UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Departamento de Engenharia de Alimentos

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Processamento e estabilidade de alimentos líquidos

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Texto sistematizado apresentado à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para o concurso de provas visando à obtenção de título de Livre Docente na área de **Processamento e estabilidade de alimentos líquidos**, junto ao Departamento de Engenharia de Alimentos, em conformidade com o edital ATAC/FZEA 16/2020.

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Carinhosamente dedico este trabalho à Cintia, Bernardo, Pipoca, Guaraná e Jade.

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SÍNTESE DA TRAJETÓRIA CIENTÍFICA DO AUTOR

A trajetória científica do autor iniciou-se em 1994, no segundo ano do curso de graduação em Engenharia de Alimentos, como aluno de Iniciação Científica. Os programas de Mestrado (1998-2000) e Doutorado (2000-2004) realizados subsequentemente revelaram a vocação para a pesquisa e ensino na fascinante área da Tecnologia de Alimentos. Vocação esta que se concretizara com o ingresso no corpo docente do Departamento de Engenharia de Alimentos (ZEA) da Faculdade de Zootecnia e Engenharia de Alimentos (FZEA) da Universidade de São Paulo (USP), no campus de Pirassununga/SP, no ano de 2005.

O curso de Engenharia de Alimentos da FZEA/USP completara apenas 4 anos de implantação. Um grande e persistente esforço foi empenhado na captação de recursos para financiar a criação da infraestrutura de pesquisa, que após 2 anos, aproximadamente, tornar-se-ia o cenário para condução de projetos em planta de processamento piloto, culminando com a criação da linha investigativa "batizada" como **Processamento e estabilidade de alimentos líquidos**.

No transcorrer dos últimos 13 anos processaram-se leite, caldo de cana, soro de queijo, bebida isotônica, néctar de fruta, sucos de laranja e uva, nas instalações do Departamento de Engenharia de Alimentos, e no *High Pressure Processing Validation Center* do *Department of Food Science and Technology* da *Cornell University/USA*, durante a realização de um estágio sabático. Este trabalho está lastreado nos resultados de maior significância produzidos ao longo do período de engajamento do autor com as atividades de pesquisa.

As pesquisas desenvolvidas no Departamento de Engenharia de Alimentos da FZEA/USP, essencialmente de natureza aplicada, foram idealizadas para privilegiar microescalas de produção, com o propósito de desenvolver e aprimorar tecnologias de processamento e envase, acessíveis a microempreendedores do segmento de alimentos líquidos. Ademais, ancorou-se esta linha de investigação ao emergente conceito "*clean label* ". Ou seja, no planejamento dos projetos de pesquisa optou-se, prioritariamente, pela aplicação combinada de tecnologias físicas de conservação de alimentos em detrimento de intervenções químicas. Neste contexto, os alimentos são

processados preservando-se a sua composição original, evitando-se o emprego de ingredientes artificiais e/ou aditivos sintéticos.

RESUMO GERAL

O presente trabalho foi elaborado como parte dos requisitos para obtenção do título de Livre Docente junto ao Departamento de Engenharia de Alimentos da FZEA/USP. Para tanto, compilaram-se sete artigos com escopo ancorado na combinação de tecnologias de processamento e estabilidade de alimentos líquidos. Conduziram-se pesquisas com leite pasteurizado, caldo de cana, soro de ricota, bebida isotônica, sucos de maçã e uva. O estudo com leite pasteurizado visou à avaliação de sua estabilidade em diferentes embalagens e temperaturas de estocagem. As investigações com caldo de cana foram focadas no impacto da acidificação, temperatura de pasteurização e sistema de embalagem no tempo de vida útil da bebida. Outro estudo foi conduzido para avaliar a cinética de escurecimento do caldo *in natura* extraído de diferentes cultivares. O soro de ricota foi utilizado como base para o desenvolvimento de uma formulação isotônica comercialmente esterilizada. Os sucos de maçã e uva tinta foram empregados como matrizes para a validação e otimização do processamento a altas pressões hidrostáticas. Em síntese, destacam-se as seguintes descobertas. No estudo reportado no Capítulo 1 – *Microbiological shelf life of pasteurized milk in bottle and pouch* – demonstrou-se a extensão do impacto da temperatura de estocagem, em ampla faixa, no tempo de vida útil de leite pasteurizado tipo A. O material de embalagem exerceu “discreto” efeito na conservação do produto sob refrigeração. Idealmente, este alimento deve ser mantido a temperaturas iguais ou inferiores a 4 ºC e na ausência de luz. No Capítulo 2 – *Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage* – constatou-se a viabilidade tecnológica de produção de caldo de cana acidificado com maracujá. O aumento da temperatura de pasteurização exerceu um impacto positivo na vida de prateleira da bebida. Os resultados do trabalho apresentado no Capítulo 3 – *Development of a ricotta cheese whey-based sports drink* – apontaram as intervenções tecnológicas necessárias para transformação de soro de ricota em um suplemento hidroeletrolítico comercialmente esterilizado, com destacado potencial de aplicação industrial. Os resultados produzidos a partir da condução do estudo que consta do Capítulo 4 – *Cultivar affects the color change kinetics of sugarcane juice* – evidenciaram a importância da escolha do cultivar da matéria-prima no que tange a cinética de escurecimento enzimático de caldo de cana destinado ao consumo direto.

Os dados gerados na pesquisa apresentada no Capítulo 5 – *Sugarcane juice stability in plastic bottles treated with silver and zinc oxide* – não confirmaram o potencial antimicrobiano de micropartículas de prata e óxido de zinco (Ag/ZnO), incorporadas ao material de embalagem, na estabilidade de caldo de cana integral pasteurizado, contrariando as expectativas do estudo. Os ensaios com a aplicação de altas pressões hidrostáticas conduzidos com suco de uva tinta reportados no Capítulo 6 – *Searching for high pressure processing parameters for Escherichia coli O157:H7, Salmonella enterica and Listeria monocytogenes reduction in Concord grape juice* – demonstraram que a aplicação de pressões moderadas e inferiores àquelas comercialmente empregadas foram efetivas na redução de patógenos de referência inoculados no suco, em conformidade com as diretrizes da *Food and Drug Administration* (FDA). O estudo reportado no Capítulo 7 – *The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice* – revelou que a combinação de dimetil dicarbonato (DMDC) e níveis de pressão moderados foi altamente efetiva na destruição de patógenos de referência inoculados em suco de maçã clarificado.

ABSTRACT

The academic assay herein is presented as part of the requirements for receiving the title of “Associate Professor” from the Department of Food Engineering at School of Food Engineering and Animal Science of the University of São Paulo/Brazil. This assay encompasses seven articles anchored in the combination of processing technologies and stability of liquid food products. The experimental studies were conducted with pasteurized milk, sugarcane juice, ricotta whey (scotta), sports drink (isotonic), apple and grape juices. The research work performed with milk evaluated its stability in different packages and storage temperatures. Studies with sugarcane juice focused on the impact of acidification, pasteurization temperature and packaging system on the juice´s shelf life. The browning kinetics of freshly extracted whole cane juice extracted from different cultivars were also evaluated. Scotta was used for formulating a shelf stable isotonic drink. Apple and Concord grape juices were tested as matrices for the validation and optimization of processing at high hydrostatic pressures. In summary, the following findings stand out. In Chapter 1 – *Microbiological shelf life of pasteurized milk in bottle and pouch* – the storage temperature exhibited a meaningful impact on milk stability. Conversely, the type of packaging showed a mild effect on the product´s shelf life. In Chapter 2 – *Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage* – the technological feasibility for producing cane juice acidified with passion fruit was demonstrated. The increase in pasteurization temperature played a positive effect on the shelf life of the end product. The findings presented in Chapter 3 – *Development of a ricotta cheese whey-based sports drink* – pointed out the technological interventions to formulate a shelf stable isotonic drink. The findings from study reported in Chapter 4 – *Cultivar affects the color change kinetics of sugarcane juice* – showed the influence of raw material cultivar upon the kinetics of enzymatic browning triggered in ready-to-drink cane juice. The study presented in Chapter 5 – *Sugarcane juice stability in plastic bottles treated with silver and zinc oxide* – did not confirm the antimicrobial potential of silver and zinc oxide microparticles (Ag/ZnO), incorporated into plastic bottle, in the stability of pasteurized whole cane juice. The experimental assays addressing the application of high hydrostatic pressures performed with Concord grape juice reported in Chapter 6 – *Searching for high pressure processing parameters for Escherichia coli O157:H7,*

Salmonella enterica and Listeria monocytogenes reduction in Concord grape juice – demonstrated that the application of moderate pressures, lower than those commercially used, were effective in reducing pathogens of reference inoculated into the food matrix. The study reported in Chapter 7 – *The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice* – revealed that the combination of dimethyl dicarbonate (antimicrobial) and moderate pressure levels was highly effective in destroying pathogens of reference in clarified apple juice.

SUMÁRIO

1 INTRODUÇÃO

A estrutura dos elementos textuais deste trabalho foi norteada pelo modelo de "Texto Sistematizado", em conformidade com as diretrizes estabelecidas para concurso de Livre-Docente da Faculdade de Zootecnia e Engenharia de Alimentos (FZEA) da Universidade de São Paulo (USP). Para tanto, foram compilados sete artigos, publicados em periódicos científicos de circulação internacional, que representam a linha de investigação do docente-candidato, direcionada para o processamento e estabilidade de alimentos líquidos. Os artigos, apresentados nos capítulos, foram introduzidos em ordem cronológica crescente com o propósito de demonstrar a evolução da trajetória do docente no âmbito da pesquisa.

O Capítulo 1 apresenta o artigo ***Microbiological shelf life of pasteurized milk in bottle and pouch*** publicado no periódico *Journal of Food Science*, em 2010. Este trabalho de pesquisa foi conduzido com o suporte de alunos de Iniciação Científica, com o objetivo de investigar a extensão do impacto da temperatura de estocagem, em uma ampla faixa, na estabilidade microbiológica de leite pasteurizado acondicionado em garrafas de polietileno de alta densidade (PEAD) e saquinhos de polietileno de baixa densidade (PEBD). Os ensaios experimentais foram realizados no Departamento de Engenharia de Alimentos da FZEA/USP.

O Capítulo 2 apresenta o artigo ***Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage*** publicado no periódico *Ciência e Agrotecnologia*, em 2014. Neste trabalho testaram-se diferentes temperaturas para pasteurização de caldo de cana acidificado com polpa de maracujá, visando à estimativa do tempo de vida útil da bebida estocada sob refrigeração. Combinaram-se quatro obstáculos: (1) alta acidez, (2) tratamento térmico, (3) envase ultra limpo e (4) estocagem refrigerada. Os resultados apresentados compuseram uma dissertação de Mestrado intitulada "Processamento e estabilidade de caldo de cana acidificado", desenvolvida na FZEA/USP.

No Capítulo 3 é apresentado o manuscrito ***Development of a ricotta cheese whey-based sports drink*** publicado no periódico *Advances in Dairy Research*, no ano de 2016. Este estudo teve seu objetivo centrado no desenvolvimento de uma formulação isotônica a partir do soro de ricota, e inseriu-se em uma tese de Doutorado

intitulada “Aproveitamento de soro de ricota para elaboração de suplemento hidroeletrolítico”. Os ensaios experimentais também foram realizados no Departamento de Engenharia de Alimentos da FZEA/USP.

O Capítulo 4 é representado pelo artigo ***Cultivar affects the color change kinetics of sugarcane juice*** publicado em 2019, na revista *Food Science and Technology*. Objetivou-se um estudo cinético do escurecimento enzimático em caldo de cana *in natura* extraído de diferentes cultivares de matéria-prima. Os experimentos inseriam-se em uma tese de Doutorado intitulada “Avaliação sensorial descritiva de caldo de cana extraído de diferentes cultivares” e foram realizados na FZEA/USP.

O Capítulo 5 consta do manuscrito ***Sugarcane juice stability in plastic bottles treated with silver and zinc oxide***, publicado em 2019 no periódico *Packaging Technology and Science*. Este artigo integrou a dissertação de Mestrado “Estabilidade de néctar de jabuticaba e caldo de cana acondicionados em garrafa plástica incorporada com agentes inorgânicos microestruturados de prata e óxido de zinco”, desenvolvida na FZEA/USP. Reportou-se no referido artigo a avaliação do desempenho da prata e óxido de zinco na estabilidade microbiológica de caldo de cana pasteurizado.

O Capítulo 6 resultou de ensaios conduzidos no *High Pressure Processing (HPP) Validation Center* do *Department of Food Science and Technology* da *Cornell University/USA*, durante a realização de um estágio sabático. No referido capítulo é apresentado o artigo ***Searching for high pressure processing parameters for Escherichia coli O157:H7, Salmonella enterica and Listeria monocytogenes reduction in Concord grape juice***, publicado no ano de 2019 no periódico *British Food Journal*. Pretendeu-se por meio deste estudo otimizar os parâmetros de processamento e avaliar se patamares inferiores de pressão aos industrialmente aplicados seriam efetivos na destruição de patógenos inoculados em suco de uva tinta.

O 7° e último capítulo consta de um manuscrito que reporta os resultados de ensaios também conduzidos no *HPP Validation Center* de *Cornell*, intitulado ***The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice***, publicado no *Brazilian Journal of Microbiology*, em 2019. Este trabalho avaliou o efeito combinado de altas pressões e

dimetil dicarbonato visando à destruição de patógenos de referência inoculados em suco de maçã.

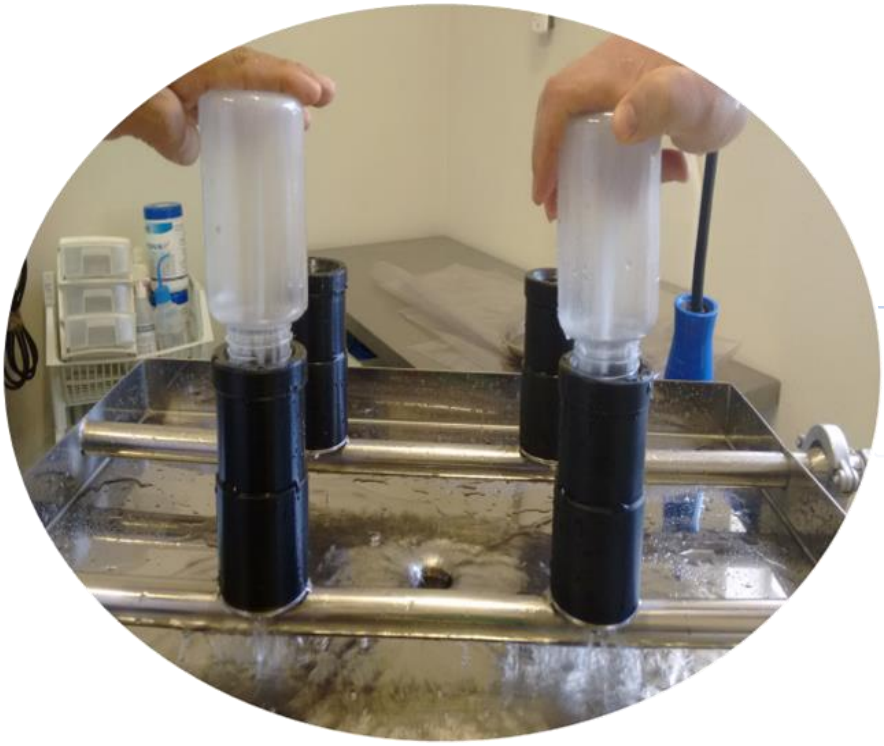
As imagens das instalações utilizadas na execução dos ensaios experimentais que compuseram os capítulos do presente texto sistematizado estão ilustradas pelas Figuras 1 e 2, mostradas a seguir.

A Figura 1 apresenta imagens da planta piloto do Departamento de Engenharia de Alimentos da FZEA/USP, idealizada e instalada pelo autor, para desenvolvimento das pesquisas neste descritas.

Figura 1 - Imagens da planta piloto instalada no Departamento de Engenharia de Alimentos da FZEA/USP.

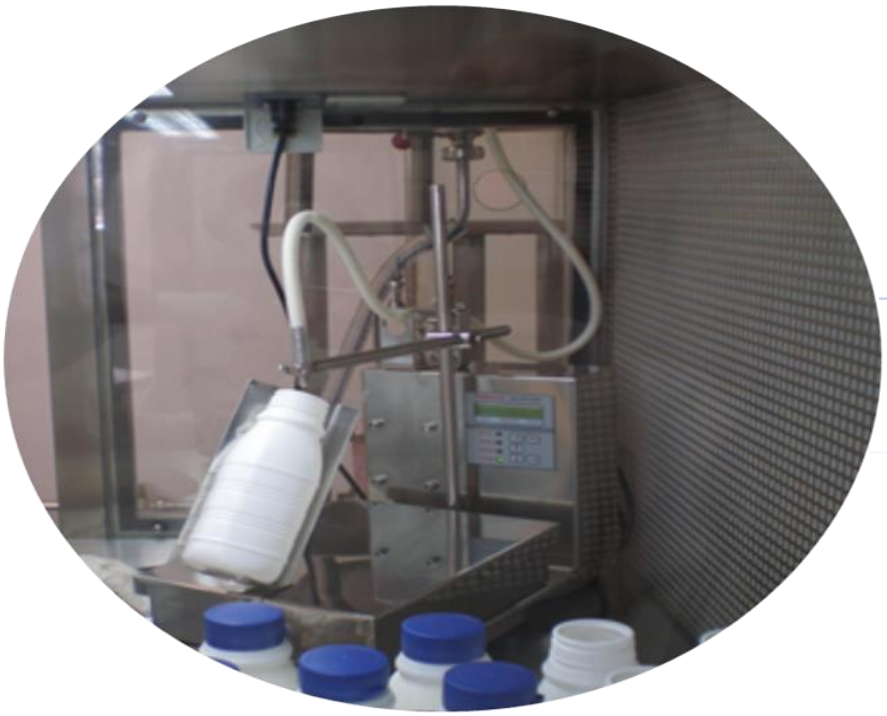
vista parcial

câmara de fluxo
de ar unidirecional



assepsia de embalagens

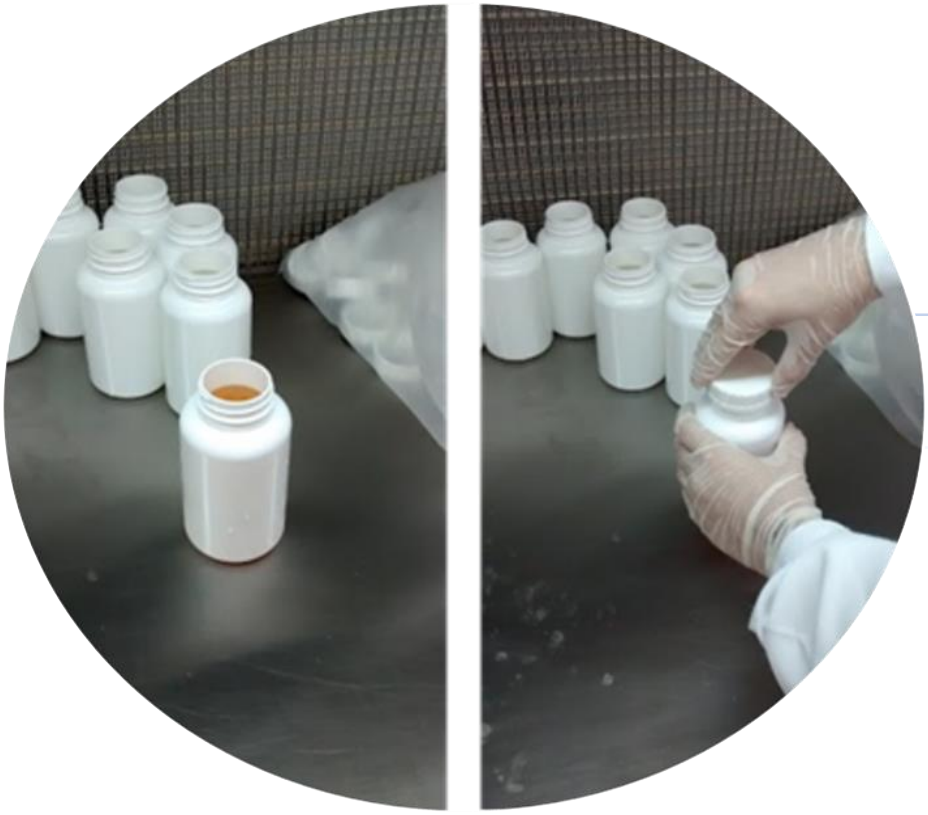
envase ultra limpo de leite pasteurizado



dosagem gravimétrica automática

extração de caldo de cana

envase de caldo de cana com polpa de maracujá



fechamento das garrafas

selagem por indução

matéria-prima e produto final

A Figura 2 reúne imagens do HPP *Validation Center* de *Cornell*, que abrange uma área de processamento onde está instalado o equipamento de alta pressão e um laboratório de biossegurança nível 2, localizado no campus *Cornell AgriTech*, na cidade de Geneva/NY. O HPP *Validation Center* realiza análises microbiológicas para avaliar se os parâmetros do processamento que se pretende aplicar em escala industrial são efetivos para assegurar uma redução superior a 5 ciclos logaritmos na população de patógenos de referência (*Escherichia coli* O157:H7*, Salmonella enterica* and *Listeria monocytogenes*), inoculados no produto de interesse, segundo as diretrizes da *Food and Drug Administration* (FDA).

Figura 2 - Imagens do HPP *Validation Center* de *Cornell*.

vista parcial

vista frontal do equipamento de alta pressão hidrostática

vista posterior com reservatório de água gelada

painel de controle

suco de maçã inoculado com *cocktail* de patógenos e dimetil dicarbonato

amostras acondicionadas

transportador de amostras para câmara de alta pressão

dispositivo de carga/descarga da câmara de alta pressão

equipamento em operação

saída da câmara de alta pressão

2 CAPÍTULO 1

Contextualização

O tempo de vida útil de leite pasteurizado é influenciado pela qualidade microbiológica da matéria-prima, tecnologia e parâmetros de processamento, sistema de embalagem e sobretudo, da temperatura de estocagem do produto. Parte significativa do potencial de qualidade de alimentos refrigerados são consumidos pela precariedade da cadeia do frio disponível no Brasil. O trabalho apresentado a seguir foi conduzido com o propósito de investigar a extensão do impacto da temperatura de estocagem, em ampla faixa, na estabilidade microbiológica de leite pasteurizado acondicionado em garrafas de polietileno de alta densidade (PEAD) e *pouches* de polietileno de baixa densidade (PEBD).

Microbiological shelf life of pasteurized milk in bottle and pouch

(PETRUS, R. R.; LOIOLA, C. G.; OLIVEIRA, C. A. F., 2010)

Abstract

The shelf life of pasteurized milk in Brazil ranges from 3 to 8 days, mainly due to poor cold chain conditions that prevail throughout the country and subject the product to repeated and/or severe temperature abuse. This study evaluated the influence of the storage temperature on the microbiological stability of homogenized whole pasteurized (75 °C/15 s) milk packaged in a high density polyethylene (HDPE) bottle and a low density polyethylene (LDPE) pouch, both with monolayer materials pigmented with titanium dioxide (TiO_2). Storage temperatures investigated were 2, 4, 9, 14 and 16 °C. Microbiological evaluation was based on mesophilic and psychrotrophic counts with 7 log CFU/mL and 6 log CFU/mL, respectively, set as upper limits of acceptability for maintaining the quality of milk. The microbiological stability for pasteurized milk packaged in HDPE bottle and stored at 2, 4, 9, 14 and 16 °C was estimated in 43, 36, 8, 5 and 3 days, respectively. For milk samples packaged in LDPE pouch, the shelf life was estimated in 37, 35, 7, 3 and 2 days, respectively. The determination of Q_{10} and z values demonstrated that storage temperature has a greater influence on microbiological shelf life of pasteurized milk packaged in a LDPE pouch compared to the HDPE bottle. Based on the results of this study, HDPE bottle was better for storing pasteurized milk as compared to LDPE pouch.

Keywords: milk stability, packaging, microbiology.

1 Introduction

Brazil is 6th internationally in milk production, exceeding 25 billion L annually with an estimated consumption of 140 L/inhabitant/year (EMBRAPA, 2008). Pasteurized milk in Brazil represents approximately 24% of the total milk market (ABLV, 2008).

Because of improper refrigeration, low quality of raw material and inadequate packaging system, the maintenance of the quality of milk is a problem in many countries (RANKIN, 2002). Raw milk deteriorates in only a few days even when stored under refrigeration temperatures. By contrast, pasteurized and refrigerated milk has a shelf life which may range from 7 up to 28 days (MEUNIER-GODDIK; SANDRA, 2002). The shelf life of pasteurized milk in Brazil varies between 3 and 8 days due to the low quality of raw material and precarious cold chain which exposes the product to abusive conditions during distribution and commercialization.

Bacterial spoilage is the biggest limiting factor in extending the shelf life of pasteurized milk beyond 14 days. Microbial growth shortens the shelf life of milk by producing undesirable changes in aroma and flavor which ultimately influence the product´s acceptance (FROMM; BOOR, 2004). Nevertheless, technologies are available to extend the shelf life of pasteurized milk such as ultra clean bottling. Good Manufacturing Practices (GMP) and storage temperature near 2-3 °C are also relevant hurdles (CROMIE, 1991). Sepulveda et al. (2005) found that under ideal conditions of processing and storage, pasteurized milk can have a shelf life of 3 weeks depending on the microbiological quality of the raw milk. Rysstad and Kolstad (2006) state that the shelf life of pasteurized milk can be extended by introducing better hygienic transfer from processing to filling machines and the filling process.

Cromie (1991) reported the factors that influence the shelf life of pasteurized milk including the quality of raw material, parameters of heat treatment, resistant microorganisms to pasteurization (particularly psychrotrophs), presence and activity of post pasteurization contaminants, packaging system and storage temperature. Among all of them, the storage temperature showed the greatest impact on the product´s stability. Vulnerability of milk's fat and protein to Physicochemical changes may also lead to deterioration.

Temperature dependence has been expressed as Q_{10}, a quotient of the reaction rate constants at temperatures differing by 10 °C, or the change of shelf life. Q_{10} reflects the change in rate for a 10 °C rise in temperature. Another term used for temperature dependence of microbial inactivation kinetics of food quality loss is the z value, which is the temperature change that causes a 10-fold change in the reaction rate constant (TAOUKS; LABUZA; SAGUY, 1997).

Clearly, the poor cold storage chain is one of the main factors responsible for the short shelf life of pasteurized milk in Brazil. The present study evaluated the microbiological stability of pasteurized milk in high density polyethylene (HDPE) bottle and low density polyethylene (LDPE) pouch, stored at 2, 4, 9, 14 and 16 °C.

2 Materials and methods

2.1 Milk samples

Bulked raw milk was treated at 75 °C for 15 s in a HTST plate system, homogenised in a one-stage homogenizer (15 MPa) and then cooled to 4 °C. The pasteurized milk was filled into 1-L HDPE bottles and 1-L LDPE pouches, both pigmented with titanium dioxide (TiO_2). Milk samples were stored at 2, 4, 9, 14 and 16 °C in the dark; they were procured from a local dairy plant in Pirassununga, state of São Paulo/Brazil.

2.2 Microbiological assays

Three pasteurized milk samples in bottle and pouch were analyzed for the growth of aerobic mesophiles and psychrotrophs to evaluate the microbiological stability of the product. Mesophilic counts of 7 log CFU/mL and psychrotrophs of 6 log CFU/mL were set as maximum accepted levels for determining the quality of milk, based on Moyssiadi et al. (2004), Cromie (1991), and Craven and Macauley (1992).

Enteric bacteria quantification was carried out to determine the effectiveness of pasteurization to eliminate pathogenic bacteria commonly found in milk. Coliforms and *Salmonella* spp. were determined as indication of failures in processing and/or contamination post pasteurization. The microbiological assays were carried out in agreement with protocols described by Frank and Yousef (2004). *Salmonella* spp. test was performed by the immune precipitation technique using the VIP® *Salmonella* test kit - AOAC Official Method 999,09 VIP (Biocontrol/USA).

2.3 Statistical analysis

Data were statistically processed through analysis of variance (ANOVA) and Tukey´s test at 5% of significance, using the software SAS 9.1.

3 Results and discussion

3.1 Microbiological evaluation

3.1.1 *Salmonella* spp. and coliforms

All milk samples were negative for *Salmonella* spp. Table 1 exhibits the coliforms counts.

Table 1 - Counts of total coliforms (MPN/mL) in pasteurized milk filled into different packaging materials.

Storage temperature (°C)	LDPE pouch			HDPE bottle		
	P_1	P_2	P_3	B_1	B_2	B_3
16	< 0.03	0.04	0.07	< 0.03	0.04	< 0.03
14	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
9	0.93	0.43	0.93	< 0.03	< 0.03	< 0.03
4	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
2	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03

Standard set by the Brazilian Ministry of Agriculture, Livestock and Supply (BMALS/MAPA, 2002): < 1.00 MPN/mL.

Table 1 demonstrates that all samples met the Brazilian regulation. Although the samples in pouch stored at 9 °C achieved upper levels of coliforms, they had counts within the legal standards.

3.1.2 Microbiological stability

Table 2 exhibits the mesophilic counts in pasteurized milk during storage. The assays were stopped as counts were equal to or greater than 7 log CFU/mL (10^7 CFU/mL). The highlighted values point out counts exceeding the pre-set limit.

Table 2 - **Mesophiles** counts in homogenized whole pasteurized milk over time.

Storage temperature (°C)	log CFU/mL									
16	0		2 d		4 d					
	b	P	b	p	b	p				
	3.8 ± 0.1 Ca	3.5 ± 0.8 Ba	5.4 ± 0.2 Bb	6.6 ± 0.0 Aa	**7.4** ± 0.1 Aa	**8.5** ± 1.5 Aa				
14	0		2 d		4 d		7 d			
	b	P	b	p	b	p	b	p		
	3.7 ± 0.1 Cb	4.0 ± 0.0 Da	3.6 ± 0.4 Cb	4.6 ± 0.4 Ca	6.1 ± 0.2 Bb	6.6 ± 0.0 Ba	**7.6** ± 0.7 Aa	**7.5** ± 0.1 Aa		
9	0		2 d		4 d		7 d		9 d	
	b	P	b	p	b	p	b	p	b	p
	4.0 ± 0.1 Da	4.0 ± 0.1 Ca	4.4 ± 0.1 Ca	4.7 ± 0.4 Ca	6.1 ± 0.1 Ba	6.2 ± 0.2 Ba	6.2 ± 0.1 Bb	6.4 ± 0.0 Ba	**7.0** ± 0.0 Ab	**7.8** ± 0.3 Aa
4	0		10 d		21 d		28 d		43 d	
	b	P	b	p	b	p	b	p	b	p
	3.3 ± 0.1 Ba	3.3 ± 0.0 Ba	3.3 ± 0.0 Ba	3.2 ± 0.3 Ba	4.0 ± 1.5 Ba	3.4 ± 0.7 Ba	3.9 ± 1.5 Ba	5.4 ± 0.2 Ab	**7.0** ± 0.2 Aa	**7.2** ± 0.2 Aa
2	0		14 d		28 d		42 d		49 d	
	b	P	b	p	b	p	b	p	b	p
	3.4 ± 0.1 Ba	3.4 ± 0.0 Ba	3.4 ± 0.1 Ba	3.7 ± 0.4 Ba	5.6 ± 2.3 ABa	6.6 ± 0.4 Aa	6.5 ± 2.2 ABa	**7.7** ± 0.8 Aa	**7.3** ± 0.1 A	-

[a]Mean values of 3 replicates ± standard deviation. HDPE bottle (b); LDPE pouch (p); d (days). Means followed by the same lower case letter (comparison between bottle and pouch) and same upper case letter (comparison between storage times) within same row are not significantly different ($p > 0.05$). Upper limit considered for keeping quality of milk: 7 log CFU/mL.

Moyssiadi et al. (2004) and Karatapanis et al. (2006) evaluated the stability of pasteurized milk in monolayer HDPE bottle pigmented with TiO_2. At time 0, mesophilic counts were above 4 log CFU/mL and after 7 days of storage at 4 °C the counts increased to 6 log CFU/mL. The findings of this study showed a greater stability; the mesophilic counts in the milk in HDPE bottle did not reach 4 log CFU/mL after 10 d of storage at 4 °C (Table 2).

Simon and Hansen (2001) evaluated the microbial growth in pasteurized milk in cartooned multilayer packages and reported mesophilic counts ranging from 0 to 2 log CFU/mL and psychrotrophic counts of approximately 1 log CFU/mL after 4 weeks of storage at 2 °C. The counts of the present study ranged from 3 to 6 log CFU/mL for the same temperature after 28 days of storage. This difference is most likely correlated with the quality of raw material.

Zygoura et al. (2004) showed that pasteurized milk in HDPE monolayer bottle pigmented with TiO_2 had mesophiles counts ranging from 4 log CFU/mL (at time 0) to 7 log CFU/mL after 7 days of storage at 4 °C. Psychrotrophic counts varied from 3 to 6 log CFU/mL. Counts of mesophiles and psychrotrophs in this research work (Tables 2 and 3) meaningfully differed from data reported by Zygoura et al. (2004).

Vassila et al. (2002) evaluated the microbiological stability of pasteurized milk in LDPE pouches. Mesophilic and psychrotrophic counts varied from 4 (time 0) to 6 log CFU/mL and from 3 (time 0) to 5 log CFU/mL, respectively, after 7 days at 4 °C. These levels were considerably upper than those found in this study.

Moyssiadi et al. (2004) investigated the effect of different packaging materials on pasteurized milk stored at 4 °C for 7 days. The initial aerobic mesophilic count was approximately 5 log CFU/mL, and the count achieved 6 log CFU/mL after 7 days. The initial psychrotrophic counts ranged from 3 to 4 log CFU/mL and reached levels ranging from 4 to 6 log CFU/mL after 7 days at 4 °C.

Cromie, Schimidt and Dommett (1989) reported the effect of pasteurization temperature on microbiological quality of pasteurized milk (72 °C/15 s,) aseptically filled in flexible metallized polyester packages. The mesophilic and psychrotrophic counts were 5 and 4 log CFU/mL, respectively, after 35 days at 3 °C. In the present study, similar counts were achieved after 28 days at 4 °C.

Sepulveda et al (2005) found mesophilic counts of approximately 4 log CFU/mL in pasteurized milk at 72 °C/15 s after 40 days at 4 °C which were lower than those exhibited in this study.

Table 3 gathers psychrotrophic mean counts. The assays were stopped as the counts were equal to or greater than 6 log CFU/mL. Highlighted values indicate counts exceeding the pre-set limit.

Table 3 - **Psychrotrophic** counts in homogenized whole pasteurized milk over time.

Storage temperature (°C)	log CFU/mL									
16	0		2 d		4 d					
	b	P	b	p	b	p				
	0.6 ± 0.3 Ca	0.9 ± 0.1 Ba	4.7 ± 0.7 Bb	**6.3** ± 0.1 Aa	**7.1** ± 0.1 A	-				
14	0		2 d		4 d		7 d			
	b	P	b	p	b	p	b	p		
	1.1 ± 0.1 Da	1.1 ± 0.1 Ca	3.0 ± 0.3 Cb	4.1 ± 0.3 Ba	5.5 ± 0.1 Bb	**6.9** ± 0.1 Aa	**7.2** ± 0.9 A	-		
9	0		2 d		4 d		7 d		9 d	
	b	P	b	p	b	p	b	p	b	p
	0.1 ± 0.2 Ca	0.5 ± 0.4 Ca	0.2 ± 0.2 Cb	1.5 ± 0.1 Ca	0.3 ± 0.1 Cb	4.4 ± 0.3 Ba	5.7 ± 0.6 Ba	5.9 ± 0.8 Aa	**6.7** ± 0.0 Aa	**6.8** ± 0.2 Aa
4	0		10 d		21 d		28 d		43 d	
	b	P	b	p	b	p	b	p	b	p
	0.1 ± 0.1 Da	0.1 ± 0.0 Da	1.1 ± 0.2 Ca	1.4 ± 0.5 Ca	3.2 ± 0.1 Bb	1.5 ± 0.4 Ca	5.0a ± 0.1 CA	5.0 ± 0.0 Ba	**6.0** ± 0.5 Aa	**6.7** ± 0.7 Aa
2	0		14 d		28 d		42 d			
	b	P	b	p	b	p	b	p		
	0.1 ± 0.1 Ca	0.1 ± 0.1 Ba	0.7 ± 0.4 Ca	2.0 ± 1.8 Ba	4.5 ± 0.5 Bb	**6.1** ± 0.1 Aa	**7.0** ± 1.7 A	-		

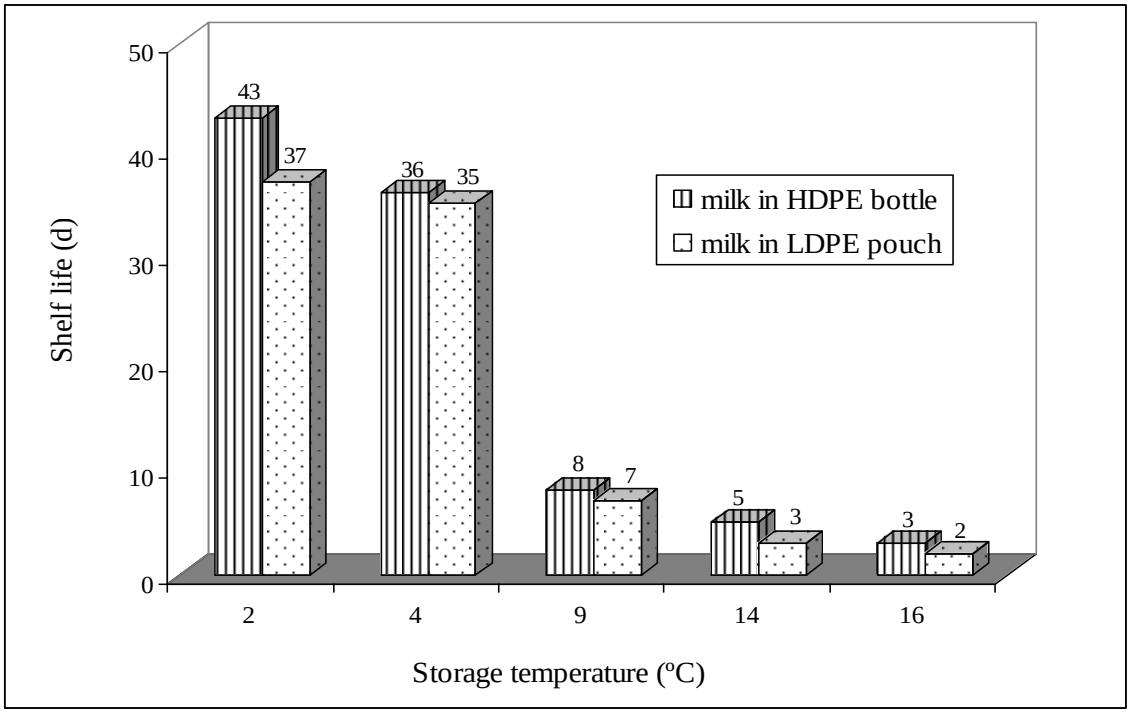
[a]Mean values of 3 replicates ± standard deviation. HDPE bottle (b); LDPE pouch (p); d (days). Means followed by the same lower case letter (comparison between bottle and pouch) and same upper case letter (comparison between storage times) within same row are not different ($p > 0.05$). Upper limit considered for keeping quality of milk: 6 log CFU/mL.

Moyssiadi et al. (2004) and Karatapanis et al. (2006) evaluated the stability of pasteurized milk in HDPE bottles. Psychrotrophic counts were greater than 3 log CFU/mL at time 0; the counts exceeded 5 log CFU/mL after 7 days at 4 °C. In this study, pasteurized milk in the same package had similar psychrotrophic counts after 28 days at 4 °C (Table 3).

Low psychrotrophic counts in milk are of utmost importance for its quality; the metabolic activity of these microorganisms triggers biochemistry changes in the milk composition. Psychrotrophic bacteria in milk stored under refrigeration have metabolic activities that spoil the flavor, odor and appearance; proteolysis and lipolysis catalyzed by microbial enzymes shorten the shelf life of milk products.

Figure 1 depicts the estimated microbiological stability for pasteurized milk, in compliance with the preset microbiological criteria. It demonstrates that the bottle gave superior performance over the pouch by conferring better stability to milk during storage at temperatures ranging from 2 to 16 °C. This can be explained by the lower oxygen permeability of the bottle, rendering lower growth of aerobic microorganisms.

Figure 1 - Microbiological shelf life for homogenized whole pasteurized milk.



Simon and Hansen (2001) stated that pasteurized milk (72 - 74 °C/16 s) possesses a shelf life between 15 and 10 days at 4 to 8 °C. In this study, the microbiological stability varied from 36 to 7 days when stored at 4 and 9 °C, respectively. Zygoura et al. (2004) evaluated the influence of packaging material on shelf life of pasteurized milk in Greece. The product was stored at 4 °C with exposure to fluorescent lighting. Under these conditions, the stability was only 5 days. Although the product had been exposed to light, Zygoura et al. (2004) used multilayered plastic bottles with a high barrier. Stepaniak (1991) holds that the shelf life of pasteurized milk may vary from 5 the 17 days when stored in a range of 11 to 2 °C, far different from the findings of this study.

Q_{10} and z values

The shelf life herein estimated was valuable to determine Q_{10} and z values. Equations 1 and 2, demonstrated by Taouks et al. (1997), were used to this end.

Q_{10} value

(Eq. 1) $$Q_{10} = \frac{shelflife_{T(^oC)}}{shelflife_{T+10(^oC)}} = \frac{shelflife_{4^oC}}{shelflife_{14^oC}}$$

$$Q_{10\,pouch} = \frac{35 days}{3 days} = 11.7 \qquad Q_{10 bottle} = \frac{36 days}{5 days} = 7.2$$

z value

(Eq. 2) $$z_{bottle} = \frac{10}{\log Q_{10}} = \frac{10}{\log 7.2} = 11.7^oC \qquad z_{pouch} = \frac{10}{\log Q_{10}} = \frac{10}{\log 11.7} = 9.4^oC$$

The calculation of Q_{10} value demonstrated that the microbiological stability of pasteurized milk in HDPE bottle varied 7.2 fold as the storage temperature varied in

10 ºC. Regarding the product in the LDPE pouch, its shelf life varied by 11.7 fold. The greater Q_{10} value so the greater is the dependence of shelf life to the temperature. The greater z value the lower is the dependence of the shelf life in relation to the temperature. Therefore, the storage temperature had a greater impact on the microbiological stability of milk when it was filled in pouch and stored in the range of 2 to 16 °C. Finally, the temperature during storage and distribution is critical for milk´s stability. A rule of thumb for pasteurized milk is that for every 2 °C increase in storage temperature, the stability is reduced by 50% (RYSSTAD; KOLSTAD, 2006).

4 Conclusions

The determination of Q_{10} and z values demonstrated that storage temperature had a greater influence on microbiological shelf life of pasteurized milk packaged in the LDPE pouch compared to a HDPE bottle. The findings of this study demonstrated that HDPE bottle had a better performance over the LDPE pouch in terms of microbial growth at storage temperatures ranging from 2 to 16 °C.

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3 CAPÍTULO 2

Contextualização

O caldo de cana é uma bebida muito consumida no mercado informal brasileiro, majoritariamente constituído por vendedores ambulantes, quase sempre em precárias condições higiênico-sanitárias. Quando o projeto-gênese que resultou neste trabalho foi elaborado, em 2009, a comercialização de caldo de cana industrializado ainda era inexistente no País. Este fato, aliado a grande disponibilidade da matéria-prima na região de Pirassununga/SP, motivou sobremaneira o desenvolvimento de pesquisas tendo esta bebida como alvo. Algumas vantagens proporcionadas pela industrialização do caldo de cana são: (1) disponibilização de uma bebida segura, nutritiva e com elevada aceitação sensorial, em qualquer época do ano, independente do período de safra, (2) aumento do tempo de vida útil, (3) extensão do consumo a locais distantes da região de plantio, com a diminuição do volume de matéria-prima transportado, reduzindo custos de logística. A adição de sucos de frutas ácidas em pequenas proporções ao caldo de cana, como limão, abacaxi e maracujá, tem o potencial de aprimorar a qualidade sensorial da bebida, conferindo um sabor refrescante e suavizando seu dulçor. Ademais, a acidificação promove a estabilização microbiológica e enzimática do produto.

Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage

(KUNITAKE, M. T.; DITCHFIELD, C.; SILVA, C. O.; PETRUS, R. R., 2014)

Abstract

The shelf life of fresh sugarcane juice is meaningfully limited due to the high rates of microbiological and enzymic reactions which take place just after extraction. To evaluate the impact of pasteurization temperature on quality and stability of cane juice with passion fruit pulp, nine batches of juice with 4 g/100 g passion fruit pulp were processed at 85, 90 and 95 °C for 30 s, in triplicate. The pasteurized beverage was ultra clean filled in polyethylene terephthalate (PET) bottles and stored at 7 °C in the dark. The finished product was characterized through Physicochemical tests. Activities of polyphenol oxidase (PPO) and peroxidase (POD) were determined before and after processing. Coliforms and *Salmonella* spp. assays were carried out to assure the beverage´s safety. Color parameters were measured in the processed juice throughout the storage period. Fifty panelists evaluated the beverage's appearance, aroma, flavor, and overall impression using a seven-point hedonic scale. Sensory stability was estimated by setting mean scores above four and percentages of acceptance above 60%. The pH, soluble solids and titratable acidity of the end product ranged from 3.96 to 4.19, 19.7 to 20.1 °Brix, and 0.163 to 0.175 g/100g citric acid, respectively. The three processing binomials were effective for PPO inactivation; however, full POD inactivation was reached at 95 °C/30 s only. The sensory shelf lives of the mixed juice processed at 85, 90 and 95 °C/30 s were 30, 40 and 50 days, respectively. In conclusion, the increase of pasteurization temperature had a positive effect on the product's stability.

Keywords: shelf life, hurdle technology, ultra clean filling.

1 Introduction

Sugarcane juice has low acidity (pH > 4.6), high water activity (a_w ~ 0.99), high sugar content (~ 20%), and therefore deteriorates rapidly even when refrigerated (YUSOF et al., 2000). Poor sanitary conditions during extraction also contribute to a rapid deterioration of the product´s quality rendering undesirable changes in appearance and flavor. To extend the product's shelf life is necessary to employ technologies to control the factors responsible for juice's deterioration.

The chemical, physical, sensory and nutritional attributes of cane juice are affected by several factors that can be physical (light, heat), chemical (O_2), biochemical (enzymes) and/or biological (microorganisms, insects). Reduction of pH below 4.6 is an important intervention to inhibit growth of most pathogenic microorganisms. The addition of high-acid fruit acts as a hurdle, preserving the beverage (SILVA et al., 2007), though the growth of filamentous molds, yeasts and aciduric bacteria, found in the raw material, gives rise to microbiological concerns.

Adoption of proper handling practices by processors is essential to avoid contamination with both pathogenic and spoilage microorganisms (VANZO; AZEVEDO, 2003). Darkening of cane juice occurs by formation of brown pigments by enzymic (oxidation of phenolic compounds forming melanin) and non-enzymic reactions (Maillard reaction, thermal and alkaline degradation and sugar condensation). During juice extraction exposure to oxygen initiates enzymic browning caused by the activity of peroxidase (POD) and polyphenol oxidase (PPO) (QUDSIEH et al., 2002; VANZO; AZEVEDO, 2003). Both enzymes can be denatured by heat, reducing their activity and thus improving the beverage´s stability; the POD has been found to be more resistant to thermal treatment than PPO in cane juice (BUCHELI; ROBINSON, 1994). Prati, Moretti and Cardello (2005) pasteurized cane juice with and without addition of fruit juices, but the impact of pasteurization temperature on the sensory stability of juice, ultra clean filled in plastic bottles has not been addressed. This study was primarily undertaken to evaluate the effect of three pasteurization temperatures on the sensory stability of cane juice acidified with passion fruit pulp and stored at 7 °C as determined by the sensory scores obtained for appearance, aroma, flavor and overall impression.

2 Material and methods

2.1 Raw material

Sugarcane *(Saccharum officinarum)* cultivar SP813250 was supplied by Tecnocana Technologia em Cana Ltda (Santa Cruz das Palmeiras, SP, Brasil). The juice was extracted and processed in a pilot plant at the Department of Food Engineering, University of São Paulo, located approximately 30 km from the cultivation site. Pasteurized and frozen yellow passion fruit pulp (*Passiflora edulis*) was procured from the local market, processed by De Marchi Indústria e Comércio de Frutas Ltda (Jundiaí, SP, Brasil).

2.2 Processing

The raw material was cut, scraped and then immersed in a sodium hypochlorite (NaClO) solution containing 30 mg/L free residual chlorine for 20 min at approximately 25 °C. Subsequently, the cane juice was extracted in a stainless steel electric cylinder mill and 4 g/100 g passion fruit pulp was added to the extracted juice, thus obtaining an acidified juice with a pH of approximately 4.0.

Acidified juice was pasteurized in a plate heat exchanger (Sumá Indústria e Comércio Ltda, Campinas, SP, Brasil) consisted of regeneration, heating, holding and cooling sections, with a nominal capacity of 300 L/h. The pasteurization binomials were 85, 90 and 95 °C for 30 s and three alternated replicates were conducted for each condition. The juice was filtered, preheated to a temperature of approximately 50 °C, homogenized at 150 Bar in a single stage homogenizer (Artepeças P&B Indústria e Comércio Ltda São Paulo, SP, Brasil), heated to the pasteurization temperature, cooled to approximately 8 °C and pumped to an insulated buffer tank. A stainless steel, ISO class 5 unidirectional air-flow cabin (Veco, Campinas, SP, Brasil) was used for the beverage's ultra clean filling into transparent 320 mL PET bottles, which were previously decontaminated by dipping in a 0.05% (v/v) peracetic acid solution at 50 °C for 30 min.

2.3 Physicochemical assays

Physicochemical tests were performed as determined by AOAC International (AOAC, 2007). The pH was determined using a digital meter Analyzer model 300 M;

the soluble solids content (expressed as °Brix) was determined using a Reichert AR 200 portable digital refractometer, and the titratable acidity (expressed as % citric acid) determined by titration with 0.1 N NaOH.

Samples of processed mixed juice, kept at 7 °C for about 60 days, were subjected to Physicochemical assays, determination of PPO and POD activities, coliforms and *Salmonella* spp. analysis. Measurement of color parameters and sensory analysis, to estimate the product´s stability, were monthly performed.

2.4 Determination of enzymic activity

The protocols adapted from Campos and Silveira (2003) were used to determine the polyphenol oxidase (PPO) and peroxidase (POD) activities.

2.4.1 Polyphenol oxidase

Five and half mililiters of 0.2 M phosphate buffer solution (pH 6.0) and 1.5 mL of 0.2 M catechol were added into a test tube and maintained at 25 °C 10 for min. Then 1.0 mL of the diluted sample in deionized water (1:10) was added. The tube was stirred for 15 s and returned to the water bath at 25 °C for 30 min. The absorbance was read in a spectrophotometer at 425 nm. The blank was prepared by diluting the sample in deionized water.

2.4.2 Peroxidase

Seven mililiters of 0.2 M phosphate buffer solution (pH 5.5) and 1.0 mL of the diluted sample (juice) in deionized water (1:10) were added to a test tube and maintained in a heat bath at 35 °C for 10 min. Then 1.5 mL of 0.05% guaiacol and 0.5 mL of 0.1% hydrogen peroxide were added. The tube was magnetically stirred for 15 s and returned to the bath at 35 °C for 15 min. Finally, the absorbance was read in a spectrophotometer at 470 nm.

One (1) unit of enzyme activity (U) was defined as the amount of enzymic extract capable of increasing absorbance at 425 and 470 nm for PPO and POD, respectively, at rates of 0.001 unit per minute.

2.5 Microbiological assays

Coliforms and *Salmonella* spp. tests were carried out in compliance with Brazilian Regulation - resolution 12 issued in January 2nd 2001 - which requires that pasteurized and chilled whole cane juice must have less than 10 coliforms at 45 °C/mL. *Salmonella* spp. must not be detected in 25 mL of sample (Brasil, 2001).

Coliforms tests were performed according to the most probable number (MPN) method. For *Salmonella* spp. analysis, the 1–2 Test® from BioControl was used. This test is a rapid analysis kit for the detection of mobile species of *Salmonella* spp. in food, and it has been endorsed by the Association of Official Agricultural Chemists (AOAC) official method 989.13. The test is based on *Salmonella* immobilization in motility media using polyvalent H (flagellar) antibodies. The immobilization of mobile species of *Salmonella* results in the formation of a visible and well-defined band.

2.6 Measurement of color

Color parameters were measured in the processed juice throughout the storage period. A colorimeter (Ultra Scan model SN7877, Hunterlab, Hunter Associates Laboratory Inc., Reston, VA, U.S.A.) and the CIELab coordinates were measured using D65 illuminant and standard observer (10°). Three measurements were taken for each sample. Results for samples processed at different temperatures were compared as well as the variations observed along the storage period.

2.7 Sensory evaluation

The finished product was subjected to sensory evaluation tests, using a seven-point hedonic scale, as described by Dutcosky (2013). The attributes appearance, aroma, flavor and overall impression were evaluated by 50 panelists, who were consumers of cane juice. The juice was served at a temperature of approximately 12 °C. To estimate the sensory stability, mean scores of above 4 (neither like nor dislike), and at least 60% of acceptance were set as limit values. Sensory tests were carried out in approximately 10 day-intervals during the 60 days of storage at 7 °C. Samples, used as the control, kept at -18 °C throughout the sensory stability study, were thawed in a fridge at 4-5 °C 24 h before sensory evaluation.

2.8 Statistical analysis

The statistical software program SAS 9.1 was used to perform the analysis of variance with a 95% confidence level. Comparison between means was carried through the Tukey´s test.

3 Results and discussion

As previously mentioned, nine batches of cane juice with passion fruit pulp were processed, as follows: R1 (batches pasteurized at 85 °C/ 30 s), R2 (batches pasteurized at 90 °C/ 30 s) and R3 (batches pasteurized at 95 °C/ 30 s).

3.1 Physicochemical characterization

Table 1 exhibits the yield extraction and Physicochemical parameters.

Table 1 - Physicochemical parameters of the acidified cane juice beverage processed in each replicate.

	R1	R2	R3
Whole fresh juice			
Extraction yield[1] (%)	54.7a ± 5.5	54.9a ± 5.5	52.1a ± 5.9
pH	5.16a ± 0.16	5.27a ± 0.08	5.27a ± 0.31
Soluble solids (°Brix)	21.1a ± 2.2	18.3a ± 4.0	20.3a ± 3.2
Titratable acidity (% citric acid)	0.052a ± 0.015	0.050a ± 0.009	0.047a ± 0.011
Acidified juice			
pH	4.19a ± 0.16	4.07a ± 0.21	3.96a ± 0.17
Soluble solids (°Brix)	20.1a ± 2.6	20.1a ± 4.6	19.7a ± 2.5
Titratable acidity (% citric acid)	0.175a ± 0.033	0.163a ± 0.013	0.167a ± 0.046

R1 - Means and standard deviations of batches 1, 4 and 7 (pasteurized at 85 °C/30 s). R2 - Means and standard deviations of batches 2, 5 and 8 (pasteurized at 90 °C/30 s). R3 - Means and standard deviations of batches 3, 6 and 9 (pasteurized at 95 °C/30 s). [1]In relation to peeled cane. Mean values followed by the same letter in the same row are not different (p>0.05).

As shown in Table 1, there were no statistic differences among mean values obtained from both freshly extracted and acidified juices, in terms of the extraction yield, pH, soluble solids and titratable acidity. Some differences in the characteristics of the juice may be attributed to variations in the plant composition due to different

periods of planting, harvesting times, climatic conditions and maturation stages (STONE; SIDEL, 1985), in spite of the fact that the raw material batches belonged to the same cultivar and originated from the same production site.

Mao, Xu and Que (2007) reported an average yield during extraction of 70.8%. The difference between this result and that found in this study may be explained by the variety of raw material, maturation stage and also the extraction method. Upper levels are usually obtained on industrial scale and are made possible by the enzymic treatment of the bagasse.

A pH below 4.6 classifies the cane juice with passion fruit pulp added as an acidified beverage. This enhances the microbiological stability and inhibits enzymic action, mainly the PPO system which has an optimum pH close to 7.2. Moreover, the citric acid in the passion fruit pulp interacts with the copper (Cu^{2+}) in the PPO active site, diminishing enzymic browning (GOMES et al., 2001).

The addition of acid fruit pulps, such as passion fruit, to cane juice in small proportions also improves its sensory quality. The acid fruit changes the ratio between soluble solids content and titratable acidity, thus diminishing the perception of sweetness and giving a refreshing flavor (THÉ et al., 2001; MATSUURA et al., 2004).

3.2 Enzymic activity

The PPO and POD activities in freshly extracted juice varied between 40.3 and 40.9 U, and from 100.2 to 226.1 U, respectively. The addition of passion fruit reduced PPO activity to 17.2 - 27.8 U while POD activity decreased to 107.9 - 163.4 U. PPO was fully inactivated by all treatments (85, 90 and 95 °C); no residual activity was detected in processed samples. Conversely, POD showed residual activities between 0.1 and 17.9 U. To fully inactivate POD the most severe condition (95 °C/ 30 s) was needed. This finding agrees with Brito et al. (2005). The variation in enzymic activities herein observed may be explained by the variability of the raw material composition, intrinsic to plant-based foods.

Mao, Xu and Que (2007) reported that PPO activity converts phenolic compounds into brown colored polymers, which causes darkening as cane is crushed. Despite the PPO inactivation during pasteurization, changes in color were still observed, suggesting that other mechanisms, like chlorophyll degradation also occur.

The raw material composition is highly variable and this gives rise to processors concerns. The juice browning is initiated by enzymic oxidation of phenolic compounds, which forms dark, insoluble pigments. The quantity and availability of phenolic compounds, and oxygen level are critical for browning. Additionally, high enzymic activity increases the browning rate, decreasing the beverage´s acceptance. Thus the inactivation of enzymes is crucial for the juice´s stabilization.

3.3 Microbiological assays

Table 2 gathers the results for the analysis of coliforms and *Salmonella* spp. in the pasteurized beverage.

Table 2 - Enterobacteria counts in acidified pasteurized cane juice (pH ~ 4.1).

Replicate	R1	R2	R3
Coliforms at 35 °C (MPN/mL)	3.9 ± 1.6	3.8 ± 1.4	3.0 ± 0.0
Coliforms at 45 °C (MPN/mL)	< 3.0	< 3.0	< 3.0
Salmonella spp. (/ 25 mL)	-	-	-

R1 – Mean count of batches 1, 4 and 7 (pasteurized at 85 °C/30 s). R2 – mean count of batches 2, 5 and 8 (90 °C/30 s). R3 – mean count of batches 3, 6 and 9 (95 °C/30 s). - not detected.

The mean counts of coliforms at (35 and 45) °C were lower than 4 MPN/mL for replicates 1, 2 and 3. All processed batches complied with Brazilian Regulation, which states that pasteurized must show coliforms count at 45 °C lower than 10 MPN/mL. *Salmonella* spp. must not be detected in 25 mL of sample (Brasil, 2001). Pshychrotrophic counts varied from (1 to 47) CFU/mL, while yeasts and molds ranged from (1 to 23) CFU/mL throughout 60 day-storage. These results indicate that the product of this study achieved a good microbiological stability when stored at 7 °C.

3.4 Color parameters

Table 3 exhibits the variation in color parameters as compared to the unprocessed beverage for different heat treatments.

Table 3 - Comparison of the variation in CIELab color parameters in relation to unprocessed beverage.

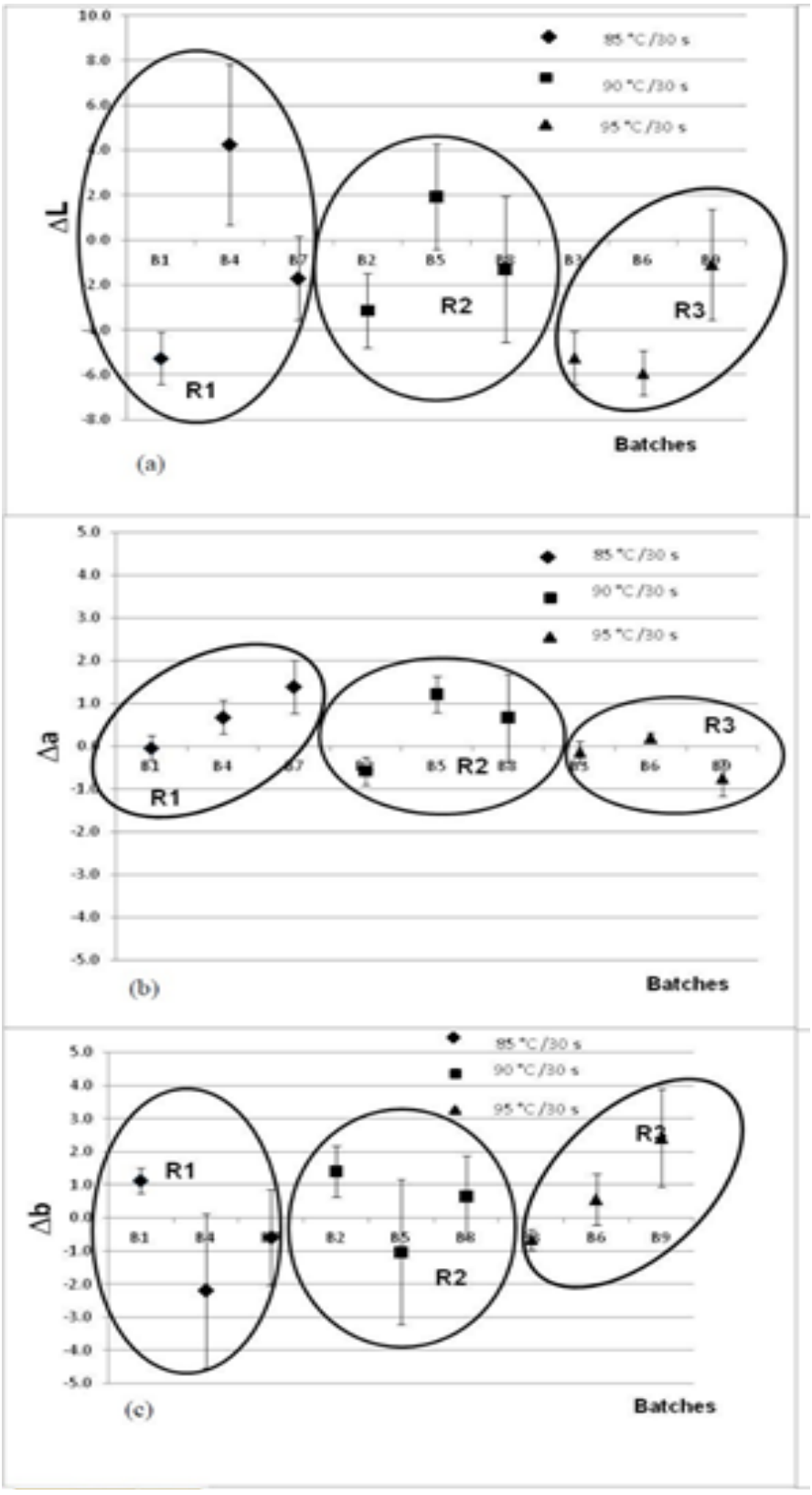
Heat treatment (°C)	ΔL	Δa	Δb
85 °C / 30 s	$2^{a} \pm 2$	$-0.1^{a} \pm 0.5$	$0.6^{a} \pm 1.8$
90 °C / 30 s	$6^{b} \pm 4$	$-0.3^{a} \pm 0.3$	$1^{a} \pm 1$
95 °C / 30 s	$6^{b} \pm 2$	$-0.1^{a} \pm 0.6$	$0.0^{a} \pm 0.5$

Means of three batches followed by the same exponent, in the same column, are not different (p>0.05). L (lightness), a (green–red), b (blue-yellow).

Processed samples showed upper L* values than the unprocessed ones, indicating that those had a lighter color. Processing at (90 or 95) °C results in a significant increase in the L value as compared to the beverages processed at 85 °C. This may be due to a greater enzyme inactivation at higher temperatures. In regard to a* and b* values significant differences were not found for different temperatures. A great variability was most likely observed because of the variations in the raw material composition.

Figure 1 demonstrates the variation in color parameters of the processed beverage throughout storage.

Figure 1 - Variation in color parameters after 4 week-storage in relation to freshly processed beverage. R1, R2 and R3 are replicates of pasteurization temperatures.



All batches, except 4 and 5 were darker after 4 weeks. Variations in Δa were slight and a definite trend could not be observed. For replicates 2 and 3 an increase in processing temperature results in higher Δb values, indicating a slightly stronger yellow color. Comparison of L values for batches 1, 2 and 3 shows that the beverage was significantly darker after 4 weeks and further increase in the storage period maintained this difference. The a* and b* color parameters both tend to present a slight increase during storage but a definite trend could not be established. Enzyme activity after processing, particularly for peroxidase (higher thermal resistance) may be responsible for the color changes observed. Beverage appearance scores seem to be related to the color parameter values. Lower sensory scores were observed for beverages with upper a* values (shift towards red color). For batch 6, the average appearance scores were statistically similar to the freshly processed beverage. The color parameters indicate a significant decrease in the L value after 4 weeks but no significant difference for a* and b* values. After 8 week-storage, the L* value maintained the difference, the a* value increased significantly and the b* value showed no significant difference (with respect to the freshly processed beverage). However, the mean scores for appearance were significantly lower after 8 weeks. The same trend can be observed for all processed batches. Clearly, the evaluation of appearance involves other attributes like homogeneity of sample and also that color change is a combination of the L*, a* and b* values.

Food stability is commonly affected by many intrinsic and extrinsic factors, and the sensory assay is an essential and rapid consumer response tool to evaluate the effect of procedures and treatments performed on the product. Table 4 demonstrates the results obtained through the 7-point hedonic scale tests for the three replicates carried out at the beginning, middle and end of the storage. The highlighted values indicate mean scores equal to or less than 4, and percentages of acceptance equal to or less than 60%.

Table 4 - Mean scores assigned for cane juice with passion fruit pulp in a 7-point hedonic scale.

Treatment	Storage at 7 °C (days)	Attribute (average scores / % acceptance[1])							
		Appearance		Aroma		Flavor		Overall impression	
		Sample	Control	Sample	Control	Sample	Control	Sample	Control
85 °C/30 s	0	6.0 / 92		6.3 / 97		5.8 / 95		5.9 / 99	
	30	5.7 / 87	5.8 / 85	5.7 / 92	5.8 / 91	5.4 / 92	5.6 / 86	5.5 / 94	5.6 / 89
	60	4.7 / 54	6.1 / 96	4.6 / **54**	6.1 / 92	4.1 / **44**	5.8 / 90	4.4 / **48**	5.9 / 96
90 °C/30 s	0	6.1 / 92		6.4 / 97		5.8 / 90		5.9 / 93	
	30	5.8 / 86	6 / 92	5.8 / 81	5.8 / 88	5.2 / 74	5.5 / 82	5.4 / 80	5.7 / 88
	60	5.6 / 79	6.0 / 92	5.3 / 73	6.0 / 93	4.8 / **60**	5.4 / 80	5.2 / 73	5.7 / 86
95 °C/30 s	0	6/ 91		6 / 91		5.5 / 82		5.8 / 88	
	30	5.6 / 83	6.3 / 96	5.6 / 82	6.0 / 95	5.0 / 67	5.8 / 93	5.2 / 72	6.0 / 94
	60	5.5 / 81	6.0 / 93	5.4 / 81	5.9 / 86	5.0 / **60**	5.7 / 84	5.0 / 70	5.7 / 92

[1]Percentage of panelists that assigned scores above 4. Control - juice stored at -18 °C; 1 = dislike extremely; 4 = neither like/nor dislike; 7 = like extremely.

Table 4 demonstrates that the batches processed at 85, 90, and 95 °C for 30 s initially achieved high scores. The acceptability of the beverages processed in all three conditions initially varied from 82 to 99% for all attributes. According to Teixeira, Meinert and Barbetta (1987) and Dutcosky (2013), approval scores above 70% indicate high acceptance.

The mean scores and acceptance percentages of the samples stored at 7 °C and of the control samples were similar up to 30 days. After a 60 day-storage, the acceptance scores significantly decreased, especially for the batches pasteurized at 85 °C, suggesting a greater quality degradation of the beverage processed at this temperature.

Sensory shelf life

The sensory shelf lives for product stored at 7 °C in the dark, based on percentage of acceptance (Table 4), were 30, 40 and 50 days for treatments at 85, 90 and 95 °C/ 30 s, respectively. In this way, the sensory stability was positively impacted by the pasteurization temperature, most likely due to enzyme inactivation (Qudsieh et al., 2002). It seems evident that the upper the pasteurization temperature the longer shelf life. Nevertheless, for some food products such as pasteurized milk, the reverse situation applies; shelf life is adversely affected (CROMIE, 1991).

Finally, the treatment at 95 °C/ 30 s proved to be effective in maintaining an acceptable level of quality of the juice, under refrigeration, for a period compatible with a possible consumer demand. The shelf life of cane juice is variable and depends on the Physicochemical and microbiological characteristics of the raw material, processing technology, packaging system, barrier properties of the packaging material and fundamentally, the temperature of distribution, storage and commercialization. The browning and the onset of off flavors are the main factors that lead to cane juice´s rejection, due to chemical, enzymic and microbiological mechanisms (BUCHELI; ROBINSON, 1994; YUSOF et al., 2000; QUDSIEH et al., 2002). Apart of this, the choice of a raw material cultivar less susceptible to enzymic browning, along with the use of proper processing technologies are highly recommended interventions.

4 Conclusions

The full inactivation of peroxidase in the acidified cane juice required a pasteurization temperature of 95 °C for 30 s. Conversely, the three binomials tested (85, 90 and 95 °C for 30 s) inactivated the PPO. The low coliforms count and the absence of *Salmonella* spp. assured the end product´s safety. All processed batches showed a high sensory acceptance, particularly in the initial period of the stability tests. Pasteurization temperature affected the sensory stability, in terms of appearance, aroma, flavor and overall impression to a large extent. An increase in temperature resulted in longer stability and a gain in the product´s shelf-life.

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4 CAPÍTULO 3

Contextualização

O soro da ricota, também denominado *scotta*, é um co-produto da indústria de laticínios destinado à alimentação animal, ou simplesmente descartado. Este estudo foi conduzido com o propósito de desenvolver tecnologias para o aproveitamento da *scotta*, como base para elaboração de um suplemento hidroeletrolítico, popularmente conhecido como bebida isotônica. Como aspectos positivos e hipóteses desta pesquisa destacam-se: (1) o aproveitamento de um co-produto de baixo custo para produção de uma bebida com alto potencial de agregação de valor, (2) a bebida elaborada pode ser consumida por indivíduos intolerantes a lactose e (3) a hidrólise enzimática da lactose amplia o poder edulcorante da bebida sem incremento calórico.

Development of a ricotta cheese whey-based sports drink

(VALADÃO, N. K.; GEREMIAS-ANDRADE, I. M.; JORY, J. C.; GALLO, F. A.; PETRUS, R. R., 2016)

Abstract

Ricotta cheese whey (RCW) is a by-product of the dairy industry; it is either destined to the feeding of swine or simply disposed of. This study focused on RCW to develop a sports drink. Sensory tests were performed for this purpose. Measurements of pH, soluble solids content, osmolality, and sodium and potassium levels were also carried out. The sports drink was pasteurized at 85 °C/30 s, ultra clean filled into plastic bottles and subjected to commercial sterility test. Nine-point hedonic scale tests were conducted to evaluate appearance, aroma and flavor. The results of the Physicochemical tests were in compliance with the Brazilian Food Regulation (pH 3.1, 6.3 °Brix, 306 mOsm/kgH_2O, 500 mg/L sodium and 650 mg/L potassium). Also commercial sterility was achieved. The mean ratings assigned to appearance, aroma and flavor were 6.9, 6.5 and 6.0, respectively. The findings indicate that RCW could be a technologically feasible alternative to produce a shelf stable sports drink.

Keywords: dairy product, isotonic drink, by-product, sensory evaluation, product development.

1 Introduction

Whey is a raw material for making ricotta cheese. The principle of its production is based on the flocculation of whey proteins through heat associated with acidification. In the production of ricotta cheese, 40 to 50 g/L of the yield is cheese (Albuquerque, 2002) and 950 to 960 g/L is ricotta cheese whey. According to the most recent data on ricotta cheese production found in the Brazilian annual milk production in 2004 (ZOCCAL, 2011) and 2014 (production estimates) (USDA/FAS, 2013), government sources estimate that in 2014 230,000 tons of ricotta cheese whey were produced. Such a large amount of effluents may cause environmental concerns connected with their disposal.

Sansonetti et al. (2010) state that ricotta cheese whey is mostly formed by water (932 to 938 g/L), the remaining whey constituents are proteins from the flocculation process (1.5 to 2.2 g/L), lactose (48 to 50 g/L), minerals (10 to 13 g/L) and organic acids (2.0 to 2.5 g/L).

The mineral level in electrolyte drinks, popularly known as sports drinks, is similar to that found in blood plasma (~ 300 mOsm/kg). Such beverages are meant to restore the water and electrolytes lost during physical exercise when the body metabolizes huge amounts of water and electrolytes (sodium, potassium, chloride, etc.) as it loses heat to the environment. If those losses are not replenished, the body lingers in a state of low water level, which leads to increased risks of exhaustion and thermal shock, complications in kidney function and muscle cramps (MONTEIRO; DE MARCHI, 2010).

The Brazilian Association of Soda and Non-Alcoholic Beverages Industries (ABIR, 2011a) reported that the consumption of sports drinks increased by 20% in 2011, a significant increase compared to the average (13.2%) between 2005 and 2009.

The Resolution 18 (09/27/2010) (BRASIL, 2010) states that an electrolyte drink is a product designed to aid hydration and must meet the following identity standards:

- The level of sodium must range from 460 to 1150 mg/L; only inorganic salts generally added to food must be used as a source of sodium.
- Potassium may be added to the product up to 700 mg/L.
- Osmolality must range between 270 and 330 mOsm/kg of water.
- Carbohydrates may represent up to 80 g/L of the beverage.
- Other nutrients and non-nutrients must not be added to the product.

- Dietary fibers, starches and polyols must not be added to the product.

Some studies seek alternatives to the use of ricotta cheese whey, such as the production of bio-ethanol (SANSONETTI et al., 2009; SARACENO et al., 2011; SANSONETTI et al., 2011; BITELLO et al., 2013; ZOPPELARI; Bardi, 2013). As for the use of ricotta cheese whey in human food, Gerhardt et al. (2013) developed a fermented milk-based beverage using ricotta cheese whey and hydrolysed collagen. Chávez (2001) developed a sport drink and Fontes et al. (2015) developed an electrolyte repository with permeated milk ultrafiltration, flavors strawberry and lemon, respectively. Notably, none of these studies mentions the use of ricotta cheese whey in sports drinks production as described in the investigation herein reported.

An off-the-record survey of the four largest dairy farms in the country producing ricotta cheese revealed that ricotta cheese whey is either destined to the feeding of swine or simply disposed of. The aim of this study was therefore to investigate and propose a method which could be implemented to utilize the ricotta whey for developing a sports drink.

2 Material and methods

2.1 Defining the sports drink flavor

To define the sports drink´s flavor, rank preference tests were performed on sixty assessors averaging 22 years old. The sensory test followed the protocol described by Meilgaard, Civille and Carr (1999).

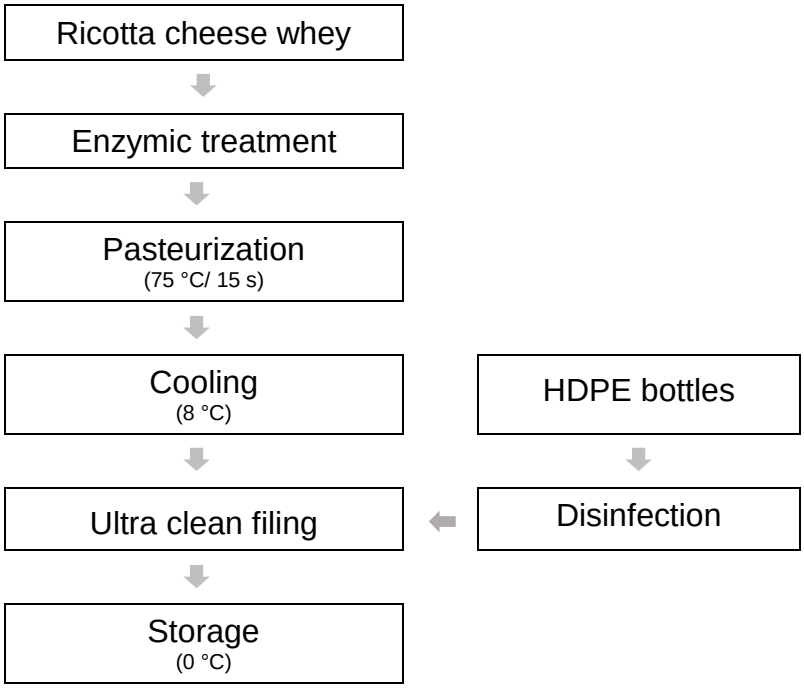
The assessors were served with seven samples of different flavors of a leading brand of sports drink, as follows: grape, passion fruit, strawberry with passion fruit, orange, lime, mandarin and citrus (composed of orange and grapefruit). A randomized complete block design was presented. The samples were served simultaneously in individual booths lighted by fluorescent white lamp. Approximately 50 mL of drink at a temperature of 4 °C was poured into plastic cups coded with random 3-digit numbers for identification. Given the light flavoring of the drink, seven samples simultaneously served were not considered to be a major factor for test fatigue. Meilgaard, Civille and Carr (1999) state that rank preference tests are carried out with three or more samples. The sensory analyses were approved by the Committee of Ethics in Research of the University of São Paulo (Protocol 359.879).

2.2 Processing the ricotta cheese whey

The ricotta cheese whey (RCW) was produced in the dairy plant at University of São Paulo, at Pirassununga campus, in accordance with the methodology described by Albuquerque (2003). The enzyme lactase (β-D-galactoside galactohydrolase/EC 3.2.1.23) Granolact M 7500 (Granolab, Brazil) was added to the RCW in the ratio of 1.05 g enzyme to each liter whey. The RCW powered with the enzyme was kept for 24 hours at 8 °C in order for the lactose to hydrolyze.

After the enzymic treatment, the RCW was pasteurized in an electric plate heat exchanger, with a nominal capacity of 300 L/h (Sumá Indústria e Comércio Ltda, Brazil) at 75 °C/ 15 s, and ultra clean filled in high density polyethylene (HDPE) bottles (Usicomp, Brazil) decontaminated beforehand. The bottles were sprayed with a peracetic acid solution (Thech Desinfecção Ltda, Brazil) (0,05%v/v/15 s/45 °C). The filling was made using a semi-automatic gravimetric filling machine (Polienva-Movitron, Brazil) installed inside of a ISO class 5 horizontal unidirectional airflow cabinet (Veco do Brasil, Brazil). The batch was stored in the dark at 0 °C to preserve the quality of the whey during the time necessary for the development of the sports drink formulation. Figure 1 depicts the flowchart for experimental processing of ricotta whey.

Figure 1 - Flowchart of the processing of the ricotta cheese whey used in the development of the sports drink formulation.



2.3 Microbiological analysis of the ricotta whey

To evaluate the microbiological stability of the pasteurized whey kept at 0 °C, standard mesophilic aerobic plate counts were carried out during storage period (eight months). The protocol described by Silva et al. (2010) was followed.

2.4 Developing the sports drink formulation

After processing the ricotta whey, the levels of sodium and potassium were analyzed in a B462 flame photometer (Micronal, Brazil). The soluble solids content (°Brix) was determined in a portable digital refractometer AR 200 (Reichert, USA). The pH was measured in a 300M Analyzer pHmeter (Hanna Instruments, Romania), and the osmolality measured in a PLZ 1000 osmometer (PLZ Tecnologia, Brazil). From the results, numerous sports drink formulations were developed with variations in the levels of ingredients and additives (ricotta cheese whey, deionized water, sodium chloride, citric acid, sucrose, mandarin flavor and twilight-yellow coloring). Physicochemical and sensory assays were also performed to guide the drink´s formulation.

2.4.1 Sensory evaluation

Just about right and hedonic scales tests were performed, following the procedures described by Meilgaard, Civille and Carr (1999), and Ferreira et al. (2000). Sixty panelists with ages ranging from 17 to 50 years old were recruited. Each panelist was served 40 mL of sample at approximately 4 °C.

Based on the results obtained in the first test, a new formulation was developed in which the concentration of ingredients and additives were changed based on the panelists' responses. During the development, which lasted approximately six months, ten sensory analysis sessions were held. The responses obtained in the sensory and Physicochemical analyses led to the development of a palatable drink in compliance with the Legislation standards.

2.4.2 Physicochemical tests

Physicochemical tests were carried out both on the ricotta whey and sports drink based on the methodology of the Association of Official Analytical Chemists (AOAC, 2012), as follows: (1) total soluble solids, (2) pH, (3) titratable acidity, (4) osmolality, and (5) sodium and potassium concentration.

2.5 Processing the new product

The sports drink was pasteurized at 85 °C for 30 s, immediately cooled to approximately 10 °C and ultra clean filled into 330 mL-white pigmented (TiO_2) polyethylene terephthalate (PET) bottles. The bottles were hermetically closed with aluminum seals and polypropylene (PP) screw caps by induction using a sealer model Super Seal Jr., made by Enercon Industries Corporation (Wisconsin, USA). A batch of 180 packages was obtained.

2.6 Commercial sterility test of the finished product

The processed batches were incubated at 25 °C for 10 days in the dark. The pre-incubation technique was performed in an attempt to increase the number of viable microorganisms if present. Under these conditions, if the product spoils, the presence of contaminants is determined. The methods described by Deibel and Jantschke (2001), and Denny and Parkinson (2001) were followed to evaluate the commercial sterility of the sports drink (pH ~ 3,0).

2.6.1 Inspection of the samples

After incubation, the bottles were examined for abnormal conditions such as leakage, swells, flippers etc. After the bottles were opened, changes in appearance, odor and flavor were investigated prior to pH determination. If the package is defective and/or spoilage is evident the commercial sterility is not achieved. Otherwise, the samples are subjected to microbiological assays.

2.6.2 Determination of pH

After the inspection of the product, the pH values of samples from ten packages were determined. A variation of less than 0.2 pH units in relation to the determination performed immediately after processing was tolerated.

2.6.3 Microbiological assays

Five samples of the product that showed no evidence of spoilage or pH variation were subjected to microbiological assays. After the samples were homogenized, 1 mL-aliquot was aseptically transferred to sterilized plates where thermoacidurans agar (TAA), acidified potato dextrose agar (PDA) and all purpose tween agar (APT) were separately poured. TAA plates were incubated at 30 °C for 5 days under aerobic and anaerobic conditions for the purpose of identifying facultative mesophilic aerobes. PDA plates were incubated at 25 °C for 5 days under aerobic condition, for yeasts and molds detection. APT plates were incubated at 30 °C for 5 days under aerobic condition, for lactic acid bacteria.

2.7 Sensory acceptability evaluation

A panel of sixty regular consumers (age of twenty two years on average) of sports drinks evaluate the product. The panelists were asked to evaluate the sensory attributes of appearance, aroma and flavor by assigning a liking score on a 9-point Hedonic Scale (1 = dislike extremely; 5 = neither like nor dislike; 9 = like extremely) (MEILGAARD et al., 1999). Tests were accomplished in individual booths lighted with a white fluorescent lamp. The samples were served at a temperature of about 4 °C in 50 mL plastic cups labeled with a 3-digit code.

2.8 Data analysis

The data were subjected to the analysis of variance with a 95% confidence level and Tukey's test for comparison between means from the microbiological and Physicochemical assays. The data were analyzed by using the SAS (Statistical

Analysis System, SAS Institute Inc., USA) version 9.2. In regard to the rank preference tests, the 10% significance chart was used (Christensen et al., 2006).

3 Results and discussion

3.1 Defining the sports drink flavor

The mandarin, passion fruit, orange, strawberry with passion fruit and grape flavors were considered the most statistically preferred, according to the rank preference test (CHRISTENSEN et al., 2006). Lime and citrus were rejected by the assessors.

A new rank preference test was then carried out. The 10% significance level chart (CHRISTENSEN et al., 2006) showed that the significant minimal difference among the samples is 42 points. The passion fruit, the strawberry flavored with passion fruit and the mandarin were the preferred flavors. Mandarin was chosen as the sports drink flavor in developing the formulation. ABIR (2011b) holds that mandarin is one of the two best-liked flavors among those available in the market. Marins and Ferreira (2005) and Brito and Marins (2005) reported that mandarin was the sports drink flavor preferred among 420 athletes and 200 judokas, respectively.

3.2 Microbiological analysis of the ricotta whey

Table 1 shows the results of the microbiological tests performed in triplicate on the samples of the ricotta whey used in the development of the sports drink formulation.

Table 1 - Mean counts of mesophilic aerobic microorganisms in pasteurized ricotta whey during the period in which the sports drink formulation was developed.

Storage time at 0 °C (weeks)	Mean count ± standard deviation (CFU/mL)
0	$< 1_{est}$
18	$10.7^{a} \pm 1.5$
25	$13.3^{a} \pm 2.1$
36	$15.0^{a} \pm 1.0$

Means (of three samples) followed by the same exponent are not different ($p > 0.05$). est – estimated value.

Data from Table 1 demonstrates that the ricotta whey remained microbiologically stable throughout the development of the sports drink, given the fact that mean counts were lower than 16 UFC/mL.

3.3 Sports drink formulation

Table 2 exhibits the profile of the sports drink developed in this study.

Table 2 - Composition of 1L-electrolyte supplement developed from ricotta whey.

Ingredients and additives	
Deionized water (mL)	617.2
Ricotta cheese whey (mL)	382.8
Sucrose (g)	36.2
Citric acid (g)	3.2
Tangerine flavor (mL)	2.6
Twilight-yellow coloring (mg)	13
Physicochemical parameters (mean ± standard deviation)	
Osmolality (mOsm/kgH_2O)	306 ± 0.6
pH	3.15 ± 0.01
Soluble solids (°Brix)	6.40 ± 0.00
Sensory acceptance (9-point hedonic scale)	
Mean score for overall impression	6.3 ± 1.8
Percentage of acceptance (%)*	81.7

*Percentage of assessors that assigned scores above 5.

Fontes et al. (2015) developed an electrolyte drink made up of the permeate of milk ultrafiltration. The formulated drink reached values (pH of 3.4, soluble solids content of 5.9 °Brix and osmolality of 311.6 mOsm/L) which are close to those found in this study.

Monteiro and De Marchi (2010) state that isotonic drinks having 60 to 80 g/L of carbohydrates in their composition enter the bloodstream faster than water. As presented in Table 2, the formulation herein developed met the Physicochemical requisites set by the Brazilian Regulation.

Figures 2 shows the results obtained for the orange color, mandarin aroma, mandarin flavor, sour, sweet and salty tastes, respectively, in the just about right scale tests.

Figure 2 - Responses obtained in the just about right (JAR) scale tests (to be continued).

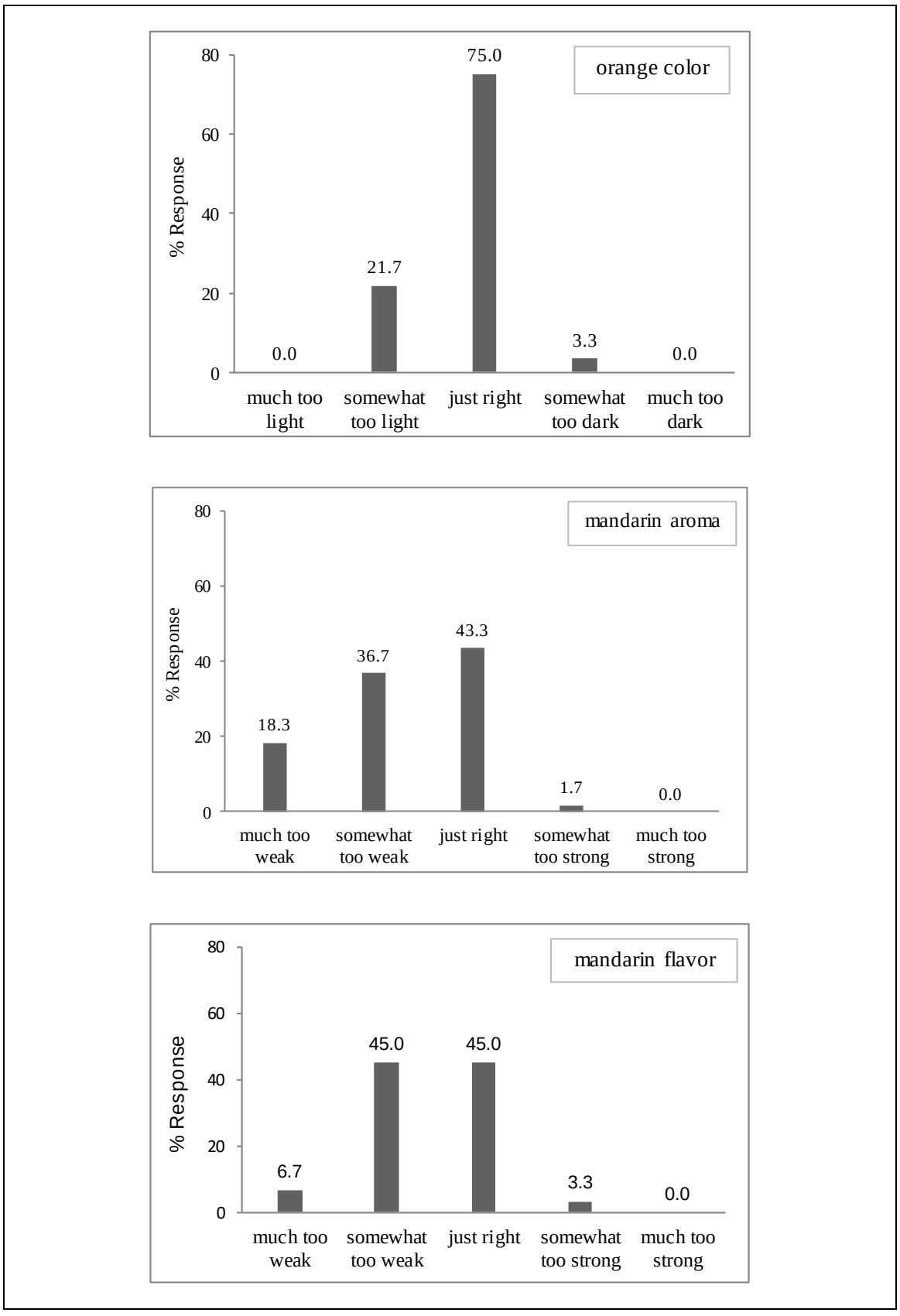
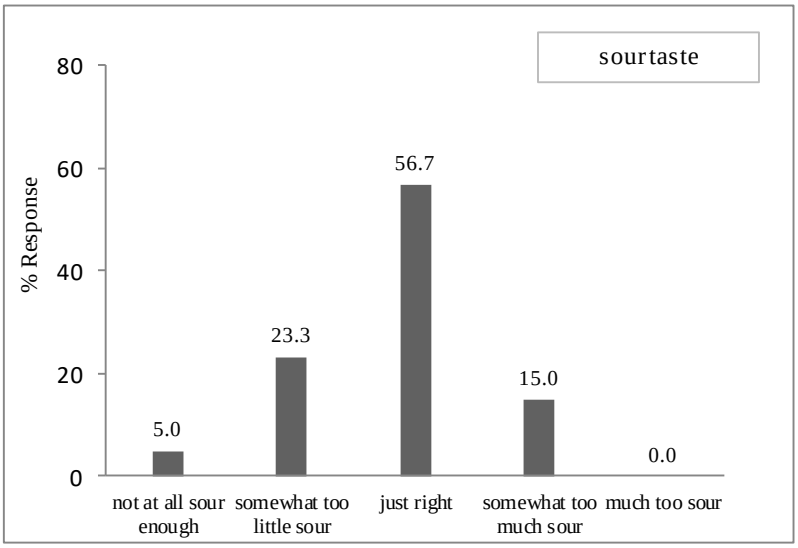
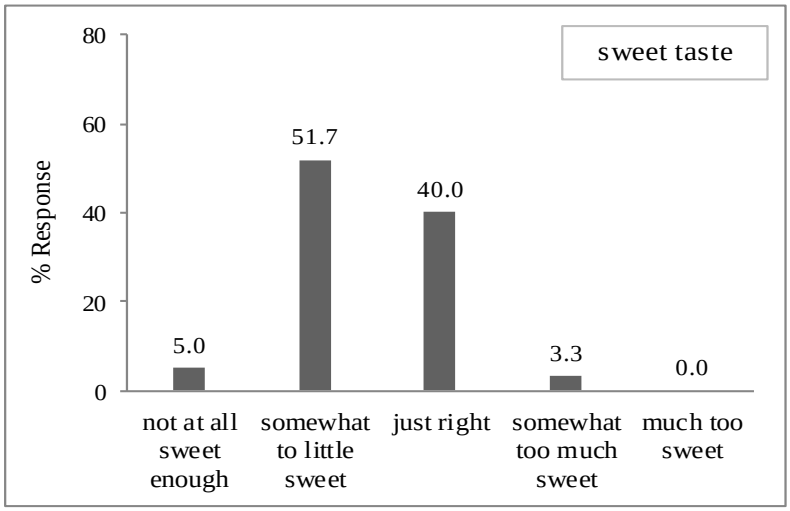
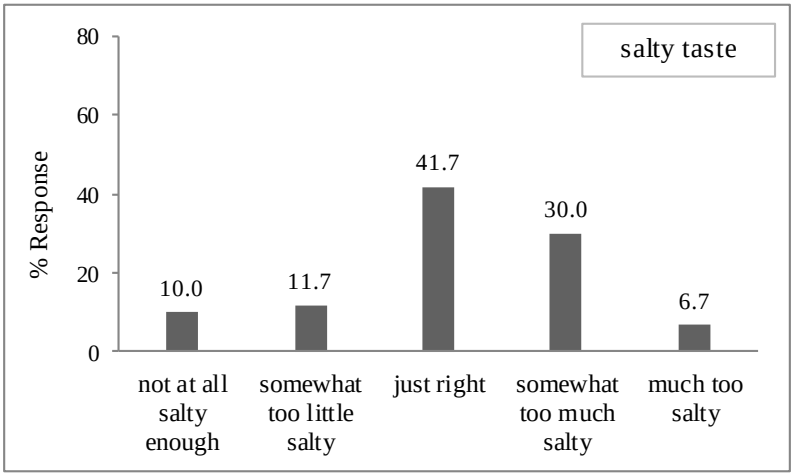


Figure 2 - Responses obtained in the just about right (JAR) scale tests.







As represented in Figure 2, only “orange color” had a minimum of 70% of responses in the “just right”, as recommended by Ferreira et al. (2000). The panelists

seemed to prefer a drink having a sweeter taste. Nevertheless, aiming at meeting the required Physicochemical standards, no more sucrose was added. In terms of salty taste, conversely, 37% of the panelists reported that the sports drink was “somewhat” and “much too salty”. The sour taste was considered below the “just right” by 28% of the assessors and above “just right” 15%; the majority (57%) considered it to be “just right”. The results suggest the concentration of mandarin flavor should be increased. However, for many preliminary formulations - with substantial increments of flavor concentration - the responses obtained in the just about right scale tests remained constant. Thus the flavor concentration was maintained. Figure 3 illustrates the by-products and the finished drink.

Figure 3 - Cheese whey (a), ricotta whey (b), and sports drink (c).

(a) (b) (c)

3.4 Commercial sterility test

3.4.1 Packages examination

After incubation at 25 °C for 10 days, no defective packages were found (such as leakage or swelling); there was no evidence of spoilage.

3.4.2 Measurement of pH

The pH values of freshly pasteurized (time 0) sports drink and stored during 10 days at 25 °C (incubation test) were 3.03 and 2.98, respectively. Thus, the variation ($|\Delta pH| = 0.05$) was less than 0.2 units, showing the Physicochemical stability of the product.

3.4.3 Microbiological assays

All five tested samples showed no microorganisms under the specific recovery media and incubation conditions (2.6.3). In this way, the sports drink achieved the commercial sterility.

Of particular relevance is the claim that the combination of intrinsic (acid pH) and extrinsic factors (pasteurization and ultra clean filling) employed in this study was effective in attaining a shelf stable sports drink. This is a very attractive achievement with respect to energy saving, since there is no need of cold chain for product´s storage, distribution and commercialization.

3.5 Acceptability of the end product

Figure 4 exhibits the histogram of ratings assigned to appearance, aroma and flavor of the shelf stable sports drink.

Figure 4 - Histogram of ratings assigned to the ricotta cheese whey-based sports drink.

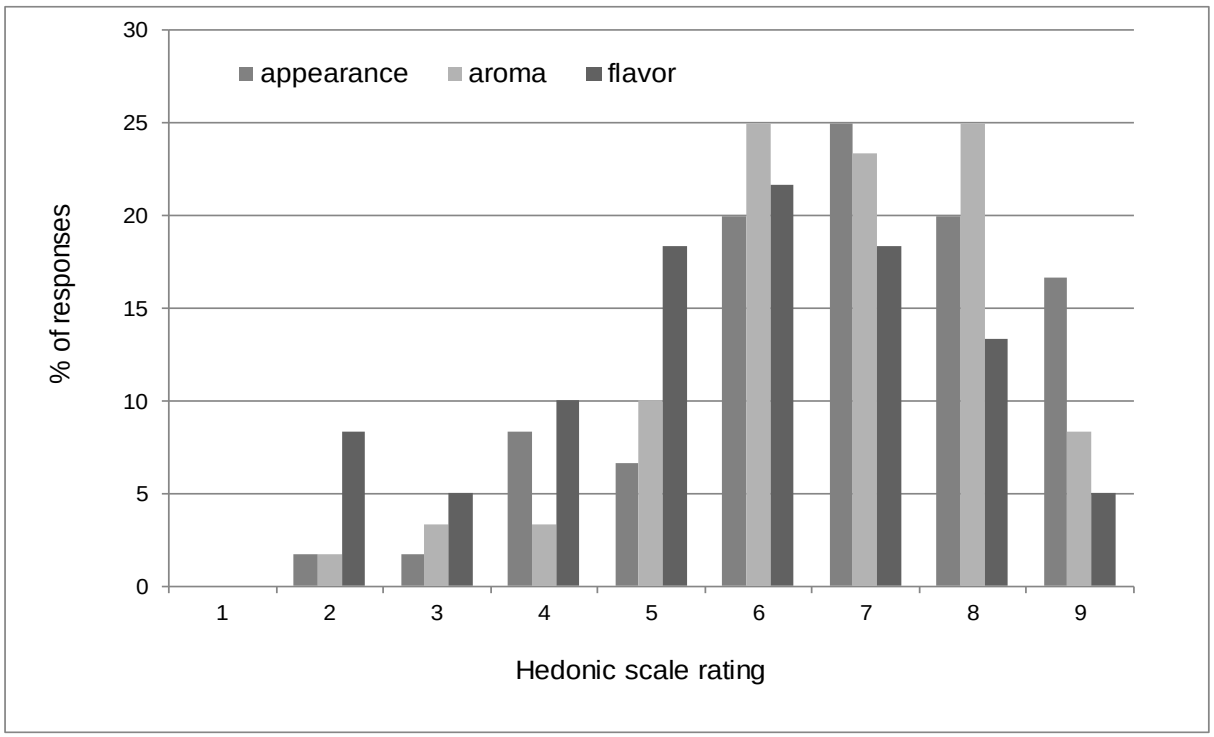


Figure 4 demonstrates a good acceptability of the drink; most ratings assigned to appearance, aroma and flavor were greater than five in the nine-point hedonic scale. The percentages of the responses equal or greater than 5 were 88.4, 91.6 and 76.6% for appearance, aroma and flavor, respectively.

Finally, some positive and appealing aspects regarding the present study are apparent: (1) a by-product of dairy industry, which is usually disposed of, may be utilized to produce a high added-value drink, (2) the developed sports drink may be consumed by lactose-intolerant individuals, since ricotta cheese whey was pre-treated with lactase, and (3) the hydrolysis of lactose into galactose and glucose enhances the sweetness of the drink with no caloric increase.

4. Conclusions

The findings of this study demonstrated that the ricotta cheese whey-based sports drink herein developed met the standards set by the Brazilian Food Regulation. The utilization of ricotta cheese whey in the production of a shelf stable sports drink is a technically feasible alternative, which adds value to a residue of the dairy industry that is usually disposed of or served as animal feed. Still, eventual adjustments to the sports drink formulation developed in this study may be necessary to enhance its sensory quality.

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5 CAPÍTULO 4

Contextualização

As reações de escurecimento enzimático incidentes no caldo de cana *in natura* representam um dos principais mecanismos de deterioração que limita seu tempo de vida útil. Com o propósito de investigar a influência do cultivar da matéria-prima na taxa de escurecimento da bebida, conduziu-se este trabalho. Dezenas de investigações científicas abordam o escurecimento em caldo de cana. Entretanto, não foram localizados estudos que reportassem a influência do cultivar de cana-de-açúcar na taxa de escurecimento do caldo *in natura* estocado sob refrigeração.

Cultivar affects the color change kinetics of sugarcane juice

(BOMDESPACHO, L. Q.; DA SILVA, B. T. R.; LAPA-GUIMARAES, J.; DITCHFIELD, C.; PETRUS, R. R., 2018)

Abstract

This study evaluated the influence of different Brazilian cultivars (RB867515/C1, SP813250/C2, RB92579/C3 and CTC02/C4) on unprocessed sugarcane juice´s quality through Physicochemical, microbiological and enzymic assays. Color parameters were determined during six days at 10 °C. The experimental design was completely randomized with four treatments (cultivars) and three replicates. Significant differences were observed ($p \leq 0.05$) among the juice samples from different cultivars. The pH values ranged from 5.09 to 5.25, soluble solids content from (14.13 to 18.60) °Brix, and titratable acidity from (0.035 to 0.120)% of citric acid. Counts of mesophiles, psychrotrophs, and molds and yeasts varied between (4.8 and 6.2) log CFU/mL, (3.2 and 4.6) log CFU/mL and (3.3 and 4.8) log CFU/mL, respectively. Polyphenol oxidase and peroxidase activities ranged from (8 to 15) U/mL and between (6 and 40) U/mL, respectively. The initial (t0) lightness varied from 21 to 37 among cultivars, and final values, after six days of storage (t6), between 22 and 41. Principal components analysis demonstrated that juice from different cultivars is characterized by different parameters. Given its lighter color and smaller color variation during storage, juice extracted from cultivar RB867515 exhibited the greatest potential for consumer acceptance.

Keywords: non-alcoholic beverage, enzymic browning, principal components analysis.

1 Introduction

The chemical composition of sugarcane (*Saccharum* spp.*)* varies considerably according to several factors like climate, maturation stage, culture physiological age, physical, chemical and microbiological soil properties, agricultural practices and cultivar. Fresh cane juice, known in Brazil as “garapa” is a popular non-alcoholic beverage and according to Yusof, Shian and Osman (2000) it can be stored at 5 °C for four days. The soluble solids content varies from (15 to 25)°Brix and its pH is between 5 and 6. Cane juice is very perishable because of the high sugar concentration, high water activity and low acidity. Contaminants are broadly transferred from soil to raw material during harvesting, such as yeasts, filamentous molds, lactic bacteria and spores. Therefore, shorter processing times in harvesting, decontamination and juice extraction allow for lower effects of microbial activity within the cane stalks and consequently higher quality juices (OLIVEIRA et al., 2007).

Cane juice is typically an opaque liquid with a brown to dark green color. The composition of the raw material affects the juice´s color due to the presence of phenolic compounds, anthocyanins, carotenoids, flavonoids, melanoidins, melanins and products from the alkaline degradation of fructose in different amounts. After extraction, several mechanisms can cause juice browning like oxidation of chlorophyll and polyphenols (PRATI; MORETTI; CARDELLO, 2005) and enzymic browning by polyphenol oxidase (PPO) and peroxidase (POD) (BUCHELI; ROBINSON, 1994 and FELLOWS, 2006). Apart from chemical and biochemical browning mechanisms, the growth of microorganisms also affects the juice´s color (OLIVEIRA et al., 2007).

Chauhan et al. (2002) studied eight cane cultivars grown in India, based on their suitability for juice production. Juice yields were recorded and samples were subjected to Physicochemical and sensory evaluation. Additives were incorporated into the juice which was then pasteurized.

Thakar (2012) reported a research work where a cultivar was selected for preparing juice beverage on the basis of yield and sensory attributes for the storage and processing. The findings demonstrated that the pasteurized cane juice could be stored safely up to 45 days and used as a nutritious packaged pasteurized juice drink alternative to other sugar-added drinks.

Nevertheless, to the best of our knowledge, no study targeting the kinetics of color change in freshly extracted cane juice was performed. Though there are studies that investigated the color variation of unprocessed cane juice, the influence of cultivar on color nor how it varies during the storage were described. In this research work, Physicochemical, microbiological and enzymic tests were carried out. The color parameters of freshly extracted and unprocessed juice from four cultivars were instrumentally measured.

2 Materials and methods

2.1 Raw material

Four sugarcane (*Saccharum* spp.) cultivars [RB867515 (C1), SP813250 (C2), RB92579 (C3) and CTC02 (C4)] were obtained from the region of Pirassununga/SP, donated by Companhia Muller de Bebidas, Porto Ferreira/SP and Baldin Bioenergia SA, Pirassununga/SP. The raw material was harvested the day before juice extraction.

2.2 Juice extraction

The stalks were selected, cut, scraped and sanitized by immersion in a peracetic acid solution 0.05 % (v/v) for 20 minutes at 25 °C. The juice was extracted in a stainless steel electric cylinder mill, manufactured by Maqtron, Joaçaba/SP, then passed through a stainless steel filter and filled into clear sterilized glass bottles with screwcaps and 1 L capacity.

2.3 Storage

The bottled juice was stored at (10 ± 1) °C in a BOD chamber in the dark for six days. Samples were daily collected for measurement of color.

2.4 Juice characterization

Freshly extracted juice was characterized by Physicochemical, microbiological and enzymic analysis.

2.4.1 Physicochemical tests

AOAC methods were employed to determine the Physicochemical parameters (AOAC, 2010). The pH was measured by a pH meter (Analyser, model 300 M, São Paulo/SP). Soluble solids content (°Brix) with a handheld refractometer (Reichert, model AR 200, New York/NY). Total titratable acidity (expressed as the percentage of citric acid) was determined with 0.1 M NaOH and phenolphthalein. The ratio of soluble solids content/titratable acidity was calculated.

2.4.2 Microbiological assays

Viable counts of aerobic mesophiles, aerobic psychrotrophs, and molds and yeasts were obtained as described by Downes and Ito (2001).

Aerobic mesophiles count

Mesophilic count was obtained by pour plating with plate count agar (PCA). Serial dilutions of a 1 mL sample of juice were plated and incubated at 35 °C for 48 h and colonies were enumerated. The results were expressed in colony forming units (CFU) per mL of juice.

Molds and yeasts

Yeasts and molds count was performed by spread plating of a 0.1 mL sample of juice onto the dichloran rose-bengal chloramphenicol (DRBC) agar. The plates were incubated at 25 °C for 5 days and colonies were enumerated.

Aerobic psychrotrophs

Psychrotrophic count was obtained by pour plating with PCA. Serial dilutions of a 1 mL-sample were plated and incubated at 7 °C for 10 days.

2.4.3 Enzymic assays

The protocols adapted from Campos and Silveira (2003) were used to determine the polyphenol oxidase (PPO) and peroxidase (POD) activities.

2.4.3.1 Polyphenol oxidase

Five and half milliliters of 0.2 M phosphate buffer solution (pH 6.0) and 1.5 mL of 0.2 M catechol were added into a test tube and maintained at 25 °C 10 for min. Then 1.0 mL of the diluted sample in deionized water (1:10) was added. The tube was stirred for 15 s and returned to the water bath at 25 °C for 30 min. The absorbance was read

in a spectrophotometer at 425 nm. The blank was prepared by diluting the sample in deionized water.

2.4.3. 2 Peroxidase

Seven milliliters of 0.2 M phosphate buffer solution (pH 5.5) and 1.0 mL of the diluted sample (juice) in deionized water (1:10) were added to a test tube and maintained in a heat bath at 35 °C for 10 min. Then 1.5 mL of 0.05% guaiacol and 0.5 mL of 0.1% hydrogen peroxide were added. The tube was magnetically stirred for 15 s and returned to the bath at 35 °C for 15 min. Finally, the absorbance was read in a spectrophotometer at 470 nm.

One (1) unit of enzyme activity (U) was defined as the amount of enzymic extract capable of increasing absorbance at 425 and 470 nm for PPO and POD, respectively, at rates of 0.001 unit per minute.

2.5 Instrumental color

A Hunterlab Ultra-Scan colorimeter (Hunter Associates Laboratory, Model SN7877 Reston, VA/USA) was used to measure the color of fresh juice and throughout the storage (6 days at 10 °C). The iluminant was D65 and observation angle at 10°. The equipment was adjusted to measure L*, a*, b* (CIELab color sytem, COMISSION INTERNATIONALE DE L'ECLAIRAGE – CIE, 1978) three times for each sample and calculate the mean values. The parameters a* and b* were used to determine Chroma (C*) and Hue angle (°Hue) with equations 1 and 2. The color coordinates were entered in the EasyRGB color calculator to obtain the color image (EASYRGB, 2016). Total color difference (TCD), in relation to freshly extracted juice, was calculated by equation 3.

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad \text{(Eq.1)}$$

$$^\circ Hue = arctan\,(b^*/a^*) \quad \text{(Eq.2)}$$

$$TCD = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2} \quad \text{(Eq.3)}$$

2.6 Data analysis

Analysis of variance and Tukey's test (95% significance level) were carried out using SAS 9.3. Principal component analysis (PCA) was performed using Statistic 13, (1984-1995, Dell Inc.). PCA is a statistical technique used to identify the smallest number of latent variables, called "principal components", that explain the greatest

amount of observed variability in a data set (MEILGAARD; CARR; CIVILLE, 1999). PCA displays the relationship among samples and suggests which parameters best characterize every sample. Each axis, in a two-dimensional representation, explains a certain percentage of the total variability that exists between samples.

3 Results and discussion

3.1 Physicochemical characterization

Table 1 exhibits the Physicochemical parameters determined in raw cane juice.

Table 1 - Physicochemical parameters of freshly extracted cane juice from different cultivars.

Cultivar	pH	Density (g/mL)	Soluble solids (°Brix)	Titratable acidity (% citric acid)	Ratio
C1	5.20^{ab}±0.00	1.071^{c}±0.00	18.60^{a}±0.00	0.120^{a}±0.00	150.5^{c}±0.5
C2	5.19^{ab}±0.08	1.164^{a}±0.00	14.1^{d}±0.2	0.035^{c}±0.00	403^{a}±9
C3	5.09^{b}±0.01	1.068^{d}±0.00	18.00^{b}±0.00	0.072^{b}±0.01	253^{b}±36
C4	5.25^{a}±0.05	1.146^{b}±0.00	16.2^{c}±0.2	0.043^{c}±0.01	377^{a}±114

Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02). Ratio: soluble solids content/titratable acidity. Mean of three replicates ± standard deviation. Means followed by the same exponent in the same column are not different ($p > 0.05$) by the Tukey test.

The juice samples extracted from different cultivars showed significant differences ($p \leq 0.05$). These differences can affect both the color variation and the juice deterioration rate. Silva (2016) reported similar pH values (5.01-5.12) for fresh cane juice. Molina et al. (2008) observed that the pH varied for different cultivars with a mean value of 5.5, close to those determined in this study. According to Sugar Engineers (2016), the density of cane juice (18 °Brix) at 25 °C is 1.0706 g/mL, close to data shown in Table 1. The soluble solids content was measured by Silva (2016) and Silva (2006); mean values of (22.3 and 18) °Brix were respectively reported. The sugar content varies with the cultivar, plant conditions and maturation degree. Cultivars C2 and C4 showed lower sugar contents, while C1 and C3 were close to the values reported by Silva (2006). Silva (2016) also determined the titratable acidity of fresh cane juice and reported values between 0.05 and 0.08% citric acid, as it was observed

for C2, C3 and C4. Conversely, C1 presented a much greater value. Prati et al. (2005) and Oliveira et al. (2007) reported a ratio of 152, close to the value determined in this study for C1. This parameter has a strong correlation to product palatability, which has an impact on consumer acceptance.

3.2 Microbiological evaluation

Table 2 shows the results from the microbiological assays.

Table 2 - Microbial counts (log CFU/mL) in raw cane juice from different cultivars.

Cultivar	Aerobic mesophiles	Molds and yeasts	Aerobic psychrotrophs
C1	6.2^{a}±0.1	3.3^{b}±0.2	4.6^{a}±0.1
C2	5.4^{b}±0.3	4.0^{ab}±0.1	3.2^{c}±0.1
C3	4.8^{c}±0.1	3.9^{ab} ±0.7	3.9^{b}±0.3
C4	5.3^{b}±0.1	4.8^{a}±0.1	3.3^{c}±0.1

Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02). Mean of three replicates ± standard deviation. Means followed by the same exponent in the same column are not different ($p > 0.05$) by the Tukey test.

As demonstrated in Table 2 there were significant differences among the microorganisms counts for the fresh juice extracted from the four cultivars. The mesophiles count in C3 was 1.4 log cycles lower than C1. In regard to molds and yeasts, and psychrotrophs, the variations were 1.5 and 1.4 log cycles, respectively. Brazilian Food Regulation (BRASIL, 2001) does not set standards for raw cane juice. Gandra et al. (2007) indicate that mesophilic counts between 6 and 7 log UFC/mL in cane juice may meaningfully alter the sensory attributes and deteriorate the product. Silva et al. (2010) reported that molds and yeasts are common in cane juice, while Oliveira et al. (2007) determined counts of 6.2 logUFC/mL, above those obtained in this study.

3.3 Enzymic assays

Table 3 presents the results for the enzymic assays in raw juice.

Table 3 - Polyphenol oxidase (PPO) and peroxidase (POD) activities in raw cane juice.

Cultivar	PPO (U)	POD (U)
C1	12^{b} ±0.1	31.2^{b}±0.8
C2	8.2^{d}±0.2	40^{a}±1
C3	14.6^{a}±0.2	26^{c}±0.3
C4	8.68^{c}±0.02	5.96^{d}±0.08

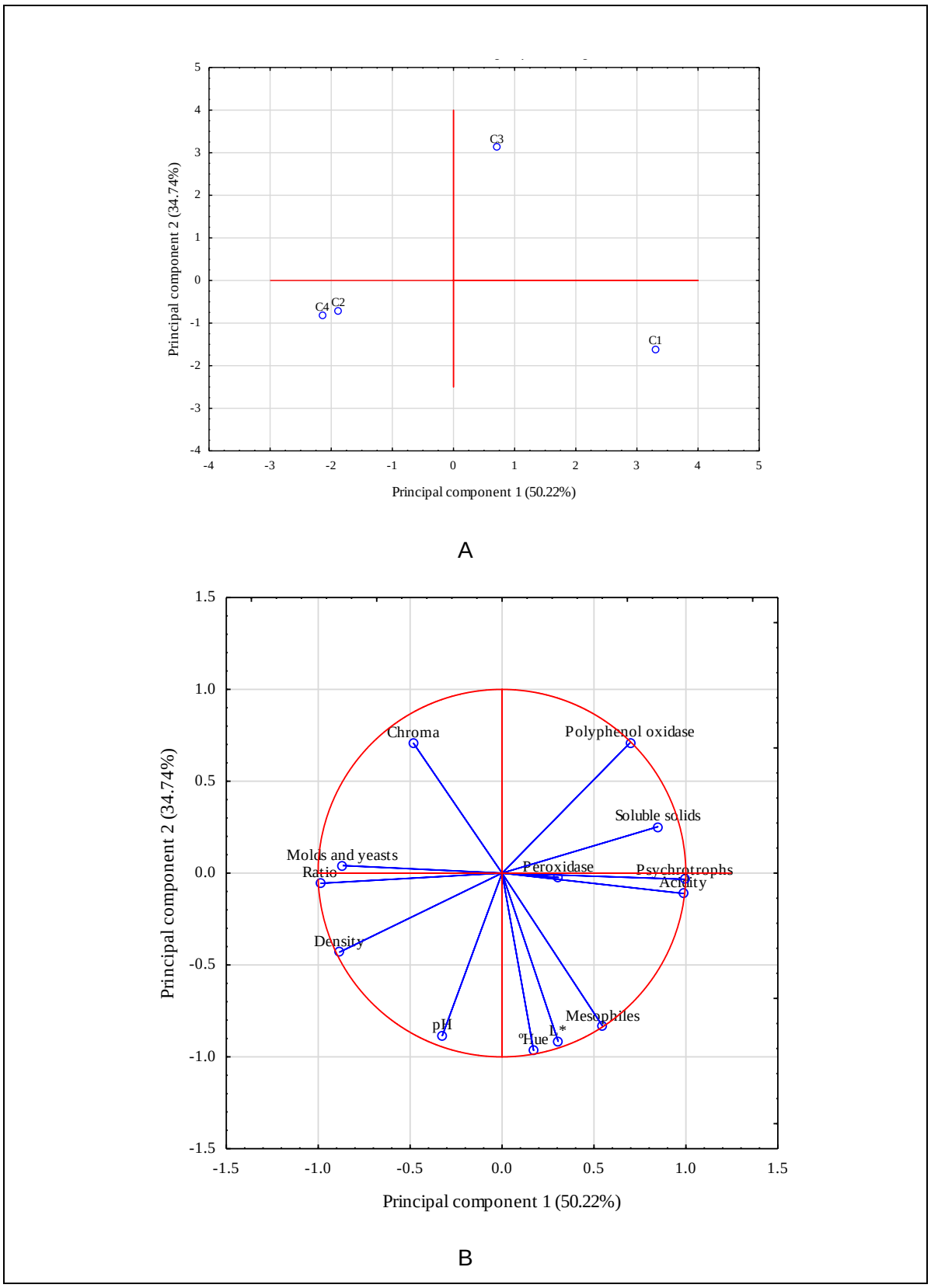
Cultivars: C1 (RB867515), C2(SP813250), C3 (RB92579), C4 (CTC02). Mean of three replicates ± standard deviation. Means followed by the same exponent in the same column are not different ($p > 0.05$) by the Tukey test.

The activities of PPO and POD were significantly different in all cultivars. C2 and C4 exhibited the lowest PPO and POD activities, respectively. POD activities were greater than PPO in C1, C2 and C3. Bucheli and Robinson (1994) stated that PPO is the main enzyme responsible for the juice browning.

3.4 Principal components analysis

Figure 1 depicts the distribution of the parameters and the samples of juice.

Figure 1 - Projection of the cases (cane juice samples from different cultivars (A) and the variables (B) on the plane. Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02).



Principal components 1 and 2 explained 84,96% (50,22 + 34,74) of the total variability among juice samples from different cultivars, and validated the two-dimensional representation of the components to describe the samples´ characteristics. The position of the four cultivars displayed in Figure 1A indicates that C2 and C4 are represented mainly by the same characteristics (ratio, density and pH). C1 and C3 showed different characteristics. C1 is in the same quadrant as °Hue, L*, mesophilic count, acidity and peroxidase activity. Meanwhile, C3 is in the quadrant with polyphenol oxidase activity and soluble solids content. The parameters acidity, psychrotrophs count, ratio and molds and yeasts count are the main contributors in PC1, while °Hue, pH and L* are the main contributors in PC2 (Figure 1B).

Parameters with longer vectors explain better the variability among samples than shorter vectors. Because peroxidase (POD) activity is represented by a short vector, this parameter does not meaningfully differ among samples, on the plane represented by PC1 and PC2 (Figure 1B). Vectors closer to each other indicate parameters that may have a high positive correlation. Vectors that form an angle close to 180° may have a negative linear correlation. In this way, Figure 1B suggests the existence of a negative correlation between acidity and ratio. Vectors that form an angle close to 90°, such as acidity and pH, may not present significant linear correlation with each other. The statistical analysis of linear correlations between data confirmed that ratio and acidity, and ratio and psychrotrophic count had a negative correlation at 5% of significance. Positive correlations ($p \leq 0.05$) were demonstrated between acidity and psychrotrophic count, and between L* and °Hue.

3.5 Kinetics of color parameters variation

Figure 2 represents the variation in color parameters throughout the storage. The parameters measured in the freshly extracted (raw) juice were used as reference for calculating the color differences (ΔL*), Chroma (ΔC), Hue angle (ΔHue*) and total color difference (TCD).

Figure 2 - Variation in lightness (L*), Chroma (C*), Hue angle (°Hue) and total color difference (TCD) in cane juice extracted from different cultivars, in relation to the initial values, throughout the storage at 10 °C in the dark. Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02).

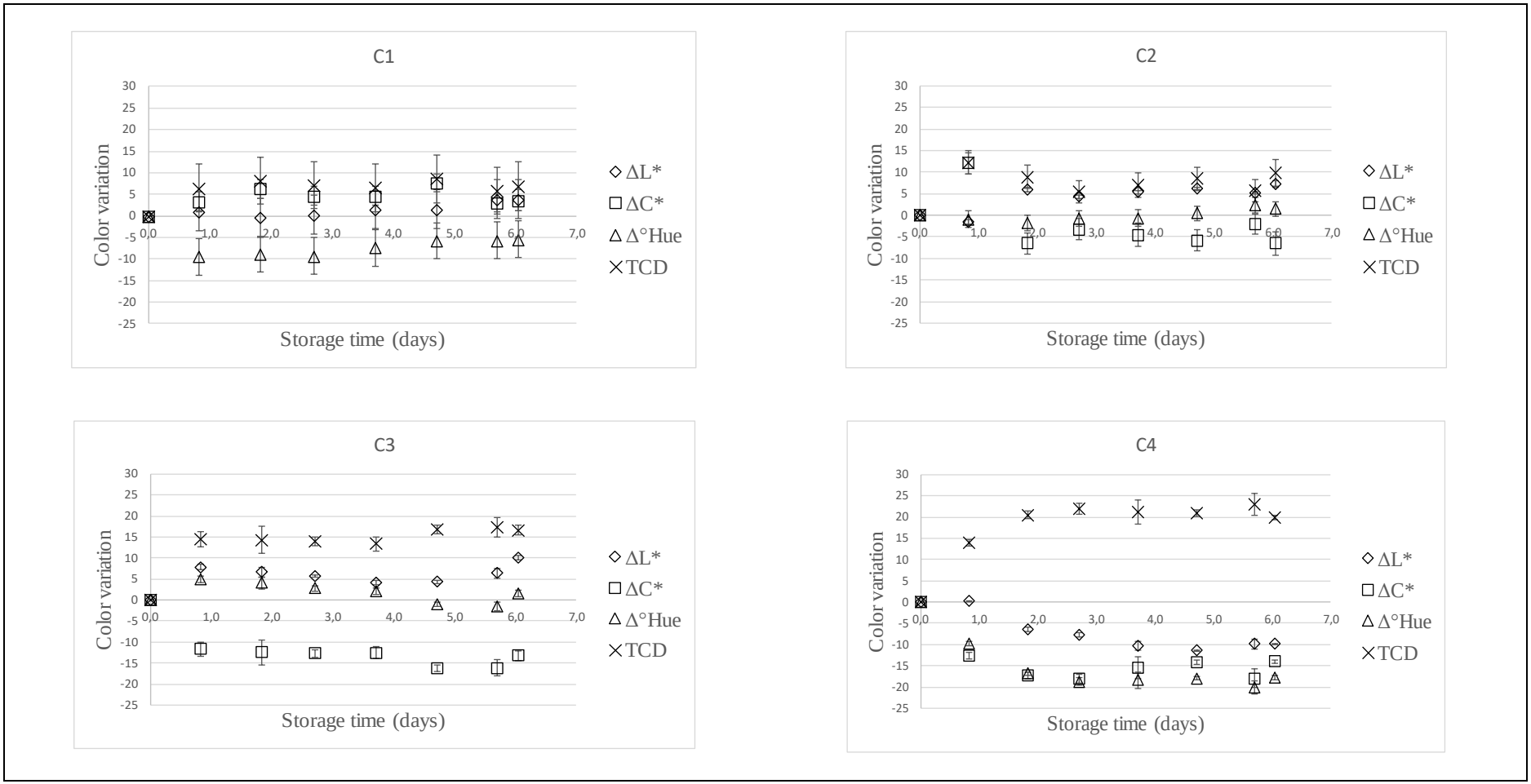
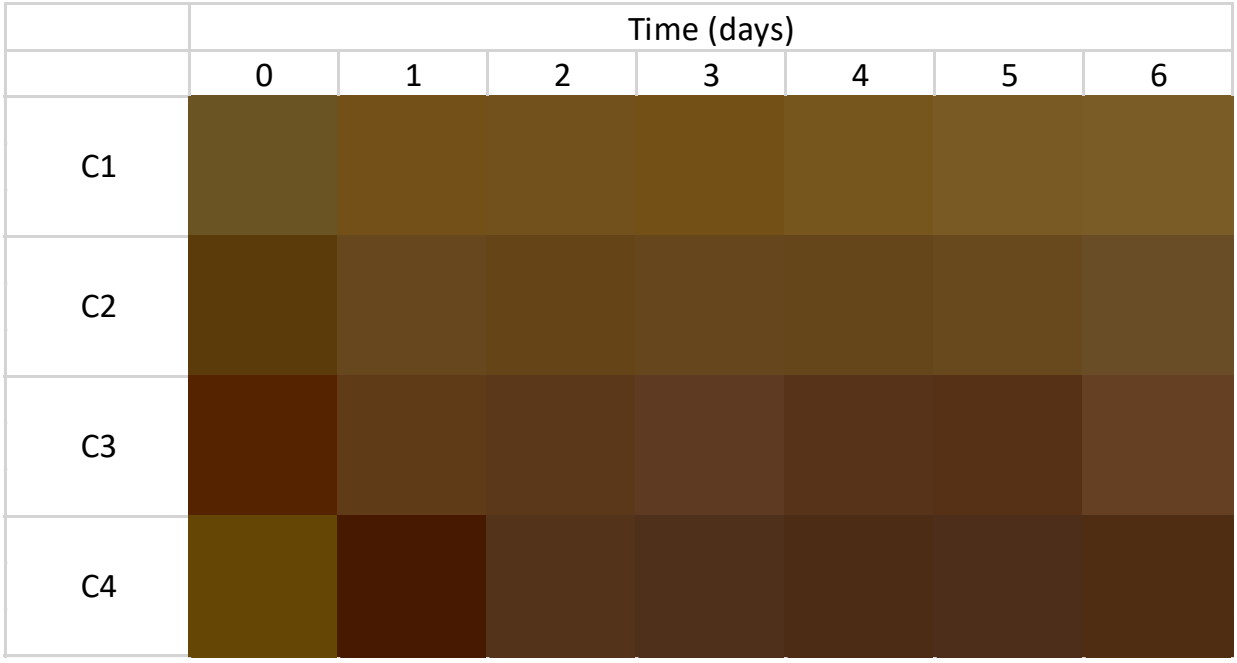


Figure 3 exhibits the color variation throughout the kinetic study.

Figure 3 - Colors obtained from the EASYRGB color calculator (EASYRGB, 2016) in cane juice stored at 10 °C in the dark. Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02).



As observed in Figures 2 and 3, the color parameters indicate that color variation in the samples extracted from cultivars C1, C2, C3 and C4 showed different behaviors. C1 exhibited the lowest variation in all parameters during storage (L^*, C*, °Hue) and consequently the lowest total color difference. Conversely, C4 had the largest total color difference. In all cases the color variation was greater during the first two days.

Figure 3 shows that color of samples clearly differed among cultivars. Samples were brown with green, yellow and reddish tones. Initially, C1 and C4 were lighter and C3 was the darkest. Juice extracted from all four cultivars changed color during storage but the intensity of change and the direction of change were different for all cultivars. The color of juice extracted from C4 darkened rapidly and after six days was much darker than the other cultivars. The peroxidase activity in C4 (Table 3) was significantly lower than that in other cultivars and the polyphenol oxidase activity was also low. This result suggests that nonenzymic mechanisms triggered juice´s browning.

Bucheli and Robinson (1994) held that PPO activity directly influences browning during juice extraction. In this work, the highest PPO activity (Table 3) was determined for C3, which initially had the darkest color. However, C1, which had the second highest PPO activity, exhibited the lightest color. Thus, the differences in color may be assigned to the concentration of phenolic compounds, carotenoids and chlorophyll that may vary between cultivars (Figure 3). Martín-Belloso and Soliva-Fortuny (2006), Marshall et al. (2000) stated that phenolic content of fruits and vegetables depend on the species, planting, maturation stage and environmental conditions. Oxygen concentration, pH and temperature also influence enzyme activity and darkening.

Filamentous fungi deteriorate cane juice, producing CO_2 and ethanol, with flocculation and biofilm formation diminishing the juice turbidity (OLIVEIRA et al., 2007). The samples herein evaluated had initial yeasts and mold counts between 3 and 5 log CFU/mL (Table 2). These microorganisms can grow during storage, and the juices will appear lighter (higher L^* values) because of the suspended solids reduction.

The comparison between the initial (t0) and final (t6) color parameters can be seen in Table 4. Color saturation also known as Chroma (C*) was determined. Low Chroma values indicate grey colors while high Chroma values, close to 60, represent vivid colors (MARINHO, 2016 and OLIVEIRA et al., 2007).

Table 4 - Comparison between initial (t0) and final (t6) color parameters in raw juice stored at 10 °C.

Color parameter	Storage (days)	C1	C2	C3	C4
L*	0	37^{Aa}±5	27.7^{Bb}±0.3	20.8^{Cb}±0.3	32.2^{ABa}±0.1
	6	41.0^{Aa}±0.6	34.9^{Ba}±0.7	30.8^{Ca}±0.4	22.3^{Db}±0.1
C*	0	32^{Ba}±2	34^{Ba}±2.	41.4^{Aa}±0.6	40.56^{Aa}±0.04
	6	35^{Aa}±2	28^{Bb}±1	28.2^{Bb}±0.9	26.6^{Bb}±0.3
°Hue	0	84^{Aa}±4	74^{Ba}±2	59.5^{Cb}±0.3	78.9^{ABa}±0.3
	6	79.0^{Aa}±0.3	74.9^{Ba}±0.4	61.1^{Ca}±0.7	61.1^{Cb}±0.5

Means followed by the same upper case exponent in the same row (comparison among cultivars) and by the same lower case exponent in the same column (comparison between storage times) are not different ($p > 0.05$) by the Tukey test. Cultivars: C1 (RB867515), C2 (SP813250), C3 (RB92579), C4 (CTC02).

Oliveira et al. (2007) determined L* values of approximately 24, relatively close to those obtained in this study. According to resuts shown in Figures 2 and 3, and Table 4, the juices from C1, C2 and C3 became lighter during storage. As for C1, the difference was not statistically significant. Only C4 showed a darker color throughout the storage. In all cases lightness tended to increase for longer storage periods which may be related to a turbidity reduction caused by growth of microorganisms. At the end of the storage, C2, C3 and C4 had significantly different lightness values ($p \leq 0.05$).

In terms of color saturation, the juice from C1 showed a slight increase in Chroma, indicating that the color became more intense (Table 4). Both a* and b* values increased, so C1 became more red and more yellow. The Chroma values decreased in C2, C3 and C4, showing less vivid colors. C2 and C3 had a decrease in a* and b* values, while a* and b* respectively increased and decreased in C4. At the beginning of the study, C1 and C2, as well as C3 and C4 were statistically similar. Nevertheless, at the end of the storage, however, only C1 differed from the others. In a study conducted by Oliveira et al. (2007), fresh cane juice showed values close to 5, indicating a neutral color. In this study, Chroma values were much greater (> 20), so the colors were much more vivid.

Regarding the ºHue (Figure 2, Table 4), the samples are located in the first quadrant (0 to 90°), that corresponds to colors varying from red-orange to yellow-orange. When freshly extracted, C1 juice color was closer to yellow, followed by C4 and C2. C3 showed a significantly lower ºHue, closer to an orange-red color. At the end of the storage, C1 and C2 did not show significant changes, maintaining a yellow-orange color. The °Hue for C3 was slightly greater indicating a shift towards yellow. The °Hue for C4 significantly decreased; the color shifted towards red as shown in Figure 2. Oliveira et al. (2007) reported °Hue close to 98° (yellow-green), notably different from those obtained in this study.

Finally, color variation in cane juice as well as its behavior during storage are triggered by the interaction of multiple factors. Physicochemical parameters, growth of microorganisms, enzyme activities, phenolic compounds content, composition of pigments among others meaningfully affect the color and the kinetics of its variation.

4 Conclusions

Sugarcane cultivar notably affected the juice´s quality regarding its Physicochemical characteristics, enzymic activity and browning reaction rates. There were significant differences among the viable microorganisms counts for the fresh juice extracted from the four cultivars. Browning was more intense in juice extracted from C4 (CTC02). C1 (RB867515) had a lighter color and its parameters did not significantly change throughout the storage, showing the best end results among the four cultivars studied. Therefore, C1 would be the most suitable cultivar for extraction of fresh sugarcane juice, exhibiting the greatest potential for consumer acceptance.

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6 CAPÍTULO 5

Contextualização

Prata (Ag) e óxido de zinco (ZnO) têm sido reportados como potenciais agentes antimicrobianos, incorporados a sistemas de embalagem. O presente estudo investigou a estabilidade de caldo de cana integral pasteurizado acondicionado em garrafas plásticas incorporadas com agentes microestruturados de Ag/ZnO. Embalagens com este perfil ativo são utilizadas no Brasil para estender o tempo de vida útil de leite pasteurizado, o que motivou a investigação do desempenho do referido sistema para caldo de cana. Assumiu-se a hipótese de que a utilização de garrafas de polietileno tratadas com Ag/ZnO ampliaria a estabilidade do caldo de cana, a exemplo do que se observa para o leite pasteurizado.

Sugarcane juice stability in plastic bottles treated with silver and zinc oxide

(RIBEIRO, A. S. C.; GOMES, J. L.; KUSHIDA, M. M.; PETRUS, R. R., 2018)

Abstract

Sugarcane juice is highly susceptible to microbial outgrowth, and the use of active packaging may enhance its preservation. In this study, the microbiological stability of pasteurized (85 °C/30 s) cane juice was evaluated. The juice was ultra clean filled into HDPE-TiO_2 bottles that had been incorporated with inorganic agents micro structured with Ag/ZnO. The processed batches were stored at 5 °C in the dark. Physicochemical, microbiological and sensory assays were conducted with both the freshly processed and stored juice to evaluate the product´s stability. The maximum thresholds set for mesophilic, psychrotrophic, and molds and yeasts counts were 5, 4 and 3 logCFU/mL, respectively. The pH values, soluble solids and titratable acidity ranged from 5.10 to 5.37, 14.3 to 24.0 °Brix, and 0.05 to 0.13% citric acid, respectively. The microbiological stability of cane juice was 50 days. The mean scores on the nine-point hedonic scale tests ranged between 5.0 and 7.7. The micro structured inorganic agents with Ag/ZnO had no influence on the microbiological stability of the end product.

Keywords**:** Active packaging, non-alcoholic beverage, small scale processing.

1 Introduction

Packaging plays a decisive role in food preservation. Originally, it was designed to act as a passive barrier to protect food products from the environment (AZEREDO, 2013). Notwithstanding, novel packaging systems have been developed to meet marketing trends. A remarkable example is active packaging, which interacts with the microenvironment and the product to extend its shelf life. Antimicrobial packaging is a promising application in this field; the incorporation of potential antimicrobial agents in the packaging material controls the deterioration of the product and its contamination by pathogens (SOYSAL et al., 2015). This technology prevents the onset of off-flavors when preservatives are directly added into the food. In contrast, the controlled release of antimicrobial agents, when incorporated into the package and in contact with food surfaces, can be significantly advantageous in extending the life life. The most commonly used inorganic antimicrobial agents in active packaging are silver (Ag) and zinc oxide (ZnO) (VAN LONG; JOLY; DANTIGNY, 2016). Of particular relevance is the claim that reducing the particle size of materials is an efficient and reliable tool for improving their biocompatibility (KIM, 2007).

Silver is used to control bacterial growth in a variety of applications, including food and beverages. Added at high concentrations, Ag^+ ions inhibit enzymic activities, reacting with electron donor groups; nevertheless, the molecular mechanism of the bactericidal effect of much lower concentrations of Ag^+ ions remains somewhat controversial (SMETANA, 2008). Silver ions and Ag-based compounds are highly toxic to microorganisms, showing strong biocidal effects (KIM, 2007). Silver ions enter the bacterial cells, are reduced, and agglomerate to form (re-form) silver metal nanoparticles inside the cell (SMETANA, 2008). Moreover, Ag^+ is reported to disrupt intracellular homeostasis and cell replication and its toxicity is related to a loss in membrane integrity due to reactive oxygen species generation (SU, 2009). Prabhu and Devarajua (2018) reported that Ag ion inhibits bacterial enzymes; it is extremely reactive and binds to the microbes and protein compounds.

In regard to ZnO, it also has antibacterial properties and is classified in a group of inorganic antimicrobial agents that are safer and extremely stable compared to organic antimicrobial agents (AMORNPITOKSUK, 2011). The dominant mechanisms of such antibacterial behavior are found to be either or both of chemical interactions

between hydrogen peroxide and membrane proteins, and chemical interactions between other unknown chemical species generated due to the presence of ZnO particles with the lipid bilayer (ASHARANI, 2008). The mechanisms of the observed antibacterial behavior of ZnO particles is the generation of radical oxygen species (ROS) and the resulting interaction between ROS and the cell. A small amount of physical damage to the cell envelope might also be induced by ZnO (ZHANG, 2010).

Cane juice is widely consumed in Brazil and Asian countries, mostly in the informal market, where the hygienic-sanitary conditions are precarious. The juice´s shelf life varies with the cultivar, processing technology, packaging system and storage conditions (ANDRADE et al., 2014).

The stability of pasteurized cane juice filled in PP packaging incorporated with nanoclay that was stored for 60 days at 28 °C was evaluated by Manikantan et al. (2013). The microbial counts exceeded 5 logCFU/mL.

Sankhla et al. (2012) studied the stability of cane juice processed at 80 °C/1 min combined with gamma irradiation (0.25, 0.5 and 1 kGy). The product was filled in glass, polyethylene terephthalate (PET), and low-density polyethylene (LDPE) bottles, and stored for 90 days at 4 °C. The glass and PET packages were more efficient in maintaining the product´s quality.

The stability of fresh orange juice and the inactivation of *Lactobacillus plantarum* in orange juice sterilized at 121 °C/15 min then filled into LDPE packaging incorporated with Ag/ZnO was evaluated by Emamifar et al. (2010, 2011). The juice was stored at 4 °C. In the first study, the authors achieved a microbiological shelf life of 28 days. As for the *Lactobacillus plantarum* inactivation, silver showed greater antimicrobial activity compared to zinc oxide (SANKHLA et al., 2012; EMAMIFAR et al., 2010). Nevertheless, the use of Ag/ZnO-HDPE bottles for cane juice has not yet been investigated.

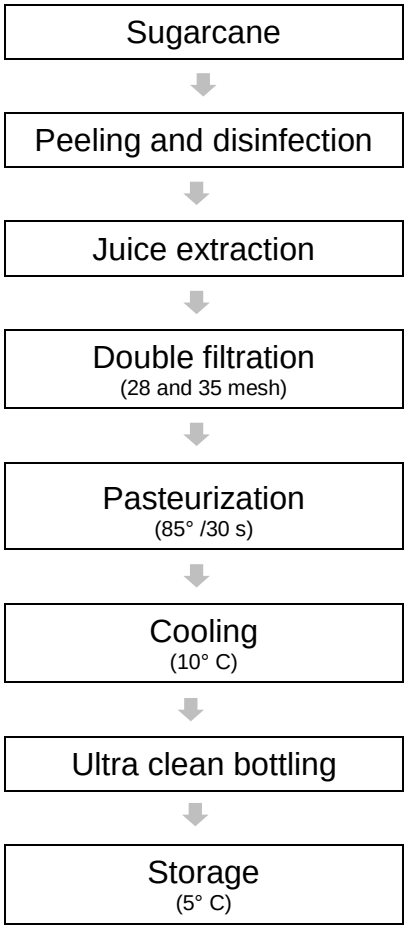
Due to the dearth of published data on the cane juice stability, packaged in potentially antimicrobial packaging, the major objective of this study was to evaluate the microbiological stability of pasteurized and refrigerated cane juice that was filled into HDPE bottle incorporated with micro structured Ag/ZnO agents.

2 Material and methods

2.1 Processing cane juice

The sugarcane (*Saccharum* spp.) RB867515 was donated by Baldin Bioenergia S/A, located in the city of Pirassununga/SP, Brazil. A few hours after harvest, the raw material was transported for approximately 5 km to the pilot plant of the Department of Food Engineering at the University of São Paulo, where it was processed. The cane was sorted, cut, scraped and decontaminated by immersion in 0.05% (v/v) peracetic acid solution for 30 minutes at approximately 25 °C. The juice was extracted in an electric stainless steel cylinder mill manufactured by Maqtron (Joaçaba/SC, Brazil). The whole juice was double filtered in 28 and 35 mesh stainless steel sieves, pasteurized at 85 °C/30 s and cooled to 10 °C. The pasteurized juice was filled into both conventional HDPE-TiO_2 and HDPE-TiO_2-Ag/ZnO bottles and stored at 5 °C in the dark. Three batches (replicates) of approximately 60 L each were processed. Figure 1 exhibits the flowchart of processing.

Figure 1 - Flowchart of cane juice processing.



2.2 Ultra clean filling

The ultra clean bottling was conducted in a semi-automatic gravimetric machine made by Polienva Movitron (São Paulo/SP, Brazil), in a unidirectional air-flow cabinet (ISO class 5) manufactured by VECO (Campinas/SP-Brazil).

2.2.1 Packaging

The 1 L-HDPE/TiO_2 bottles (conventional and Ag/ZnO treated) and LDPE caps were donated by Letti Agrindus S/A (Descalvado/SP). The microstructured Ag/ZnO agent (0.2 µm), at a concentration of 0.3% w/w, is supported on silica particles whose effect is enhanced by the catalytic action of the material. The agent is incorporated in

HDPE resin during the extrusion step. Figure 2 shows the packages used in this research work.

Figure 2 - Bottles used for cane juice packaging.

The microparticles, produced by Nanox Intelligent Materials (São Carlos/SP), are mixed as a powder in the polyethylene matrix and used to shape the plastic bottles by injection or blow, and are inert. The particles do not detach themselves from the package nor come into contact with the product. The technology consists of the deposition of silver particles on a micrometric scale on the surface of ceramic particles (silica). The adhesion of the silver particles to the ceramic matrix results in a composite (formed by two different types of materials) in micrometric scale and with potential bacterial property. The effect of combining the silver particles with the ceramic matrix is synergistic. Silver has bactericidal properties; silica does not, but it potentiates this action and helps control the release of silver particles to kill bacteria (FAPESP, 2015).

Table 1 exhibits the characteristics of the plastic bottle used in this research work.

Table 1 - Characterization of 1-L HDPE-TiO_2 bottle.

	Conventional	Incorporated with Ag/ZnO
Mass (g)	37.26	41.21
Thickness (mm)		
Neck	1.43	1.40
Shoulder	0.50	0.56
Body	0.50	0.55
Heel	0.53	0.49
Opacity (%)	57.5	67.8

2.3 Physicochemical characterization

The Physicochemical assays were performed following the protocol of the Association of Official Analytical Chemists (AOAC, 2000). The pH was measured in a pHmeter (Tecnopon model MPA-2010). The soluble solids content (expressed in °Brix) was determined in a digital refractometer (model Reichert AR- 200). The titratable acidity (expressed as % citric acid) was determined by quantifying the volume of 0.1M NaOH previously standardized until pH of the juice reached 8.3.

2.4 Microbiological evaluation

Counts of total aerobic mesophiles, psychrotrophs, molds and yeasts were carried out according to the Compendium of Methods for the Microbiological Examination of Foods (DOWNES, 2001). Analyses of coliforms and *Salmonella* spp. were conducted to assure the juice´s safety. To verify the presence of *Salmonella*, the rapid BAX System method by Du Pont was used. The procedures followed the protocol described by the manufacturer's guidelines; the end results are qualitative (presence/absence). The 3M Petrifilm™ was used for quantification of *E. coli* and coliform organisms, as directed by the manufacturer, and endorsed by AOAC.

2.5 Criteria to estimate the shelf life

2.5.1 Microbiological shelf life

Table 2 points out the microbiological criteria set in the shelf life study, based on Kilcast and Subramaniam (2000).

Table 2 - Maximum limits for microbial counting in cane juice.

Group of microorganism	Acceptable treshhold (log CFU/mL)
Aerobic mesophiles	5
Aerobic psychrotrophs	4
Molds and yeasts	3

2.5.2 Sensory shelf life

The sensory acceptance of the end product was evaluated based on sensory results of a panel consisting of 120 habitual consumers (age of 22 years on average) of cane juice. The assessors were asked to evaluate the appearance, flavor and overall impression by assigning a liking score on a 9-point hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely) (MEILGAARD; CIVILLE; CARR, 1999) Tests were accomplished in individual booths lighted with a white fluorescent lamp. Samples of juice were individually served at a temperature of approximately 10 °C in 50 mL plastic cups labeled with a 3-digit code. One sample, obtained from the same production batch and stored at -18 °C then naturally thawed in refrigerator 24 h before the sensory analysis began, was taken to serve as control. Mineral water was provided to cleanse the palate. This study was approved by the Ethics in Research Committee from the College of Animal Science and Food Engineering of the University of São Paulo (Report 66177916.3.0000.5422).

2.6 Statistical analysis

The data analysis from the three juice batches (replicates) was performed using the Sisvar program version 5.6. Analysis of variance (ANOVA) and Tukey's honest significant difference test (95% significance level) were carried out to check the difference among means.

3 Results and discussion

The results collected from the three batches of juice represent the replicates of processing and shelf life study.

3.1 Physicochemical parameters

The results from the Physicochemical analysis of both raw and processed juice are pointed out in Table 3.

Table 3 - Physicochemical parameters determined in raw and freshly processed cane juice (cultivar RB867515).

Batch	pH		Soluble solids (°Brix)		Titratable acidity (% citric acid)	
	R	P	R	P	R	P
1	$5.15^{Bc} \pm 0.02$	$5.18^{Ab} \pm 0.01$	$17.9^{Ac} \pm 0.1$	$17.6^{Bc} \pm 0.0$	$0.05^{Bb} \pm 0.01$	$0.07^{Aa} \pm 0.00$
2	$5.37^{Aa} \pm 0.01$	$5.37^{Aa} \pm 0.01$	$19.8^{Ab} \pm 0.1$	$19.5^{Bb} \pm 0.0$	$0.08^{Aa} \pm 0.00$	$0.08^{Aa} \pm 0.00$
3	$5.18^{Ab} \pm 0.00$	$5.18^{Ab} \pm 0.00$	$24.4^{Aa} \pm 0.0$	$24.0^{Aa} \pm 0.0$	$0.07^{Aa} \pm 0.00$	$0.07^{Aa} \pm 0.00$

R - raw juice. P - pasteurized juice. Mean values followed by the same upper case exponent in the same row (comparison between raw and pasteurized juice), and same lower case exponent in the same column (comparison among batches) are not different ($p > 0.05$).

The pH values of the raw juice ranged from 5.15 to 5.37. Despite presenting a statistically significant difference, the results were close to each other. Comparing the raw with the pasteurized juice, only batch 1 showed significant difference between samples. As for the soluble solids content, the raw juice exhibited a wide variation (17.9 to 24.4 °Brix). Because the experimental assays were run from April through September 2017, the raw material composition was affected by the climatic conditions. Although statistically different (batches 1 and 2), variations after processing were not relevant. Regarding the titratable acidity, the processing did not affect this parameter, remaining in the same range for the three batches. Saxena, Makroo and Sristava (2016) reported pH values and soluble solids for raw cane juice of 4.42 and 19.6 °Brix, respectively. In a study conducted by Ramachandran et al. (2017) on the optimization of cane juice's shelf life, the pH value was 5.2. Sanda et al. (2016) reported a pH of 5.28. These results are similar to those found in this study. Titratable acidity results

for raw juice greater that those found in this work (0.1 and 0.2%) were reported by Saxena, Makroo and Sristava (2016), and Sanda et al. (2016), respectively. Xiao, Liao and Guo (2017) evaluated some quality indexes of cane juice from March through December. The soluble solids values reported for the processed juice were lower (15.4 to 16.8 °Brix) than those from this study. Kayalvizhi et al. (2016) reported pH values in the range of 3.98 to 5.10. In regard to soluble solids the results were between 17 to 17.5 °Brix.

3.2 Microbiological evaluation of the processed juice

3.2.1 Pathogens analysis

Salmonella spp. was not detected in any juice samples. Thermotolerant coliforms counts were lower than 10 CFU/mL in all samples. These results are in compliance with Brazilian Regulation (BRASIL, 2001).

3.2.2 Spoilage microorganisms

The counts of aerobic mesophiles, psychrotrophs, and yeasts and molds are demonstrated in Table 4.

Table 4 - Microbial counts (log CFU/mL) in cane juice.

Batch	Aerobic mesophiles			Aerobic psychrotrophs			Molds and yeasts		
	R	P	(DR)	R	P	(DR)	R	P	(DR)
1	6.8	1.7	5.1	4.6	1.2	3.6	4.8	$< 1_{est}$	> 3.8
2	5.1	1.4	3.7	4.9	$< 1_{est}$	> 3.9	3.9	$< 1_{est}$	> 2.9
3	4.5	1.0	3.5	4.1	$< 1_{est}$	> 3.1	4.0	$< 1_{est}$	> 3

R – raw juice. P – freshly pasteurized. DR – decimal/log reduction. est – estimate count.

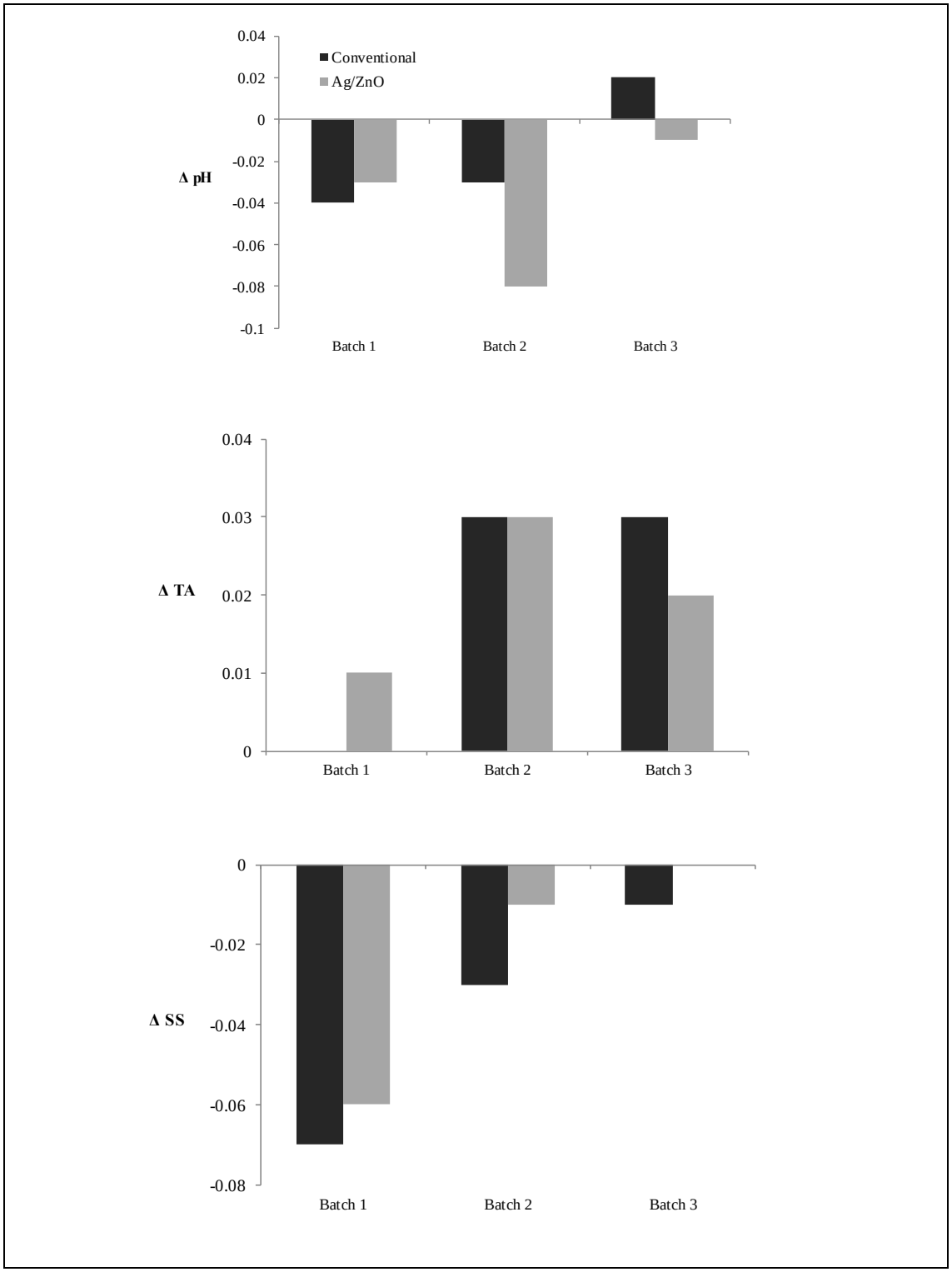
The results of Table 4 indicated that the treatment at 85 °C/30 s was relatively efficient in reducing the potential spoilage contaminants in the freshly extracted juice.

3.3 Shelf life study

3.3.1 Physicochemical tests

Figure 3 demonstrates small variations in the Physicochemical parameters during the shelf life study. The ∆values represent the differences between the parameter measured at the beginning and the end of the storage at 5 °C.

Figure 3 - Total variation in pH (ΔpH), titratable acidity (ΔTA) and soluble solids (ΔSS) during storage of cane juice in conventional and Ag/ZnO-HDPE bottles.



Despite some differences between conventional and Ag/ZnO bottles, no relevant variations in all parameters were observed throughout the storage period. The pH value, soluble solids content and titratable acidity may be used as deterioration rate indicators, because variations in these parameters may indicate the growth of microorganisms (ANDRADE et al., 2014). Manikantan et al. (2017) reported decreases in pH and soluble solids of pasteurized cane juice filled into packaging incorporated with nanoclay, after 60 days of storage. The results showed a reduction in the soluble solids values from 19.4 to 18.5 °Brix and pH from 5.18 to 5.05. The acidity values of the cane juice in both types of packaging varied between 0.05 and 0.13% citric acid. Yasmin, Massod and Abid (2010) also reported variations in the acidity of cane juice (0.6 to 0.8% citric acid) after 120 days of storage. In general, the Physicochemical parameters did not significantly change over time.

3.3.2 Microbiological assays

Table 5 exhibits the results from the microbiological assays conducted during the shelf life study. The values in bold indicate counts that exceeded the previously set limits.

Table 5 - Counts (log CFU/mL) in pasteurized cane juice filled in HDPE bottles.

Batch	Storage (days)	Mesophiles		Psychrotrophs		Molds and yeasts	
		Conv	Ag/ZnO	conv	Ag/ZnO	conv	Ag/ZnO
1	0	1.7^{Ac}	1.7^{Ac}	$< 1_{est}$	$< 1_{est}$	1.2^{Ac}	1.2^{Ab}
	30	2.5^{Ab}	2.0^{Bb}	1.6^{Ab}	1.0^{Bb}	1.5^{Ab}	1.2^{Bb}
	55	3.8^{Aa}	3.5^{Ba}	3.8^{Aa}	3.8^{Aa}	$\mathbf{4.0}^{Aa}$	$\mathbf{4,0}^{Aa}$
2	0	1.4^{Ac}	1.4^{Ac}	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$
	30	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$
	48	1.4^{Ac}	1.3^{Ac}	$< 1_{est}$	$< 1_{est}$	1.0^{c}	$< 1_{est}$
	60	2.9^{Ab}	1.8^{Bb}	3.0^{Ab}	1.2^{Bb}	2.0^{Ab}	1.7^{Bb}
	75	4.5^{Aa}	4.0^{Ba}	$\mathbf{4.4}^{Aa}$	$\mathbf{4.4}^{Aa}$	2.2^{Aa}	2.0^{Ba}
3	0	1.0^{Ac}	1.0^{Ab}	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$
	30	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$	$< 1_{est}$
	48	1.0^{Ac}	1.0^{Ab}	2.1^{Ac}	1.0^{Bc}	$< 1_{est}$	$< 1_{est}$
	60	1.7^{Ab}	1.0^{Bb}	2.5^{Ab}	1.9^{Bb}	1.8^{Ab}	1.0^{Bb}
	77	3.0^{Aa}	2.2^{Ba}	$\mathbf{4.3}^{Aa}$	$\mathbf{4.1}^{Ba}$	2.1^{Aa}	2.0^{Aa}

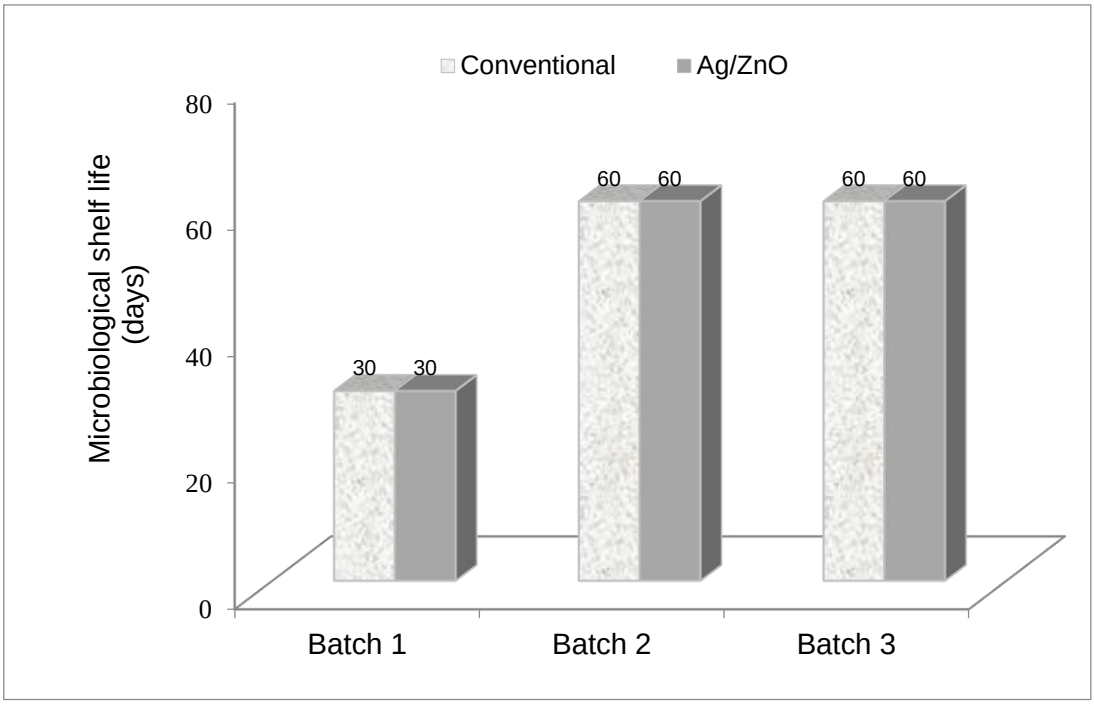
Conv - conventional bottle. Means followed by the same upper case exponent in the same raw (comparison between conventional and Ag/ZnO bottles) and same lower case exponent in the same column (comparison among storage times) are not different ($p > 0.05$).

The rate of the microbial growth in batch 1 was notably greater than batches 2 and 3, limiting the juice's shelf life to 30 days, in both types of packages. As for the batches 2 and 3, the microbiological shelf life achieved 60 days in both bottles. At some points, the counts in Ag/ZnO bottle were low and exhibited statistical differences compared to conventional bottle. For example, in batch 1, the Ag/ZnO-bottle showed a better performance, inhibiting the microbial growth until 30 days; however, at the end of the storage counts for both types of packaging remained close. Despite this observation, in terms of microbial counts those differences are not meaningful because they were all around to 0.5 log cycle.

Rodrigues and Han (2003) state that microbiostatic agents can inhibit the growth of microorganisms above a certain critical concentration. Neverthless, when the concentration is lower than the critical level, the suppressed microorganisms can grow. It is therefore critical to maintain the concentration of the antimicrobial agent above the critical value during the entire shelf life. In this study, the concentration of Ag/ZnO (0.3% w/w) may not have been high enough to inhibit the growth of spoilage microorganisms in whole cane juice.

Manikantan et al. (2017) obtained mesophiles count equal to 6.8 log CFU/mL in cane juice bottled in a package with nanoclay, after 60 days under refrigerated storage. This count was greater than that of this study. Figure 4 depicts the estimated microbiological shelf life for the three batches.

Figure 4 - Microbiological shelf life of pasteurized cane juice filled in HDPE-TiO_2 bottles stored at 5 °C in the dark.



As represented in Figure 4, the shelf life of batch 1 was limited to 30 days, which was considerably shorter than batches 2 and 3 (60 days). This result may be explained by the initial microbial counts in batch 1, which were greater than batches 2 and 3, mainly for psychrotrophs and molds and yeasts. This affects the product`s stability. As for batch 1, the psychrotrophic counts exceeded the maximum limit (4 log CFU/mL) after 55 days of storage. Therefore, the best that can be said is the juice in conventional HDPE-TiO_2 and Ag/ZnO bottles reached a microbiological shelf life of 50 days. This is a good achievement since sugar cane juice is highly susceptible to microbiological spoilage. A fact also worth highlighting is that no chemicals were added into the juice. However, the findings did not confirm the potential antimicrobial action of the Ag/ZnO microparticles incorporated in the HDPE-TiO_2 bottles, under the conditions of this research work.

3.3.3 Sensory tests

The results from the hedonic scale tests are shown in Table 6. The highlighted values point out means and percentages of acceptance which did not meet the pre-set threshold limits (cut-off point). The tests were stopped when the mean score for any attribute was less than or equal to 5 and/or the acceptance less than 60%.

Table 6 - Mean scores/percentages of acceptance[1] obtained in the nine-point hedonic scale tests for cane juice in HDPE-TiO_2 bottles during storage at 5 °C.

Batch	Storage (days)	Appearance		Flavor		Overall Impression	
		conv	Ag/ZnO	conv	Ag/ZnO	conv	Ag/ZnO
1	0	6.0^{a} / 67		7.5^{a} / 88		7.0^{a} / 85	
	30	**5.0**^{Bb} / **50**	6.0^{Aa} / 61	7.0^{Ab} / 76	6.0^{Bb} / 62	6.0^{Ab} / 75	6.0^{Ab} / 61
2	0	7.3^{a} / 87		7.7^{a} / 90		7.5^{a} / 90	
	30	6.8^{Ab}/ 83	6.7^{Ab}/ 77	6.9^{Ab} / 84	6.8^{Ab}/ 80	6.8^{Ab}/ 80	6.8^{Ab} / 84
3	0	5.6 / **53**		7.7/ 94		7.2/ 93	

Conv - conventional bottle. Means followed by the same upper case exponent in the same row (comparison between conventional and Ag/ZnO bottles) and same lower case exponent in the same column (comparison among storage times), for each batch separately, are not different ($p > 0.05$). [1]Percentage of assessors who assigned scores greater than 5.

In regard to batch 1, the mean scores for juice in Ag/ZnO-bottles ranged from 5 (“neither like/nor dislike”) to 7.5 ("like moderately") after 30 days. As for the batch 2, the scores were slightly greater, surviving until 30 days, when formation of gums occurred. This event might be due to the lactic bacteria growth in the raw material, which produces polysaccharides and increases the juice´s viscosity. At this point, the sensory analysis was stopped.

The juice´s appearance in batch 3 was determined to be a negative factor, jeopardizing its acceptability. These results may be most likely due to the large variability inherent to the raw material quality, which was obtained in different seasons of the year. The enzymic browning of the cane juice depends on the concentration of phenolic compounds and the polyphenol oxidase and peroxidase activities (KUNITAKE et al., 2014). Another browning mechanism is the Maillard reaction forming dark pigments (melanoidines). The caramelization and oxidation of chlorophyll also affect the juice´s color leading to its rejection (PRATI; MORETTI, 2010). The three batches, in both conventional and Ag/ZnO bottles, achieved high scores for flavor and overall impression.

By comparing Tables 5 and 6, one may conclude that the sensory acceptability limited the juice´s shelf life. The microbiological shelf life reached 60 days (for batches 2 and 3); conversely, the longer sensory shelf life was 30 days. As a whole, cane juice filled in both types of bottle achieved good scores for overall impression after 30 days of storage. Statistical difference was observed between means in t0 and t30 for batches 1 and 2. As for the packages, no significant difference was found.

Manikantan et al. (2017) reported scores ranging from 6.6 to 7.2 for overall impression of cane juice filled into flexible PP packages incorporated with nanoclay. The juice was stored during 30 days at approximately 28 °C. Further comparison of data from different sources has been made difficult due to a lack of standard experimental conditions.

4 Conclusions

The findings of this study did not confirm the antimicrobial potential of the micro structured Ag/ZnO incorporated in the HDPE bottles for pasteurized cane juice stored under refrigeration. However, a microbiological shelf life of 50 days, on average, was achieved. In terms of flavor and overall impression, the juice had a good acceptance; nevertheless, the appearance limited the end product's shelf life. Although the Ag/ZnO microparticles have had no effect on the juice´s stability, the combination of preservation methods (heat treatment, ultra clean bottling and refrigeration storage) was successful under the conditions of this research work.

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Os dois capítulos/artigos subsequentes foram elaborados a partir de dados de ensaios experimentais conduzidos no *High Pressure Processing (HPP) Validation Center* do *Department of Food Scicence and Technology* da *Cornell University/USA*.

Dentre os métodos emergentes não térmicos para processamento de alimentos, a tecnologia de altas pressões é atualmente a mais difundida em escala mundial. Muitas indústrias norte-americanas têm empregado altas pressões no processamento de sucos de fruta, visando, sobretudo, a maior preservação da qualidade sensorial, nutricional e funcional da bebida.

7 CAPÍTULO 6

Contextualização

Tendo em vista o limitado volume de trabalhos investigando a aplicação otimizada da tecnologia de processamento a alta pressão (HPP) na inativação de bactérias patogênicas em sucos de fruta, o estudo apresentado a seguir foi conduzido com o objetivo de otimizar a pressão e o tempo de residência para redução de *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* inoculados em suco de uva tinta. Embora a aplicação da tecnologia de alta pressão tenha demonstrado eficiência na destruição de células vegetativas de micro-organismos patogênicos, estudos reportando a inativação de patógenos de referência em suco de uva *Concord* não foram localizados na literatura.

Uma vez que os custos de manutenção dos equipamentos de alta pressão aumentam exponencialmente a partir de 600 MPa, a otimização deste parâmetro é altamente desejável. Pretendeu-se por meio deste estudo avaliar se patamares inferiores de pressão são efetivos na destruição de patógenos inoculados em suco de uva tinta.

Searching for high pressure processing parameters for *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* reduction in Concord grape juice

(PETRUS, R. R.; CHUREY, J. J.; WOROBO, R. W, 2020)

Abstract

High pressure processing (HPP) has been widely used for high-acid (pH ≤ 4.6) juices. This study was designed to investigate optimal parameters aimed at achieving at least 5-log reduction of pathogens of reference in Concord grape juice (pH 3.39). Juice samples were inoculated with five strain cocktails of *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*, separately. Eleven trials were carried out based on a central composite rotational design (CCRD). Pressures (P), ranging from 319 to 531 MPa, and dwell times (t), from 35 to 205 s, were tested. The performance of the combinations (*P* x *t*) was evaluated by pathogen challenge microbiological assays. *E. coli* O157:H7 was more resistant to HPP than *S. enterica*. *L. monocytogenes* did not grow in unprocessed juice (before HPP). Findings demonstrated that moderate pressures (~ 400 MPa) and short dwell times (~ 2 min) were effective in achieving a greater than 5-log reduction in the pathogens of reference. The results of this study can supplement the dearth of information on the applicability of high pressure as a Concord grape juice processing technology in terms of the pathogens inactivation.

Keywords: Emerging technology, food safety, challenge test, factorial design.

1 Introduction

Fruit juices are commonly pasteurized by employing heat methods. However, non-thermal technologies have been broadly investigated to preserve both sensory and nutritional quality of the product (CHEN et al., 2013). Among these technologies, the high pressure processing (HPP) has become a success in the processing of fruit juice (KNORR et al., 2011, BARBA et al., 2012), Once the raw fruit thermolabile delicate aromas and flavors are not affected by pressure as they are by thermal processing (OEY et al., 2008).

Acid fruit juices are a substrate in which foodborne pathogens can maintain their viability; several foodborne illness outbreaks involving juices have been documented (USFDA, 2004). *Escherichia coli* O157:H7 and *Salmonella enterica*, acid-resistant pathogens, are primarily involved in fruit juice-borne outbreaks (Park et al., 2017). The USFDA (2004) states that high-acid juices (pH ≤ 4.6) containing enteric bacterial pathogens such as *E. coli* O157:H7 and *Salmonella* spp. have caused serious foodborne illness outbreaks. Although outbreaks of foodborne illnesses linked to *Listeria monocytogenes* have not occurred in fruit juices, the National Advisory Committee on Microbiological Criteria for Foods has suggested that *L. monocytogenes* should be categorized as a target bacterium (RAJAURIA; TIWARI, 2018).

HPP is effective in reducing vegetative bacterial pathogens; it relies on the application of very high pressures (100–600 MPa) to the food/beverage, to inactivate spoilage andpathogenic microorganisms and guarantee the safety, as well as extend the shelf life of the product (USFDA, 2004). As long as no heat or mild heat (temperature ≤ 40 °C) is supplied, most of the original food sensory, nutrient, and functional properties are retained after processing (SILVA et al., 2012). Kan et al. (2017) held that food products with a high-acid content are particularly favorable candidates for HPP technology.

Although there have been several published studies about the high pressure inactivation of pathogens in fruit juices, there is a dearth of published data about the optimized use of HPP on a wide spectrum of pathogens, such as is addressed in this research work. The juices are usually processed at 600 MPa; nevertheless, the findings of this investigation indicate that a lower pressure may be used to kill or reduce the pathogens of reference to safe levels. The maintenance costs of the equipment

increases with pressure, so significant reductions in process pressure requirements equate to lower production costs and are therefore highly desirable. The novel approach presented herein is that relatively moderate pressures and shorter dwell times may be effectively employed to reduce pathogens in Concord grape juice. Notwithstanding, the effect of HPP on spoilage microorganisms in Concord grape juice should also be investigated. This study broadens the scope and possibilities for HPP parameters to provide improved knowledge in Concord grape juice processing techniques where safety is maintained at a potentially lower cost of production.

2 Materials and methods

This research work was conducted at the Cornell High Pressure Processing Validation Center. The facility includes the processing area and the biohazard level 2 laboratory at the Food Science Building at Cornell AgriTech, in Geneva, NY/USA.

The strains selected for this study as well as their origin were (1) *E. coli* O157:H7 (C7927/ human isolate, apple cider linked to an outbreak, ATCC 43890/human isolate, ATCC 43894/human, ATCC 43889/human and ATCC 35150/human); (2) *S. enterica* (Hartford H0778/orange juice, Typhimurium FSL R9-5494/orange juice, Muenchen FSL R9-5498/alfalfa sprouts, Javiana FSL R9-5273/tomatoes and Enteriditis FSL-R9-5505/beans sprouts); (3) *L. monocytogenes* (Lineage I serotype 4b FSL J1-108/coleslaw, Lineage I serotype 4d FSL J1-107/coleslaw, Lineage II serotype 1/2a FSL R9-0506/cantaloupe, FSL R9-5411/caramel apple and FSL R9-5506/packaged salad). The pathogen cocktails were selected on their produce related outbreaks, which best matches the food matrix being tested. The procedures for cultivation, inoculation, and enumeration are as follows.

2.1 Inoculum preparation

Frozen stocks of pathogenic *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* were kept at the biosafety level 2 laboratory at - 80 °C until used. Thawed stocks were reactivated by streaking onto trypticase soy agar (TSA) plates and incubated overnight at 36 ± 1 °C prior to use. After the streaked culture was grown, the plate was placed at 4 °C. A single isolated colony of each pathogen strain was separately transferred into 10 mL of trypticase soy broth (TSB) and incubated for 24 h at 36 ± 1 °C under shaking

(175 rpm). This procedure was carried out for all pathogens. A loop of the inoculated TSB was then transferred into a volume of 10 mL-TSB (pH 5.0) and incubated for 24 h at 35 ± 1 °C under shaking (175 rpm). After incubation, cells were concentrated by centrifugation at 10,000 rpm for 2 min using an Eppendorf 5415 C microcentrifuge (Eppendorf, Hamburg, Germany) and resuspended in 1 mL of citrate buffer (pH 4.0) diluent. A cocktail of five strains/serotypes of each pathogen was prepared by equivalent volumes of cells being combined and serving as the cocktail inoculum for the grape juice.

2.2 Food matrix

As food matrix, 2.8 L-PET bottles of shelf stable preservative free, 100% Concord grape juice (Welch´s), were procured from local supermarket in Geneva, NY. The juice was inoculated with a five strain cocktail of each of the three pathogens, separately.

2.3 Sample inoculation and processing

The HPP containers (66 mL-PET bottles) were filled with the product leaving a maximum headspace of 5%. The volume of 0.5 mL of each cocktail, required for a target concentration of approximately 10^7 CFU/mL of juice, was inoculated into each container of product. The inoculation was performed in the BL2 safety cabinet and a sample was aseptically collected to determine the initial cell count (N_0) in the juice as a positive control. After sampling, containers were immediately sealed and repackaged independently into a plastic bag. Bags were sealed using a vacuum sealer and placed in a second plastic bag with chlorinated solution that was also sealed using the vacuum packaging machine and finally packaged in a third bag. The duration between inoculation and HPP treatment was 1 h, approximately.

Samples were then placed in the HPP unit and subjected to the processing conditions indicated by experimental design (Tables 1 and 2). The pressure, dwell time, and water temperature were recorded. The HPP unit used was a Hiperbaric 55 L commercial processing unit. It has a water chiller to maintain the chamber water temperature during the HPP cycle at a temperature of approximately 5 ˚C. Once the HPP cycle was finished, the samples were immediately analyzed for the respective pathogens.

2.4 Colony enumeration

Serial dilutions of each sample were done using 0.1% sterile peptone water. One (1) mL of each dilution was pour plated in petri dishes with violet red bile agar (Alpha Biosciences™) for *E. coli*, bismuth sulfite agar (Criterion™) for *Salmonella*, and Oxford Listeria Agar Base (Alpha Biosciences™) for *L. monocytogenes*. Upon agar solidification, the Petri dishes were inverted and incubated for 48 h at 36 ± 1 °C. The colonies were enumerated using a Quebec colony counter, and the level of microbial reduction was determined.

2.5 Experimental design

Table 1 exhibits the levels used in the central composite rotational design (CCRD), carried out according to Rodrigues and Iemma (2012).

Table 1 - Coded and actual values used in the CCRD for reducing foodborne pathogens inoculated in Concord grape juice.

Variable	code	-1.41 ($-\alpha$)	-1	0	+1	+1.41 ($+\alpha$)
Pressure (MPa)	x_1	319	350	425	500	531
Dwell time (s)	x_2	35	60	120	180	205

($-\alpha$) lower axial point, (-1) lower level, (0) central point, (+1) upper level, ($+\alpha$) upper axial point. $\alpha = (2^n)^{1/4} = 1.41$. n = 2 (number of independent variables).

2.6 Data analysis

Data from the experimental design were processed through response surface methodology (RSM). The analysis of regression was firstly performed for both 1st (responses obtained only from central points) and 2nd (responses including axial points) orders, at 10% of significance. Due to the high variability of processes involving microorganisms, p-values below 10% ($p \leq 0.1$) were considered significant parameters, based on Rodrigues and Iemma (2012). Then the mathematical model was re-parameterized only considering the statistically significant coefficients. The analysis of variance was performed to evaluate if the model was statistically significant. If so, the response surface was built. Statistical tests were performed using the software Protimiza Experimental Design (experimental-design.protimiza.com.br).

3 Results and discussion

Table 2 shows the numbers of log reductions achieved by different binomials in the cocktails of *E. coli* O157:H7 and *S. enterica* inoculated in Concord grape juice (pH 3.39, a_w 0.99, 16.5 °Brix). The binomials that attained log reductions greater than 5 in both pathogens cocktails are highlighted.

Table 2 - Matrix of the experimental design and responses obtained from the shelf stable Concord grape juice inoculated with foodborne pathogens cocktail.

Trial	Independent variable		Response (log reduction)	
	Pressure (MPa)	Dwell time (s)	*E. coli* O157:H7	*S. enterica*
1	350 (-1)	60 (-1)	3.2	2.8
2	**500 (+1)**	**60 (-1)**	**6.6**	**7.6**
3	350 (-1)	180 (+1)	4.1	7.6
4	**500 (+1)**	**180 (+1)**	**6.6**	**7.6**
5	319 (-α)	120 (0)	3.6	3.2
6	**531 (+α)**	**120 (0)**	**6.6**	**7.6**
7	425 (0)	35 (-α)	4.6	3.7
8	**425 (0)**	**205 (+α)**	**6.6**	**7.6**
9	**425 (0)**	**120 (0)**	**6.6**	**7.6**
10	**425 (0)**	**120 (0)**	**6.6**	**7.6**
11	**425 (0)**	**120 (0)**	**6.6**	**7.6**

(-α) lower axial point, (-1) lower level, (0) central point, (+1) upper level, (+α) upper axial point. $\alpha = (2^n)^{1/4} = 1.41$. n = 2 (number of independent variables). Initial counts (log CFU/mL) – *E. coli*: 6.6 *S. enterica*: 7.6 *L. monocytogenes*: 0

A thought provoking finding is that *L. monocytogenes* did not show growth in any sample of grape juice, either before or after processing. Ghasemi at al. (2017) reported that grape pulp has an inhibitory effect on *L. monocytogenes*, and Kan et al. (2017) highlighted that the pressure resistance of *L. monocytogenes* exhibits significant variability. Silva et al. (2014) evaluated the antimicrobial activity of *Myrciaria cauliflora* (jabuticaba – a native Brazilian black fruit) extract and found that it inhibited

the growth of *L. monocytogenes*. Jabuticaba peels are a source of anthocyanins, the flavonoids responsible for the dark color of the fruit. These pigments possess potent antioxidant and anti-inflammatory compounds, and antimicrobial property as well. Concord grape is also an important source of anthocyanins and this is more likely to explain the inhibition of *L. monocytogenes* in grape juice, as found in this study.

As exhibited in Table 2, trials 1, 5 and 7 did not achieve 5-log reduction in both *E. coli* O157:H7 and *S. enterica* cocktails. Trial 3 (350 MPa/180 s) was effective only for *S. enterica*. This result suggests that *E. coli* O157:H7 was more resistant to HPP than *S. enterica*. The USFDA (2004) holds that *E. coli* O157:H7 is one of the most resistant microorganism to HPP of public health significance that is likely to occur in high-acid juice.

Mañas and Mackey (2004) studied changes induced by HPP in cells of *E. coli*. The authors proposed that exponential-phase cells are inactivated by irreversible damage to the cell membrane. In contrast, stationary-phase cells have a cytoplasmic membrane that is robust enough to withstand pressurization up to very intense treatments. The retention of an intact membrane appears to allow the stationary-phase cell to repair gross changes in other cellular structures and to remain viable at pressures that are lethal to exponential-phase cells.

The effect of HPP on bacterial survival is influenced by a number of interacting factors such as pressure level and duration of the treatment, temperature, bacterial species, and the bacterial development phase (SMELT et al., 2001). In this work, the temperature was kept at approximately 5 °C throughout processing.

Chang et al. (2017) investigated the impact of HPP on the microbial reduction in white grape juice. The initial populations of mesophiles, coliforms, molds and yeasts in the untreated juice were 3.5, 2.1 and 2.2 log CFU/mL, respectively. HPP at 300 MPa for 3 min resulted in a log reduction of 1.0, 0.6 and 1.1 in mesophiles, coliforms, molds and yeasts, respectively. Conversely, HPP at 600 MPa for 3 min resulted in a reduction in mesophiles by more than 2 log CFU/mL. Nevertheless, the HPP effect on pathogens was not investigated by those authors.

Yuste et al. (2004) studied the inactivation of foodborne pathogens by HPP. Cell suspensions (~ 4 log CFU/mL) were treated by combining different pressures and dwell times until complete inactivation. *S. typhimurium* and *E. coli* were totally inactivated at

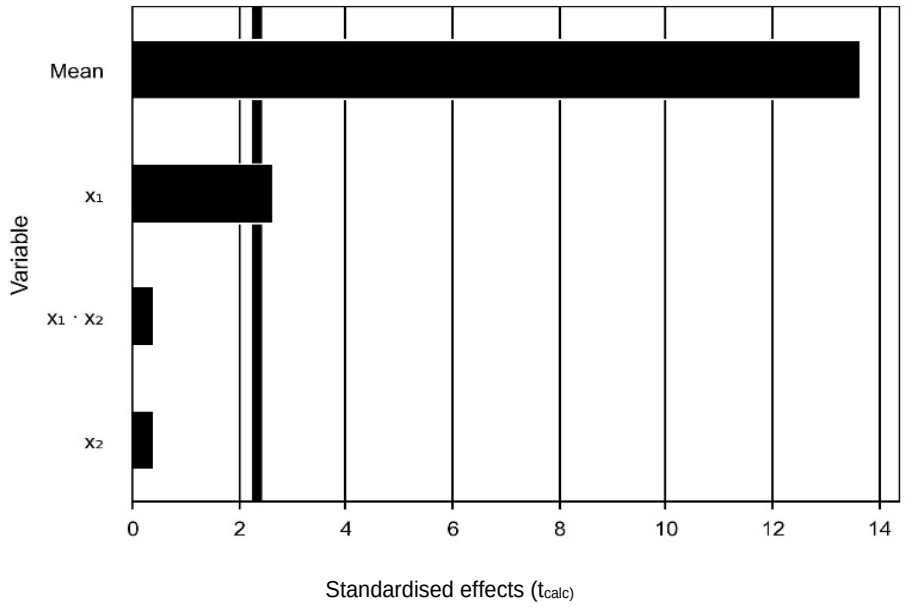
400 MPa for 1 min, and *L. monocytogenes,* at 400 MPa for 5 min, and 500 MPa for 1 min.

In this study, the combined action of high pressure (extrinsic factor), acid pH (3.39) and the phenolic compounds (intrinsic factors to Concord grape juice) may have had a significant effect on the pathogen reductions observed. Combining HPP with additional hurdles and other milder process treatments may help reduce processing costs and allow wider industrial adaptation of HPP (BALASUBRAMANIAM; Farkas, 2008). Smelt et al. (2001) states that high pressure is no different from other physical preservation methods in that its effectiveness is influenced by various intrinsic and extrinsic factors. Vegetative cells of bacteria become more sensitive to pressure in low pH conditions. Low pH not only enhances inactivation directly during treatment but also can inhibit outgrowth of cells that have been subjected to heat or pressure (SMELT et al., 2001). The hurdle technology (generally known as combined methods or barrier technology) shows synergistic effects while using various mechanisms for the inhibition or inactivation of targeted microorganisms (KHAN et al., 2017). The preservation of almost all foods is based on the combined application of several preservative methods. Therefore hurdle technology is the key for food preservation (SINGH; SHALINI, 2016).

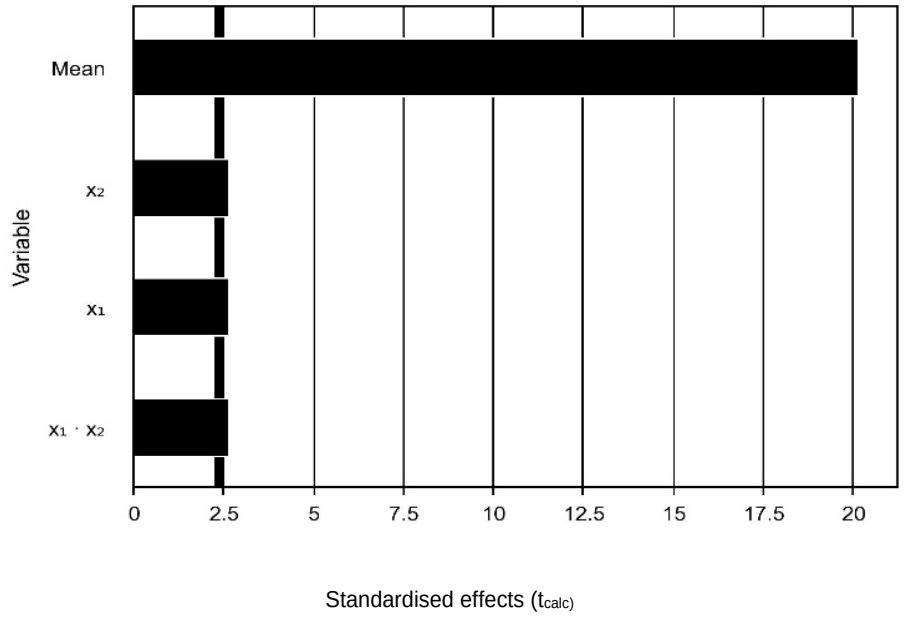
Because the maintenance costs of high pressure processing equipment exponentially increase with pressure beyond 600 MPa, significant reductions in process pressure requirements are highly desirable (BALASUBRAMANIAM; FARKAS, 2008). In this context, another relevant finding of this research work is relatively moderate pressures (~ 400 MPa) and short dwell times (~ 2 min) were highly effective in reducing the pathogens tested in Concord grape juice. Conversely, some spoilage microorganisms may have a higher pressure resistance.

Figure 1 illustrates the results from the analysis of effects (Pareto diagram) at 10% of significance. Due to the high variability of processes involving microorganisms, p-values below 10% ($p \leq 0.1$) are considered significant parameters (RODRIGUES; IEMMA, 2012).

Figure 1 - Pareto diagram for reduction of pathogens cocktail inoculated in Concord grape juice ($p \leq 0.1$). x_1 - pressure. x_2 - dwell time.



E. coli O157:H7



S. enterica

As for *E. coli* O157:H7, the Pareto diagram shows that only pressure (x_1) was significant in the pathogen reduction. With respect to *S. enterica*, pressure (x_1), dwell time (x_2) and their interaction ($x_1.x_2$) were significant.

Table 3 exhibits the analysis of variance performed with the experimental data from Table 2, only considering the statistically significant terms.

Table 3 - Analysis of variance for reduction of pathogens inoculated in grape juice ($p \leq 0.1$).

Response	Variation source	Sum of squares	Degrees of freedom	Mean square	F value F_{calc}	F value F_{tab}
E. coli O157H7 1rst order	regression	8.7	1	8.7	**10.6**	4.06
	residual	4.1	5	0.82		
	total	12.8	6			
	R^2	**0.68**				
E. coli O157H7 2nd order	regression	18.9	4	4.73	**26.28**	3.18
	residual	1.1	6	0.18		
	total	20	10			
	R^2	**0.94**				
S. enterica 1st order	regression	17.3	3	5.77	**6.95**	5.39
	residual	2.5	3	0.83		
	total	19.8	6			
	R^2	**0.88**				
S. enterica 2nd order	regression	40.2	5	8.04	**22.33**	3.45
	residual	1.8	5	0.36		
	total	42	10			
	R^2	**0.96**				

R^2 – Coefficient of determination.

By comparing the coefficients of determination (R^2) and calculated F values (F_{calc}) in both 1st and 2nd order analysis, for *E. coli* O157:H7 and *S. enterica*, one can clearly conclude that the 2nd order model better fits to the experimental data. This is mainly due to the fact that R^2 values obtained in the 2nd order analysis were much greater than those in the 1st order.

Figure 2 depicts the *experimental* versus *predicted* values, showing they are in agreement. The clusters in both charts are due to the fact that some responses (Table 2) were coincident. This occurred because the initial counts (*E.*coli: 6.6 log UFC/mL;

S. enterica: 7.6 log UFC/mL) made somewhat difficult the evaluation of the extension of pathogens reduction by the different combinations of pressure and dwell time herein tested.

Figure 2 - Experimental versus predicted log reduction of pathogens in Concord grape juice subjected to high pressure processing.

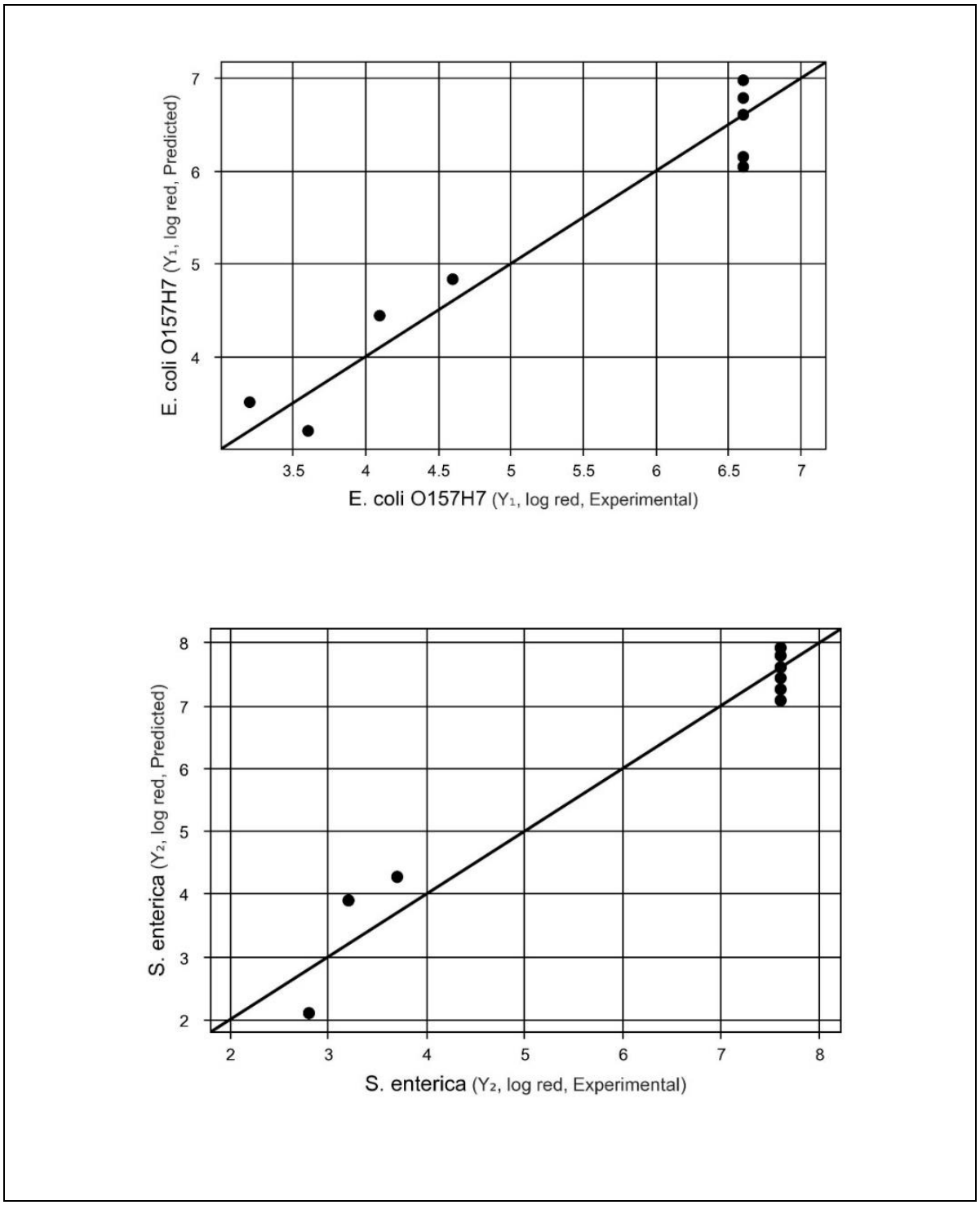
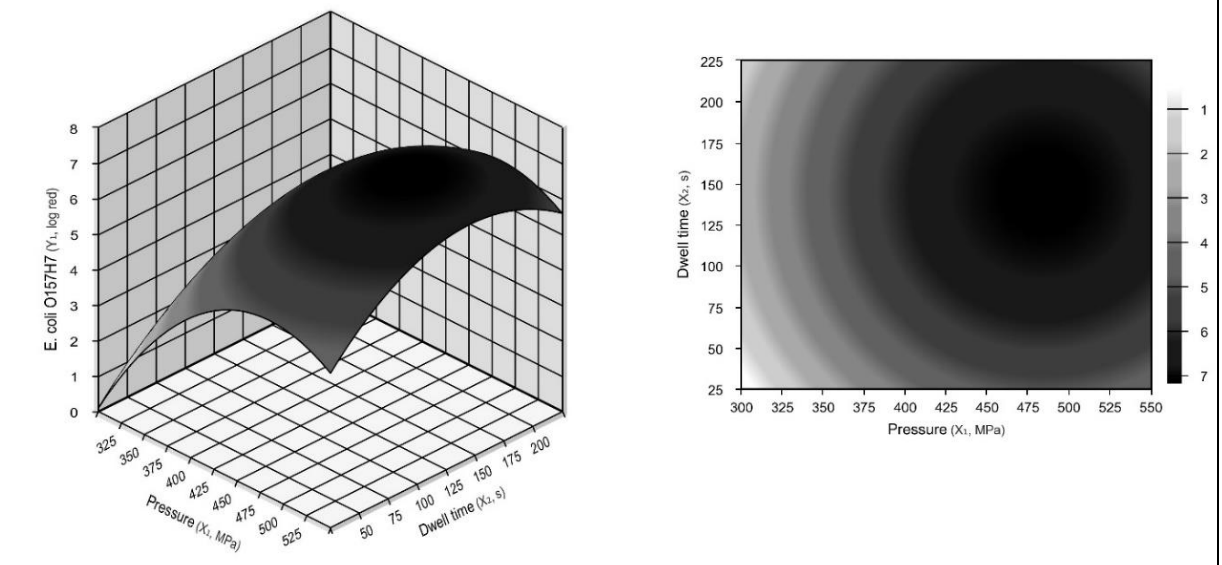
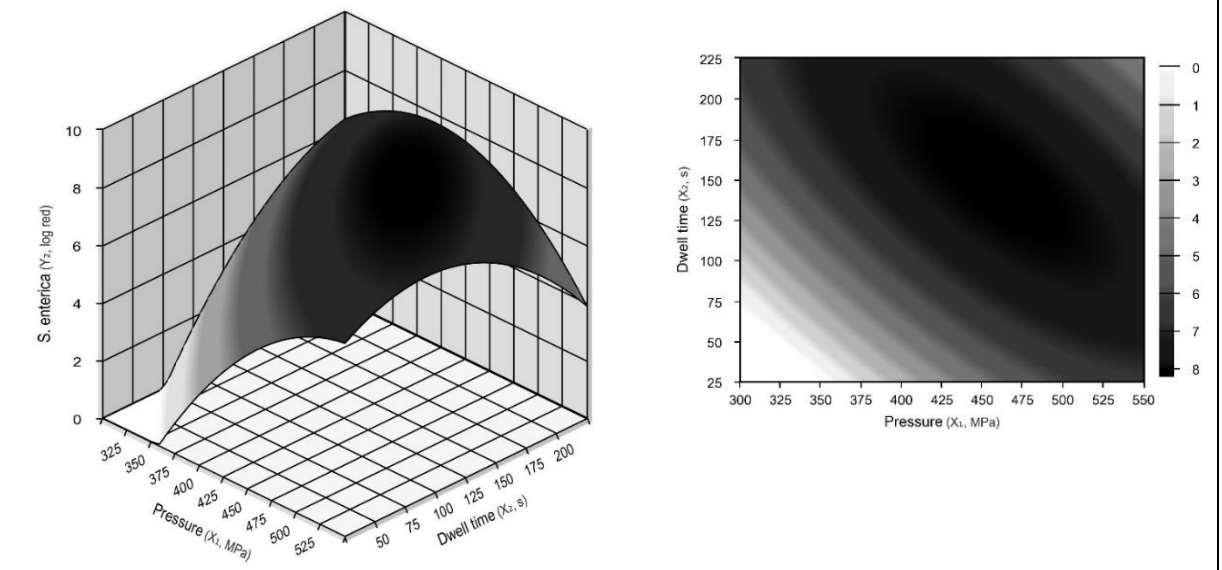


Figure 3 exhibits the response surfaces and the contour curves constructed for reducing *E. coli* and *S. enterica* inoculated in Concord grape juice and subjected to HPP.

Figure 3 - Response surfaces and contour curves in function of pressure and dwell time for pathogens reduction in high pressure processed grape juice. Y_1 = log reduction. x_1 = pressure. x_2 = dwell time.



$$Y_1 = 6.60 + 1.27\ x_1 - 0.81\ x_1^2 + 0.47\ x_2 - 0.56\ x_2^2 \quad \text{(coded Eq 1)}$$



$$Y_1 = 7.60 + 1.38\ x_1 - 0.88\ x_1^2 + 1.29\ x_2 - 0.76\ x_2^2 - 1.20\ x_1\ x_2 \quad \text{(coded Eq 2)}$$

By analyzing the curve for *E. coli* O157:H7 log reduction, one can identify the existence of an optimal range for the pressure (430 – 530 MPa) and dwell time (100 – 190 s). With regard to *S. enterica* reduction, the optimal ranges were 380 – 530 MPa and 80 – 210 s. This is of much greater interest than a simple point value, because it provides information about the “robustness” of the process, and most notably, it is the variation in pressure and dwell time that may be permitted around optimal values which still maintains the process under optimized conditions. This finding is fundamental for the control engineer to define and maintain the pressure and time sensors and controller levels. This directly affects viability and process implementation (RODRIGUES; IEMMA, 2012).

Equations 1 and 2 express the models with coded values that represent the log reduction (Y_1) of pathogens, using statistically significant parameters ($p \leq 0.1$). The terms that were not statistically significant were incorporated into the lack-of-fit to calculate the coefficient of determination (R^2). The R^2 values of 0.94 (2^{nd} order for *E. coli* O157:H7) and 0.96 (2^{nd} order for *S. enterica*) were both considered very good for this type of experiment. Still coded Equations 1 and 2 can be used to predict the number of log reductions that can be achieved in the cocktails of pathogens tested in Concord grape juice, processed under the same conditions of this study. The coded model (Equation) is that whose regression coefficients are obtained from the matrix of coded variables (-α, -1, 0, +1, +α). In this way, to obtain a predicted value from the model one must replace the values in the coded equation. In contrast, if using real values for the variables in the model, the predicted value may be incorrect and even absurd. The mathematical models hereby presented are only valid in a range of pressure from 319 to 531 MPa, and dwell time from 35 to 205 s (Table 1), as well as for the pH and soluble solids content such as those determined in Concord grape juice. The use of factorial design and response surfaces proved to be advantageous, allowing optimization of the pathogen reductions.

Finally, a fact worth highlighting is the novel thermal processing interventions employed by the fruit juice industry for controlling foodborne pathogens involve the utilization of sophisticated systems, which enable reduced processing times and temperatures to prevent loss of nutritional and sensory quality while still securing outstanding bactericidal efficacy (PARK et al., 2017).

4 Conclusions

From the present study it was observed that *E. coli* O157:H7 was more resistant to high pressure processing than *S. enterica*. A relevant finding is that relatively moderate pressures (~ 400 MPa) and short dwell times (~ 2 min) were effective in reducing the pathogens tested in Concord grape juice. Another compelling outcome is that *L. monocytogenes* did not show growth in any sample of grape juice, either before or after HPP. The response surface methodology was successfully used to predict optimal parameters for grape juice high pressure processed, in terms of pathogen reduction. The combined action of moderate pressure, acid pH, and, presumably, phenolic compounds content, intrinsic to Concord grape juice, was highly effective at achieving a greater than 5 log reduction in the pathogens used for this study.

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8 CAPÍTULO 7

Contextualização

O controle de patógenos é de grande relevância para a indústria de sucos de fruta, e a tecnologia de altas pressões (HPP) tem sido considerada uma das intervenções microbicidas mais promissoras. Complementarmente, à luz da "Tecnologia de Obstáculos" o efeito combinado de altas pressões e inibidores microbianos como dimetil dicarbonato (DMDC) requer novas abordagens investigativas. O capítulo apresentado a seguir pretende demonstrar que o emprego combinado de HPP e DMDC pode ser uma via alternativa às tecnologias clássicas de processamento térmico. O *design* experimental objetivou a redução de pelo menos 5 ciclos logaritmos em *cocktails* de patógenos de referência – *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* – inoculados em suco de maçã. Os resultados da pesquisa demonstraram que níveis de pressão relativamente moderados e tempos de retenção reduzidos, combinados com baixas concentrações de DMDC podem ser efetivamente empregados na destruição de patógenos em suco de maçã.

The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice

(PETRUS, R. R.; CHUREY, J. J., HUMISTON, G. A., CHENG, R. M.; WOROBO, R. W., 2020)

Abstract

Novel processing technologies can be used to improve both the microbiological safety and quality of food products. The application of high pressure processing (HPP) in combination with dimethyl dicarbonate (DMDC) represents a promising alternative to classical thermal technologies. This research work was undertaken to investigate the combined effect of HPP and DMDC, which was aimed at reaching at least 5-log reduction in the pathogens of reference *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* separately inoculated in apple juice. Different strains of each species were tested. Pressures (ranging from 100 to 600 MPa), dwell times (26 – 194 s), and DMDC (116 – 250 mg/L) were tested based on a central composite rotational design. The dwell time, in the studied range, did not have a significant effect ($p > 0.1$) on pathogens reduction. All treatments achieved a greater than 5-log reduction for *E. coli* O157:H7 and *L. monocytogenes*. The reductions for *S. enterica* was also greater that 5-log for almost all tested combinations. The results for *S. enterica* indicated that it is more resistant to HPP and DMDC compared to *E. coli* O157:H7 and *L. monocytogenes*. The findings of this study demonstrated that DMDC at low concentrations can be added to apple juice to reduce the parameters conventionally applied in HPP. The combined use of HPP and DMDC was highly effective under the conditions of this study.

Keywords: Hurdle technology, nonthermal processing, microbial inhibitor, factorial design.

1 Introduction

Hurdle technology, known as combined methods or barrier technology, exhibits interaction effects while using various mechanisms for the inhibition or inactivation of targeted microorganisms (KHAN et al., 2017). Food preservation frequently relies on the combined application of several preservation methods (SINGH; SHALINI, 2016). Because consumers are more demanding for products which have natural flavor and higher nutritive value, high pressure processing (HPP) has been considered one of the preferred technologies to meet these consumer preferences.

Balasubramaniam and Farkas (2018) states that HPP offers a commercially viable and practical alternative to thermal processing by allowing industry to process foods at or near ambient temperature. HPP can effectively inactivate pathogenic and spoilage bacteria, yeasts, and molds, but has limited effectiveness against spores and enzymes. Kan et al. (2017) hold that food products with a high acid content are particularly favorable candidates for HPP technology. The pressure resistance of *Escherichia coli* and *Listeria monocytogenes* exhibits significant variability, which is biotype/strain specific. For food applications, the minimum and maximum limits of HPP are 200 MPa and 600 MPa, respectively (KHAN et al., 2017). Notwithstanding, effective preservation using HPP may be achieved at moderate pressures in combination with other hurdles, which result in synergistic effects of two or more suitable antimicrobial factors at moderate doses.

Huang et al. (2017) reported that HPP eliminates food pathogens at ambient temperature, and extends the shelf life of foods circulated through the cold chain. HPP maintains the sensory properties and nutritional value of the foods, which is not possible using traditional thermal pasteurization. The U.S. Food and Drug Administration (FDA) has officially recognized HPP as a nonthermal pasteurization technology that can replace traditional thermal processing in the food industry, primarily for acid foods.

Combining HPP with additional hurdles and milder processing treatments may help to reduce processing costs and allow wider industrial adaptation of HPP. Once the maintenance costs of the HPP equipment exponentially increases with pressures beyond 600 MPa, significant reductions in processing pressure requirements are highly desirable (BALASUBRAMANIAM; FARKAS, 2008).

In regard to chemical inhibitors, dimethyl dicarbonate (DMDC) is primarily used as a yeast inhibitor, but also has inhibitory activity against molds and bacteria (BASARAN-AKGUL, 2009). DMDC, a dicarbonic acid ester, is a colorless, transparent liquid with a fruity aroma. It is a powerful antimicrobial agent due to its potentially high reaction capacity with nucleophilic groups of enzymes from microorganisms, such as imidazoles, amines, or thiols, which results in the rapid inactivation of microorganisms (YU et al., 2013). DMDC in water solutions can be rapidly and completely hydrolyzed into low levels of methanol and carbon dioxide, which are both natural constituents of fruits and vegetables, proven to be harmless (WANG et al., 2013). Because the hydrolysis products are nontoxic to consumers, DMDC is not required to be declared in the product label (ASSATARAKUL, 2017). DMDC is FDA approved for use as a microbial inhibitor with the maximum limit of 250 mg/L. In this context, the combined use of HPP and DMDC may represent a promising hurdle approach to reduce the high pressure processing conditions. Lindner et al. (2017) added DMDC as preservation method to a functional fruit/vegetable beverage (pH 3.5). *Alicyclobacillus acidoterrestris* and *E. coli* were inoculated into the beverage to induce spoilage. The DMDC reduced *E. coli* to undetectable levels and exhibited a greater reduction of *A. acidoterrestris* vegetative cells, molds and yeasts.

Limited reports are available on the combined use of HPP and DMDC in the inactivation of pathogenic bacteria in juices. This study was conducted to identify the optimal combination of pressure, dwell time, and DMDC concentration to attain at least 5-log reduction in cocktails of *Escherichia coli* O157:H7, *Salmonella enterica,* and *Listeria monocytogenes* inoculated into shelf stable apple juice.

2 Material and methods

The experiments were conducted at Cornell High Pressure Processing Validation Center. The facility includes the processing area and the biohazard level 2 laboratory in the Food Science Building at Cornell AgriTech Campus, in Geneva, NY, USA. The strains were selected based on their produce related outbreaks, and are described in Table 1.

Table 1 - Serotypes/strains and origin of the pathogens used in the study.

Pathogen	Strain or serotype	Origin
E. coli O157:H7	C7927	Human isolate, apple cider linked to an outbreak (date of outbreak unavailable)
	ATCC 43890	Human isolate, date of outbreak unavailable
	ATCC 43894	Human isolate, date of outbreak unavailable
	ATCC 43889	Human isolate, date of outbreak unavailable
	ATCC 35150	Human isolate, date of outbreak unavailable
S. enterica	Hartford H0778	Orange juice, US outbreak in 1995
	Typhimurium FSL R9-5494	Orange juice, multistate US outbreak in 2005
	Muenchen FSL R9-5498	Alfalfa sprouts, multistate US outbreak in 2016
	Javiana FSL R9-5273	Tomatoes, multistate US outbreak in 2002
	Enteriditis FSL-R9-5505	Beans sprouts, multistate US outbreak in 2014
L. monocytogenes	Lineage I, serotype 4b FSL J1-108	Coleslaw, US outbreak in 1981
	Lineage I, serotype 4d FSL J1-107	Coleslaw, US outbreak in 1981
	Lineage II, serotype 1/2a FSL R9-0506	Cantaloupe, US outbreak in 2011
	FSL R9-5411	Caramel Apple, multistate US outbreak 2014-2015
	FSL R9-5506	Packaged Salad, multistate US outbreak in 2016

2.1 Inoculum preparation

Frozen stocks of pathogenic *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes* were kept at - 80 °C until used. Thawed stocks were reactivated by streaking onto Trypticase Soy Agar (TSA) plates and incubated overnight at 36 ± 1 °C. After the streaked culture was grown, the plate was placed at 4 °C. A single isolated colony of each pathogen strain was separately transferred into 10 mL of Trypticase

Soy Broth (TSB) and incubated for 24 h at 36 ± 1 °C under shaking (175 rpm). This procedure was carried out for all pathogens used in the study. A loop of the inoculated TSB was then transferred into a volume of 10 mL-TSB (pH 5.0) and incubated for 24 h at 35 ± 1 °C under shaking (175 rpm). Acid adaptation of the cells with citric acid was performed based on the protocol reported by Usaga (2014). After incubation, cells were concentrated by centrifugation at 10,000 rpm for 2 min using an Eppendorf 5415 C microcentrifuge (Eppendorf, Hamburg, Germany) and resuspended in 1 mL of citrate buffer (pH 4.0) diluent. A cocktail of five strains of each pathogen was prepared by equivalent volumes of cells being combined and serving as inoculum for apple juice.

2.2 Food matrix

As food matrix, 1.9 L-PET bottles of shelf stable preservatives free, 100% apple juice (Wegmans Food Markets, Inc.), were procured from local supermarket in Geneva, NY. The apple juice was separately inoculated with five-strain cocktails of each of the three pathogens (Table 1).

2.2.1 Physicochemical measurements

The juice´s pH was measured with the pH meter Edge (Hanna® instruments). The soluble solids content was measured using a digital refractometer (model 300034, Sper Scientific). Total titratable acidity was determined with a 848 Titrino Plus Autotitrator (Ω Metrohm). The water activity (a_w) was measured with the Water Activity Meter Aqualab 4TE (Decagon). All Physicochemical assays were performed in triplicate.

2.3 Sample inoculation and HPP treatment

The HPP containers (66 mL-PET bottles) were filled with apple juice leaving a maximum headspace of 5%. A volume of 0.5 mL of each cocktail, required for a target concentration of approximately 10^7 CFU/mL of juice, was inoculated into each container of product. The inoculation was conducted in a bio level 2 safety cabinet and one sample was aseptically collected to determine the initial cell count (N_0) as positive control. DMDC was added immediately after the pathogens were inoculated. Containers were sealed and repackaged independently into a plastic bag. Bags were sealed using a vacuum sealer and placed in a second plastic bag with chlorinated solution that was also sealed using the vacuum packaging machine and finally

packaged in a third bag. Samples were placed in the HPP unit and subjected to the processing conditions accordingly to experimental design (Tables 2 and 4). The pressure, dwell time, and water temperature were recorded. The HPP unit used was a Hiperbaric 55 L commercial processing unit. It has a water chiller to maintain the chamber water temperature during the HPP cycle; the apple juice was kept at a temperature of approximately 8 °C. Once the HPP cycle was finished, the samples were analyzed for the respective pathogens. Duplicate inoculated and HPP treated samples were placed in a temperature-controlled incubator at 4 °C. These samples were enumerated for pathogen levels over a course of 30 days to observe for any pathogen regrowth during that period.

2.4 Dimethyl dicarbonate solution

Dimethyl dicarbonate (Velcorin™, 99.8%, Bayer Corp., Pittsburgh, PA) solution was freshly prepared by performing a 1:4 dilution in 100% (v/v) ethanol, and immediately added to 66 g of apple juice to obtain the final concentrations of 116, 143, 183, 223, and 250 mg/L (Table 2).

2.5 Colony enumeration of pathogens

Serial dilutions of each sample were done using 0.1% sterile peptone water. One (1) mL of each dilution was pour plated in petri dishes with violet red bile agar (Alpha Biosciences™) for *E. coli* O157:H7, bismuth sulfite agar (Criterion™) for *S. enterica*, and Oxford Listeria agar base (Alpha Biosciences™) for *L. monocytogenes*. Upon agar solidification, the petri dishes were incubated for 48 h at 36 ± 1 °C. The colonies were enumerated using a Quebec colony counter, and the level of microbial reduction was determined.

2.6 Experimental design

Table 2 exhibits the levels used in the central composite rotational design (CCRD).

Table 2 - Coded and actual values used in the CCRD for reducing foodborne pathogens inoculated in apple juice.

Variables	Code	-1.68	-1	0	+1	+1.68
Pressure (MPa)	x_1	100	201	350	499	600
Holding time (s)	x_2	26	60	110	160	194
DMDC (mg/L)	x_3	116	143	183	223	250

(-1.68) lower axial point, (-1) lower level, (0) central point, (+1) upper level, (+1.68) upper axial point. $\alpha = (2^n)^{1/4} = 1.68$. α = axial point. n = number of variables.

2.7 Data analysis

Data from the experimental design were processed through response surface methodology. The analysis of regression was first performed for both 1st (responses obtained only from central points) and 2nd (responses including axial points) orders, at 10% of significance. Due to the high variability of processes involving microorganisms, p-values below 10% ($p \leq 0.1$) were considered significant parameters (RODRIGUES; IEMMA, 2012). Then the mathematical model was re-parameterized considering the statistically significant coefficients only. The analysis of variance was carried out to ensure the model is statistically significant. If so, the response surface was built. Statistical tests were conducted using the software Protimiza Experimental Design (http://experimental-design.protimiza.com.br).

3 Results and discussion

Table 3 gathers the results from the Physicochemical assays of apple juice.

Table 3 - Physicochemical parameters of the shelf stable clarified (filtered) apple juice.

pH	a_w	Soluble solids (˚Brix)	Titratable acidity (%malic acid)
3.76 ± 0.02	0.99 ± 0.01	12.5 ± 0.02	0.34 ± 0.00

Means from three measurements ± standard deviation.

Basaran-Akgul et al. (2009) reported pH values, soluble solids content and titratable acidity for apple cider (unclarified apple juice) ranging from 3.24 to 3.91, 11.2

to 13.8 ºBrix, and 0.295 to 0.650%, respectively. The results from this study are within the range of those. High-acid food products, such as apple juice, are particularly favorable candidates for HPP technology (KHAN et al., 2017). The pH of acid solutions decreases as pressure increases and it has been estimated that, in apple juice, there is a pH drop of 0.2 unit per 100 MPa. As the pressure is released, the pH reverts to its original value. The pH and pressure can act synergistically leading to increased microbial inactivation (USAGA; WOROBO; PADILLA-ZAKOUR, 2014).

A common element to all decontamination strategies by HPP is the need to account for the food matrices hosting these microorganisms. Food matrices are complex environments which may offer shelter to microorganisms, even under harsh treatment conditions (GEORGET et al., 2015).

The results from the combinations of different levels tested in this study are exhibited in Table 4. Only trials which achieved equal to or greater than 5-log reductions were considered effective, as required by the FDA for fruit and vegetable juices.

Table 4 - Matrix of the experimental design and responses obtained from the shelf stable apple juice inoculated with foodborne pathogen cocktails.

Trial	Variable			Response (log reduction)		
	Pressure (MPa)	Dwell time (s)	DMDC (mg/L)	*E. coli* O157:H7	*S. enterica*	*L. monocytogenes*
1	201 (-1)	60 (-1)	143 (-1)	7.4	4.8	6.4
2	499 (+1)	60 (-1)	143 (-1)	7.4	7.2	6.4
3	201 (-1)	160 (+1)	143 (-1)	7.4	5.4	6.4
4	499 (+1)	160 (+1)	143 (-1)	7.4	7.2	6.4
5	201 (-1)	60 (-1)	223 (+1)	7.4	6.6	6.4
6	499 (+1)	60 (-1)	223 (+1)	7.4	7.2	6.4
7	201 (-1)	160 (+1)	223 (+1)	7.4	6.5	6.4
8	499 (+1)	160 (+1)	223 (+1)	7.4	7.2	6.4
9	350 (0)	110 (0)	183 (0)	7.4	7.2	6.4
10	350 (0)	110 (0)	183 (0)	7.4	7.2	6.4
11	350 (0)	110 (0)	183 (0)	7.4	7.2	6.4
12	100 (-1.68)	110 (0)	183 (0)	5.4	5.4	6.4
13	600 (+1.68)	110 (0)	183 (0)	7.4	7.2	6.4
14	350 (0)	26 (-1.68)	183 (0)	7.4	7.2	6.4
15	350 (0)	194 (+1.68)	183 (0)	7.4	7.2	6.4
16	350 (0)	110 (0)	116 (-1.68)	7.4	7.2	6.4
17	350 (0)	110 (0)	250 (+1.68)	7.4	7.2	6.4

(-1.68) lower axial point, (-1) lower level, (0) central point, (+1) upper level, (+1.68) upper axial point. $\alpha = (2^n)^{1/4} = 1.68$. Initial counts (log CFU/mL) – E. coli: 7.4, S. enterica: 7.2, L. monocytogenes: 6.4.

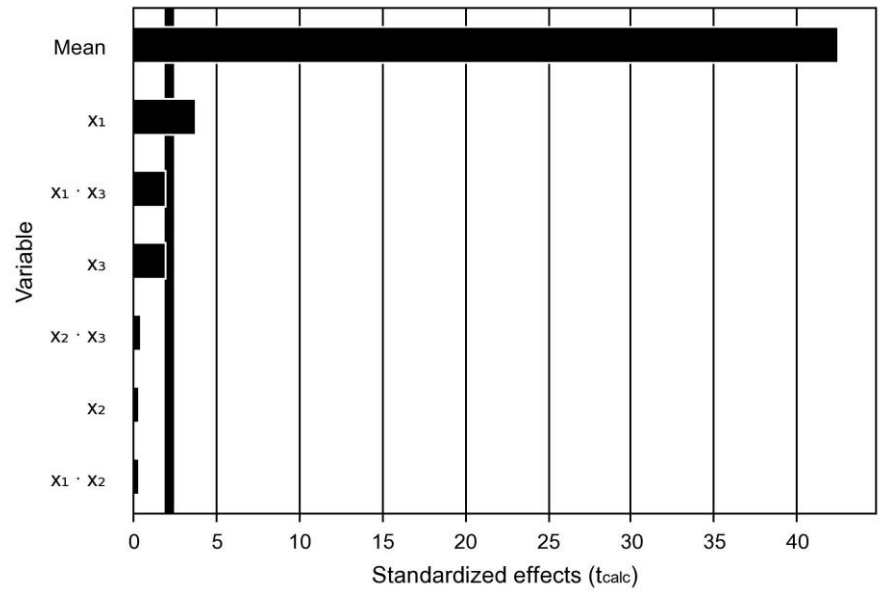
The results from Table 4 show that, regardless the levels of variables, the combination of HPP and DMDC was highly effective on all pathogens reduction. Except for trial 1, the trinomial (201 MPa / 60 s / 143 mg/L DMDC) did not reach 5-log reductions for *S. enterica*. The combination of relatively low pressure with DMDC can be successfully used as a means for reducing pathogens in apple juice. The findings also suggest that *S. enterica* was more resistant to the combination of HPP and DMDC than *E. coli* O157:H7 and *L. monocytogenes*. Gram-positive bacteria, such as *L. monocytogenes*, tend to be more pressure resistant than Gram-negatives, and cocci are more resistant than rod-shaped bacteria. However, there are some exceptions. Certain strains of *E. coli* O157:H7 have been reported to be pressure resistant (USAGA; WOROBO; PADILLA-ZAKOUR, 2014). The pathogens did not regrow in apple juice after storage at 4 ºC for 30 days.

Assatarakul (2017) studied the effect of DMDC (ranging from 0 to 300 mg/L) on *E. coli* O157:H7, *L. monocytogenes*, *S.* Enteritidis and *S. aureus* in fresh mandarin juice. The author reported that *E. coli* was the most sensitive pathogen, and the addition of DMDC could effectively aid in pathogen reduction.

Because there was no variation in the responses for *L. monocytogenes* (6.4 log reduction), and very little variation for *E. coli* O157:H7, neither the analysis of effects nor analysis of variance were carried out. This finding indicates that, within the studied range, the variables had no effect on pathogens reduction. Further studies are warranted to exploit lower DMDC concentrations (< 116 mg/L).

Figure 1 illustrates the diagram of effects (Pareto diagram) for *S*. *enterica* reduction.

Figure 1 - Pareto diagram for reduction of *S. enterica* inoculated in apple juice ($p \leq 0.1$).



x_1 – pressure. x_2 – dwell time. x_3 – DMDC concentration.

Figure 1 demonstrates that pressure (x_1), DMDC concentration (x_3), and the interaction between them (x_1. x_3), had a significant effect ($p \leq 0.1$) on *S. enterica* reduction. Conversely, dwell time had no impact on *S. enterica* reduction. This is a very compelling finding, as it shows that the lowest level, in the studied range (26 to 194 s), can be applied to result in savings of energy and time.

Table 5 shows the analysis of variance (ANOVA) for *S. enterica* reduction.

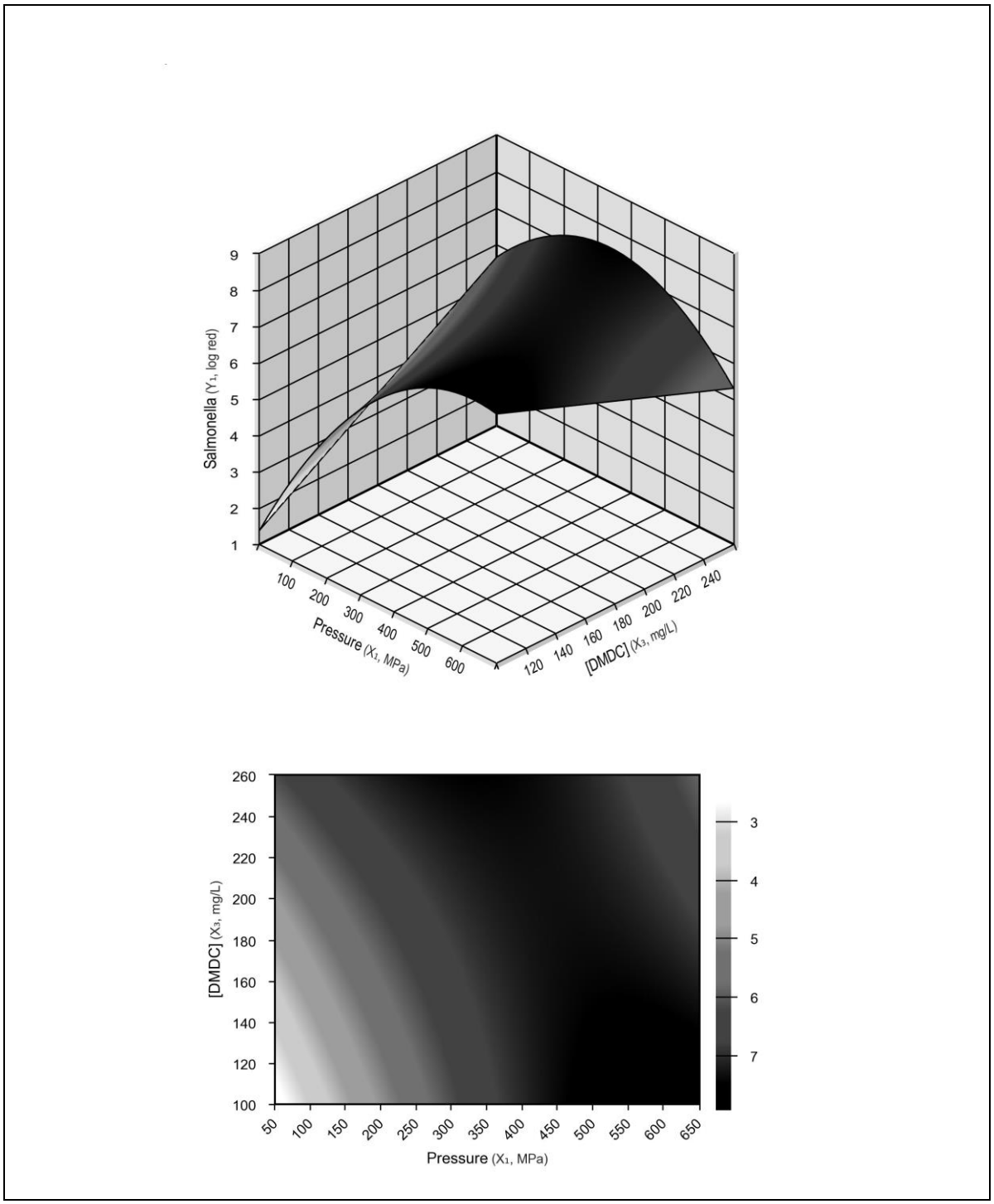
Table 5 - Analysis of variance ($p \leq 0.1$) for reduction of *S. enterica* inoculated in apple juice.

	Variation source	Sum of squares	Degrees of freedom	Mean Square	F value	
					F_{calc}	F_{tab}
1st order	Regression	5.9	3	1.97	**11.59**	3.07
	Residual	1.2	7	0.17		
	Total	7.1	10			
	R^2	**0.83**				
2nd order	Regression	8.7	4	2.18	**21.8**	2.48
	Residual	1.2	12	0.10		
	Total	9.9	16			
	R^2	**0.87**				

The ANOVA demonstrates that the 2nd order model ($R^2 = 0.87$) better fitted to experimental data than the 1st order ($R^2 = 0.83$**)**. Additionally, F_{calc} was approximately nine-fold greater that F_{tab} regarding the 2nd order analysis, and four-fold greater, for 1st order.

Figure 2 represents the response surface and the contour curves. Because dwell time had no significant effect ($p \leq 0.1$) on *S. enterica* reduction, this variable was not included.

Figure 2 - Response surface and contour curves in function of Pressure and DMDC concentration for *S. enterica* reduction in apple juice.



$$Y_1 = 7.06 + 0.62\ x_1 - 0.36\ x_1^2 + 0.21\ x_3 - 0.36\ x_1\ x_3 \quad \text{(coded Eq. 1)}$$

Y_1 = log reduction of *S. enterica*. x_1 = Pressure. x_3 = DMDC concentration.

Analyzing the curve (Figure 2), one can identify the existence of an optimal range for the pressure (380 – 600 MPa) and DMDC concentration (116 – 250 mg/L) for *S. enterica* reduction. This is of much greater interest than a simple point value, because it provides information about the "robustness" of the process, and most notably, it is the variation in pressure that may be permitted (± 100 MPa) around optimal value which still maintains the process under optimized conditions (log reductions ≥ 5.0) (RODRIGUES; IEMMA, 2012). From Figure 2, one can determine the pressure and DMDC concentration to achieve 5-log reduction of *S. enterica*. The optimum condition (≥ 5 log reduction) can be reached by combining relatively mild pressure (400 MPa) and DMDC concentration (120 mg/L), suggesting a synergistic effect between these two variables.

Coded Equation 1 can be used to predict the number of log reductions of *S. enterica* by combining different pressures and DMDC concentrations, in the studied ranges. From another perspective, by setting the number of 5-log reduction in pathogens as target of processing, one can calculate the pressure and/or DMDC concentration to attain that goal. The coded model (Equation) is that whose regression coefficients are obtained from the matrix of coded variables (-α, -1, 0, +1, +α). In this way, to obtain a predicted value from the model one must replace the values in the coded equation. If using real values for the variables in the model, the predicted value may be incorrect and even absurd. Equation 1 only contains the statistically significant terms at 10%, as depicted in Pareto diagrams (Figure 1). For practical purposes, it is desirable that the fitted model be as simple as possible and contain the smallest possible number of parameters without giving up the quality assured in the careful selection of the experimental design (RODRIGUES; IEMMA, 2012). The models were reparameterized/reduced because the parameters with little or no influence, such as dwell time, on the outcome of the final fit were excluded.

Assatarakul (2017) reported the effect of DMDC on degradation kinetics and inactivation of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *S.* Enteritidis in fresh mandarin juice with and without heat. The results pointed out that addition of DMDC can effectively aid in pathogen reduction which leads to a decrease in heat exposure of the fresh mandarin juice, possibly enhancing its quality. Therefore, DMDC can potentially be used as an additional treatment or in combination with other treatments

to improve the safety without significant effects on physical and chemical properties of mandarin juice.

Yu et al. (2016) evaluated the effect of high pressure (200 MPa) and DMDC (250 mg/L) on microbial and nutrient qualities of mulberry juice. This combined treatment offered a useful alternative to conventional heat pasteurization for controlling microbial growth and significantly extending the juice´s shelf life.

Basaran-Akgul et al. (2009) tested three stains of *E. coli* O157:H7 to investigate the effectiveness of DMDC and SO_2 in eight different unfiltered apple ciders. DMDC was extremely effective in extending the shelf life and enhancing the safety of fresh juices, not only due to its fungicidal activity but also due to its antibacterial activity against pathogens such as *E. coli* O157:H7. Two hours after DMDC addition, the authors obtained approximately 2 and 5-log cycles reduction for 125 and 250 mg/L DMDC, respectively.

Whitney et al. (2008) investigated the effect of HPP in conjunction with DMDC on *E. coli* O157:H7 inoculated in shelf stable apple juice. After inoculation, the juice was subjected to 550 MPa for 2 min at 6 °C. The most effective treatment was 125 mg/L of DMDC, which caused a 5-log reduction. The number of reductions reached in this study (Table 4) was greater than those aforementioned.

Khan et al. (2017) held that using combinations of these techniques could potentially save energy and improve food safety and quality. The combination of HPP and DMDC might reduce the cost of decontamination treatment and minimize the effect on quality, while extending the shelf life. Because almost all tested combinations (pressure x dwell time x DMDC concentration) gave pathogen reductions greater than 5 log, lower levels of pressure and DMDC might be investigated in further studies.

The results herein exhibited may be utilized by the apple juice industry for effective application of HPP combined with DMDC. Because the maintenance costs of HPP equipment increases with pressure, significant reductions in process pressure requirements are highly desirable. This study broadens the scope and possibilities for HPP parameters to provide improved knowledge in juice processing techniques where safety and quality are maintained at a potentially lower cost of production.

4 Conclusions

The combination of high pressure processing (HPP) and dimethyl dicarbonate (DMDC) was highly effective to inactivate the cocktails of *L. monocytogenes, S. enterica,* and *E. coli* O157:H7 inoculated in apple juice. Almost all tested combinations (pressure x dwell time x DMDC concentration) achieved greater than 5-log reductions in the pathogens of reference. The dwell time, in the studied range, did not have a significant effect on pathogen inactivation. The findings of this study showed that DMDC at low concentrations can be safely added to apple juice to reduce the parameters conventionally applied in HPP.

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9 CONSIDERAÇÕES FINAIS E PERSPECTIVAS PARA NOVOS ESTUDOS

As pesquisas neste reportadas constituem os pilares da linha de investigação do autor, apresentada como **Processamento e estabilidade de alimentos líquidos**. Os dados apresentados, publicados em periódicos de circulação internacional com seletiva política editorial, preenchem lacunas na literatura científica e incrementam o conhecimento tangível à Tecnologia de Alimentos, notadamente relacionado à combinação de métodos térmicos e não térmicos de conservação, e vida de prateleira de leite pasteurizado, caldo de cana, bebida isotônica e sucos de fruta.

Em síntese, destacam-se as seguintes descobertas reportadas no corpo deste texto. No estudo reportado no Capítulo 1 – ***Microbiological shelf life of pasteurized milk in bottle and pouch*** – demonstrou-se a extensão do impacto da temperatura de estocagem, em ampla faixa, no tempo de vida útil de leite pasteurizado tipo A. O material de embalagem exerceu "discreto" efeito na conservação do produto sob refrigeração. Idealmente, este alimento deve ser mantido a temperaturas iguais ou inferiores a 4 ºC e na ausência de luz. No Capítulo 2 – ***Effect of pasteurization temperature on stability of an acidified sugarcane juice beverage*** – constatou-se a viabilidade tecnológica de produção de caldo de cana acidificado com polpa de fruta. O aumento da temperatura de pasteurização exerceu um impacto positivo na vida de prateleira da bebida. Os resultados do trabalho apresentado no Capítulo 3 – ***Development of a ricotta cheese whey-based sports drink*** – apontaram as intervenções tecnológicas necessárias à transformação de um co-produto da indústria de laticínios, denominado soro de ricota, em um suplemento hidroeletrolítico comercialmente estéril, com destacado potencial de aplicação industrial. Os resultados produzidos a partir da condução do estudo que consta do Capítulo 4 – ***Cultivar affects the color change kinetics of sugarcane juice*** – evidenciaram a importância da escolha do cultivar da matéria-prima no que tange a cinética de escurecimento enzimático de caldo de cana destinado ao consumo direto. Os dados gerados na pesquisa apresentada no Capítulo 5 – ***Sugarcane juice stability in plastic bottles treated with silver and zinc oxide*** – não confirmaram o potencial antimicrobiano de micropartículas de prata e óxido de zinco (Ag/ZnO) na estabilidade de caldo de cana integral pasteurizado, contrariando as expectativas do estudo. Os

ensaios com a aplicação de altas pressões hidrostáticas conduzidos com suco de uva tinta reportados no Capítulo 6 – ***Searching for high pressure processing parameters for Escherichia coli O157:H7, Salmonella enterica and Listeria monocytogenes reduction in Concord grape juice*** – demonstraram que a aplicação de pressões moderadas e inferiores àquelas comercialmente empregadas foram efetivas na redução de patógenos de referência inoculados no suco, em conformidade com as diretrizes da FDA. O estudo reportado no Capítulo 7 – ***The combined effect of high pressure processing and dimethyl dicarbonate to inactivate foodborne pathogens in apple juice*** – revelou que a combinação de dimetil dicarbonato (DMDC) e níveis de pressão moderados foi altamente efetiva na destruição de patógenos de referência inoculados em suco de maçã clarificado.

Os ensaios experimentais conduzidos no Departamento de Engenharia de Alimentos da USP e no *High Pressure Processing Validation Center* da *Cornell University*, descritos neste texto sistematizado, exibem o seu potencial de aplicação na indústria de alimentos líquidos. Internamente, maior ênfase tem sido atribuída a projetos compatíveis com micro e pequenas escalas de produção. Salienta-se que a implantação de tecnologias de processamento e envase mais elaboradas é frequentemente restrita a médias e grandes escalas em virtude do elevado custo de investimento e manutenção. No que tange à tecnologia de altas pressões, a sua aplicação ainda é restrita a poucas plantas industriais no Brasil. Não obstante, observa-se uma tendência de disseminação deste método não térmico, não apenas para alimentos líquidos, mas também para outras categorias.

Apesar da incidência de danos colaterais à qualidade sensorial, nutricional e funcional dos alimentos, deflagrados pela ação do calor, não visualiza-se a exclusão do tratamento térmico. Mas sim, a sua combinação com tecnologias emergentes não térmicas, em intensidades mais brandas para potencializar os efeitos sinérgicos, favorecendo a preservação das características inerentes ao alimento e sobretudo, garantindo a segurança do produto.

Nos anos subsequentes pretende-se avançar nas pesquisas direcionadas para o processamento de alimentos líquidos, combinando-se tecnologias não térmicas (dióxido de carbono em estado supercrítico, plasma a frio, ozônio, alta pressão hidrostática etc) com métodos tradicionais. Com a construção de parcerias, inclusive

de áreas distintas da Tecnologia de Alimentos, planeja-se a otimização e a validação das tecnologias investigadas. Bebidas como sucos, néctares e caldo de cana permanecerão como os alvos de estudos futuros, alinhados com o conceito "*clean label*", que rotula alimentos destituídos de ingredientes artificiais e/ou aditivos sintéticos. A despeito do adverso cenário no tocante ao financiamento de pesquisas científicas no Brasil, assim como a concessão de bolsas de estudo, drasticamente limitadas, esforços têm sido empenhados visando a captação de recursos junto às agências de fomento. Projetos de pesquisa têm sido continuamente submetidos com a finalidade de captar recursos para sustentar e impulsionar a linha de pesquisa do autor. Paralelamente, buscam-se parcerias com o setor privado. Ademais, novos estágios sabáticos internacionais, idealmente em instituições de excelência em Tecnologia de Alimentos, para facilitar o acesso ao "estado da arte" e incrementar a produção científica também são vislumbrados no médio e longo prazos.