparison of means. All tests were performed with a confidence level of 95%.

3 Results and discussion

3.1 Isolation and enumeration of molds and yeasts

Figure 1 shows the results of count of molds and yeasts isolated from cloudy apple juice concentrate obtained from two varieties of apple (Granny Smith and Red Delicious).

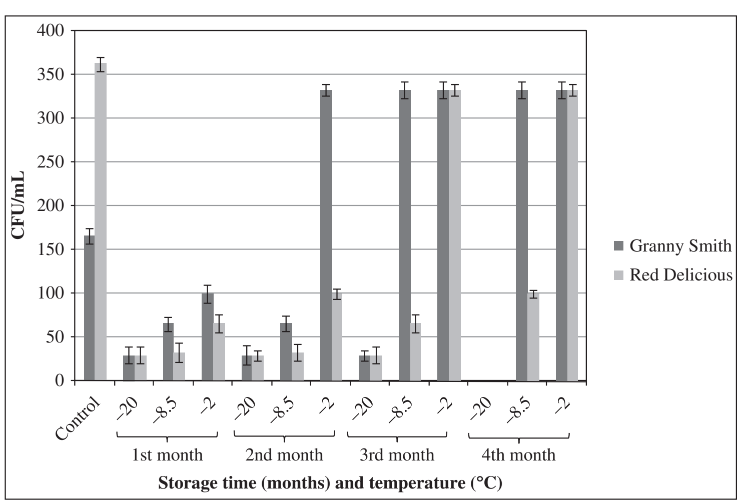

Figure 1 Count of molds and yeasts isolated from cloudy apple juice concentrate obtained from two varieties of apple (Granny Smith and Red Delicious).

In general, there is no a criterion defined between countries that import the product about acceptable limits of microorganisms (in particular, molds and yeasts), for what, to discuss our results, we take the limit of CFU/mL for fungi more strict declared by local exporters.

For cloudy juices processed from Granny Smith apples, only the growth of filamentous fungi was observed, and as the storage temperature increases, so does the counting of molds. This behavior was observed in all storage conditions. The preservation of samples at −20 and −8.5°C limited to acceptable values the growth of molds and yeasts during the first two months, exceeding this limit from the third month.

Control samples counts were higher than count in those stored for one month in both varieties. This may be due that cold storage following heat processing inactivates sub lethally injured microorganisms, and leads to the lower counts of cells in stored samples.

All the yeasts isolated from cloudy apple juice processed from Red Delicious belonged to *Zygosaccharomyces* species. In both varieties of juice, the molds isolated from the samples belonged to the genus *Cladosporium, Geotrichum, Aspergillus, Penicillium, Fusarium*, and *Alternaria*. The molds and yeasts found in the different samples are part of the normal microbiota of the apple (raw material) and/or from concentrated apple juice processing-environment.

3.2 Isolation of LAB

None of the Gram-positive bacteria obtained from the colonies in agar MRS and M17 were catalase negative. This result does not mean the absence of this group, which has been described as microbiota of the apple and juice non-pasteurizing. The results could be due to the heat treatment performed to inactivate the PPO.

3.3 Isolation of TAB

All protocols performed to evaluate growth of TAB cells showed that the apple juice was free of spores.

It is noteworthy that none of the samples showed contamination with TAB, which is of particular interest to Argentine's apple juice industry since it is one of the main microbiological problem of juice concentrates, and a major cause of rejection of countries that import the product. Three different protocols were followed to evaluate growth of TAB cells, since uncertainty exists about which media is most effective for isolation of TAB from fruit juices and fruit juice products.

Recommended culture media, incubation temperature, and incubation time differ among countries and groups representing the juice and beverage industry in various countries and geographic regions. The Japan Fruit Juice Association [31] recommends using yeast extract starch glucose (YSG) agar in samples pre-enriched in YSG broth. The American Public Health Association recommends the use of K agar as a direct plating medium [32]. Baumgart [33], in the Handbook of Culture Media for Food Microbiology, stated that in general acidified potato dextrose agar (pH 3.5) is suitable as a selective plating medium for *Alicyclobacillus*. It was recommended that plates be incubated at 46°C for two days.

In order to achieve a low microbial load in the juice and avoid contamination post-processing, a high level of sanitation in the fruit and processing equipment is required. According Mossel et al. [34], and to the Good Manufacturing Practices and Distribution (GMPD) and Sanitation Standard Operating Processes, two types of measures are essential to prevent the spoilage of foods: (a) reduce the number of contaminant microorganisms by selecting raw materials of good quality, monitor the proper functioning of the process to control microorganisms and prevent cross-contamination, and (b) limit the colonization diminishing or stopping the microbial growth through the implementation of appropriate measures for cleaning and sanitizing. Therefore, juice fruits producers should be reduce the risk of contamination by microorganisms during the cloudy apple juice processing and make packaging under strict aseptic conditions.

3.4 Physicochemical properties

The initial values obtained for pH and °Brix are shown in Table 1.

As it was observed that temperature and storage time have little effect on pH and soluble solids concentration, the values for the different storage conditions and time are omitted for simplicity. Various authors have studied the storage effect on the pH of fruit juices.

Table 1 Initial values of pH and soluble solids concentration for cloudy apple juice.

Red Delicious		Granny Smith	
pH	°Brix	pH	°Brix
3.865	46.8	3.315	47.0

Esteve et al. [4], Rivas et al. [35] and Rodrigo et al. [36], who studied the changes of pH in pasteurized orange and orange–carrot juice during storage at 4, 10, and 12°C, also report no changes.

No significant changes in °Brix were reported previously for pasteurized orange and orange – carrot juice [35, 37, 38].

To measure color, the CIE parameters L*, a*, b* were obtained, and color difference ΔE calculated. Table 2 shows the results for both juices. It is considered that a value of ΔE not exceeding 2 implies not noticeable visual difference in color for a number of situations [39, 40]. The samples under consideration showed a total color difference less than 2 during the first three months.

In Table 3, the values of turbidity are shown. It can be observed that it increases slightly with storage time and temperature. This increase was higher in cloudy apple juice obtained from Granny Smith apples.

Molds and yeasts, typical microbiota present in apple juices, can grow at refrigeration temperatures and generate gas production, turbidity increase, and swelling of the containers [41]. In this work, it was observed that as the storage temperature and time increase, so does the counting of molds. This behavior could explain the turbidity increase obtained. The high turbidity values determined during storage time indicate that both apple juice varieties are inherently stable [1].

Viscosity values at each storage time and temperature were calculated by regression of data of shear stress vs. shear rate. In all cases, data followed a linear trend ($R^2 > 0.99$) indicating that both juices behave as Newtonian fluids. Genovese and Lozano [42] also reported that cloudy apple juices at soluble solids concentrations up to 50 °Brix show such behavior.

Newtonian viscosities obtained for cloudy apple juice at different storage temperatures are listed in Table 4. No significant changes can be observed for juices stored at −20 and −8.5°C over the period considered. However, this property shows an evident decrease for juice stored at 2°C. This fact could be associated with the higher growth of molds and yeast observed at this temperature. Many types of yeast, some bacteria, and a large variety of filamentous fungi are known to synthesize pectinases, being particularly effective than those coming from the fungi of the genus *Aspergillus*. Pectinases are responsible for the degradation (hydrolysis) of long and complex molecules called pectins [43, 44]. The optimum pH values for actions of different enzymes on apple pectin were determined to be between 3.5 and 6.5. Significant changes related to the characteristics and technological properties of fruit and vegetable products, during maturation, storing, and canning, are related to the physicochemical transformations of pectins caused by the action of pectic enzymes. It was observed that the apple pectin hydrolysis releases reducing sugars and reduces the viscosity [45]. These results agree with ours for long storage times and high storage temperatures.

Table 2 CIE color parameters of Cloudy Apple Juice during storage.

t (months)	*T* (°C)	Red Delicious				Granny Smith			
		L*	a*	b*	ΔE	L*	a*	b*	ΔE
0		31.61	6.24	5.09		30.67	6.43	3.66	
1	−20	31.72	6.34	5.34	0.28	30.85	6.60	3.87	0.32
2		32.05	6.24	5.26	0.47	31.00	6.35	3.50	0.38
3		33.47	6.62	5.09	1.89	32.35	6.85	3.52	1.74
1	−8.5	31.63	6.37	5.28	0.22	30.60	6.52	3.72	0.12
2		31.64	6.47	5.19	0.25	30.65	6.47	3.60	0.08
3		32.91	6.71	4.84	1.41	31.86	6.96	3.36	1.33
1	2	31.46	6.47	5.25	0.31	30.45	6.54	3.72	0.25
2		31.37	6.50	5.26	0.39	30.32	6.61	3.84	0.43
3		32.47	6.92	4.95	1.11	31.43	7.14	3.62	1.03

Table 3 Turbidity (NTU) for different storage temperatures.

t (months)	Red Delicious			Granny Smith		
	−20°C	−8.5°C	2°C	−20°C	−8.5°C	2°C
0	1,192	1,192	1,192	926	926	926
1	1,224	1,236	1,225	945	943	934
2	1,203	1,222	1,212	912	915	921
3	1,256	1,234	1,243	938	944	965
4	(*)	1,261	1,264	(*)	1,022	1,046

Notes: (*) Not determined.

Table 4 Viscosity (mPa s) for different storage temperatures.

t (months)	Red Delicious			Granny Smith		
	−20°C	−8.5°C	2°C	−20°C	−8.5°C	2°C
0	31.59	31.59	31.59	33.73	33.73	33.73
1	32.51	31.18	29.89	34.06	33.35	33.69
2	36.09	33.67	31.77	35.76	34.53	23.89
3	32.07	32.51	22.61	34.40	34.55	17.91
4	(*)	29.91	11.63	(*)	31.55	10.44

Notes: (*) Not determined.

4 Conclusions

The results of the present work show that, from the microbiological point of view, the cloudy apple juice concentrate maintains its acceptability properties up to two months after being obtained (Granny Smith) and as long as three months (Red Delicious) at storage temperatures of −8.5 and −20°C. It is important to emphasize that thermo acidophilic bacteria were not isolated from the samples in all storage conditions.

No noticeable differences in pH and soluble solids content were observed among storage times and temperatures. Turbidity increased slightly with storage time and this increase was higher in cloudy apple juice obtained from Granny Smith apples. No significant changes in viscosity can be observed for juices stored at −20 and −8.5°C over the period considered. Low values of ΔE were obtained, which indicate color stability for all storage conditions evaluated.

This is an interesting conclusion, as it opens the door to energy saving as a consequence of a lower requirement in refrigeration for transport and short time storage purposes, with a positive impact on environment and companies' costs.

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