OPTIMIZATION OF HYDROTHERMODYNAMIC TECHNOLOGY FOR BLUEBERRY FOOD PROCESSING

by

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ABSTRACT

Traditional processing technologies can negatively affect polyphenolics of blueberry products. Optimization of hydrothermodynamic (HTD) processing has been used to minimize blueberry-based product quality degradation.

Preliminary experiments have shown that the quality of HTD blueberry purée in terms of anthocyanin concentration and tannin content was significantly higher than that of most commercial blueberry products. HTD technology provided pasteurization with significantly lower losses of anthocyanins as compared with conventional heating.

Screening of the factors has shown that holding time, temperature of pasteurization and heating rate were significant, while cavitation mode was non-significant. Response surface methodology was used to determine an optimal combination of temperature and time (92 to 95°C with holding time 0.5 to 2.0 min).

Non-linear regression analysis of the shelf-life data has provided fitted models which could be used to predict changes in anthocyanin concentration and tannins for different storage periods at 4°C and at room temperature (20 to 25°C).

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAFC	Agriculture and Agri-Food Canada
AIC	Akaike information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Aerobic plate count
C3G	Cyaniding-3-glucoside
CCD	Central composite design
CD	Color density
cfu	Colony forming unit
cs	Cold storage
CS	Compound symmetry
DF	Dilution factor
DW	Dry weight
FB	Frozen blueberry
FP	Frozen blueberry purée
FW	Fresh weight
GAE	Gallic acid equivalent
GLM	General linear model
HPLC	High-performance liquid chromatography
HTD	Hydrothermodynamic
HTDT	Hydrothermodynamic technology
HTST	High temperature short time
IQF	Individual quick freezing
LDL	Low density lipids
MW	Molecular weight
NB	New Brunswick
NID (μ, σ^2)	Normally and independently distributed random variables with mean μ and variance σ^2

NLS	Non-linear least squares
NS	Nova Scotia
NSAC	Nova Scotia Agricultural College
ORAC	Oxygen radical absorbance capacity
PEI	Prince Edward Island
PO	Peroxidase
PPO	Polyphenoloxidase
RSM	Response surface methodology
rt	Room temperature
st dev	Standard deviation
TFA	Trifluoroacetic acid
UN	Unstructured
USA	United States of America
UV	Ultra-violet
A	Absorbance
a	Number of factor levels
C	Tannin content or anthocyanin concentration
$f(x_i,\theta)$	Expectation function
i, j, k, l, m	Notations for factor levels and replications
k	Temperature-dependent rate constant
n	Number of replications
t	Storage period, days
x_1, x_2, x_1^2, x_2^2	Linear and quadratic components for response surface analysis
x_i	Vectors for non-linear regression analysis
y	Response
$\alpha\beta, \alpha\gamma, \alpha\delta, \beta\gamma, \beta\delta, \gamma\delta$	Two-way interaction effects
$\alpha\beta\gamma, \alpha\beta\delta, \alpha\gamma\delta, \beta\gamma\delta$	Three-way interaction effects
$\alpha\beta\gamma\delta$	Four-way interaction effects
$\beta_0, \beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12}$	Model parameters for response surface analysis

ε	Disturbance (error terms)
ζ_1, ζ_2	Uncoded variables for response surface analysis
$\eta(\theta)$	n-dimensional prediction vector in non-linear regression analysis
θ	Parameter in non-linear regression analysis
μ	Overall mean
σ^2	Variance
$\tau, \alpha, \beta, \gamma, \delta$	Treatment effects
φ	Incremental parameter in non-linear regression analysis

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1. INTRODUCTION

1.1 Wild Blueberry: General Information

1.1.1 Importance of Wild Blueberry for the Regional Horticulture Economy

Atlantic Canada is the major producer of wild lowbush blueberries *Vaccinium angustifolium* (together with Maine, USA). In 2010, about 15% of lowbush blueberries were harvested in Quebec, and 85% of all wild blueberries produced in Canada were harvested in the Atlantic Provinces, namely Prince Edward Island, New Brunswick, and Nova Scotia. Nova Scotia produced 35.8% of the wild blueberries harvested in Canada (Statistics Canada).

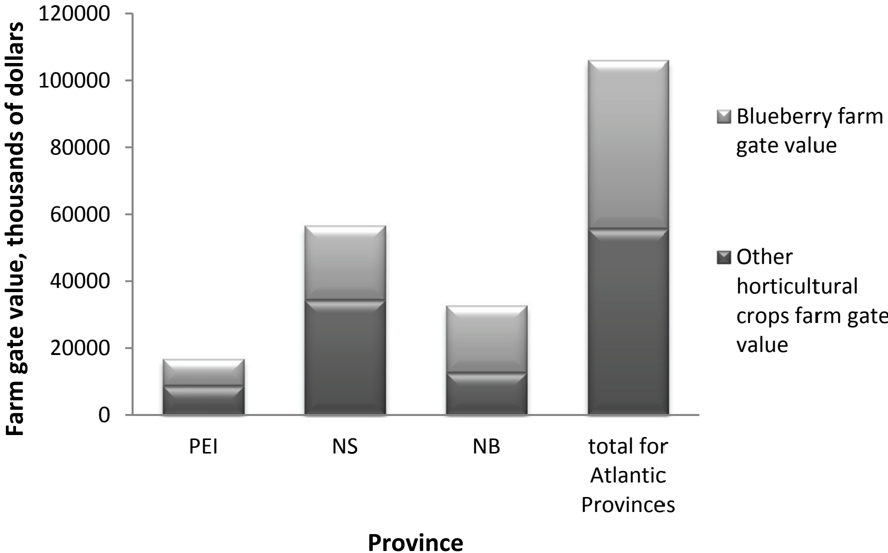


Figure 1.1 Share of blueberry farm gate in total fruit and vegetables farm production

The importance of wild blueberries for Atlantic Canada as a horticultural crop is demonstrated in Fig.1.1.The total farm gate due to blueberry production compared tothe total farm fruit and vegetable production (in thousands of dollars) in 2010 was 48.5%, 39.4%, and 61.3% for PEI, NS and NB respectively (Statistics Canada). In the past 20 years, wild blueberry production demonstrated a strong growth all across Canada: total area of farming increased 57%from 1992 till 2003 (Strik, 2005; Strik and Yarborough, 2005). In the Atlantic Provinces, total market production increased almost twice in the past 10 years, from 20,221 metric tonnes in 2001 to 35,288 metric tonnes in 2010, which gave more than a 2.5-fold increase in the farm gate value due to wild blueberry production (compare 19,800 thousand dollars in 2001 and 50,449

thousand dollars in 2011) (Statistics Canada). The increase in wild blueberry production demands new market development. Market size can be increased through the public's awareness of the health benefits of blueberries, and the availability of convenient blueberry-rich products can allow consumers to regularly eat these nutritious fruit.

In spite of the high amount of blueberries produced in Atlantic Canada, there are not many blueberry-based products on the local market. Fresh wild blueberries are available during the short harvest season and approximately 2 weeks after the end of it. Hence, only 0.4% of wild blueberries are consumed as fresh, and about 9% is used for production of juice (mainly), jams, jellies and pie filling. Some bakery and confectionary products with blueberries are also presented on the local market, such as pies, muffins, ice cream, yogurts, chocolate bars, gummy candies (Robichaud, 2006; Villata, 1998). The majority of harvested wild blueberries (90%) are individually quick frozen (IQF) and used mainly for export (Statistics Canada). Since 2000, the export of blueberries has represented 84 to 100% of all Canadian production (including re-export of some blueberries that Canada imported) (Robichaud, 2006).

Wild blueberry has a significant impact on the local horticulture economy and demonstrated a solid growth in the past years. There is a strong demand on wild blueberries overseas; the majority of Canadian blueberry is exported. Thus, the population of Atlantic Canada has a great production capacity for wild blueberries but limited access to use it. At the same time, significant changes of consumer habits and preferences towards healthier natural products were recently reported. The number of educated consumers in Atlantic Canada, and nationally and internationally interested in health-promoting foods, has increased significantly (Beaudin, 2005). However, the lack of commercially available high-quality healthy food has been specified as one of the major problems which should be resolved by producers. Locally grown wild blueberry is an excellent candidate for health-promoting foods. Development of new blueberry-based products will allow consumers to have more choices and different ways to consume blueberries, and on the other hand, will provide producers with greater market size for wild blueberries.

1.1.2 Wild Blueberry Nutrition Information

The nutrition information on blueberries is presented in Table 1.1. The nutrient content on a dry matter basis is presented on Fig.1.2.Sugars are the major component of blueberries' dissolved dry matter (65%). Lowbush blueberries contain almost equal amounts of glucose and fructose and do not contain any sucrose (Barker et al., 1963). As with all fruits and berries, blueberries are a good source of dietary fiber (2.4% of fresh product). Blueberries are not a very rich source of vitamins and minerals, however fresh blueberries contain a moderately high amount of vitamin C (9.7mg/100g FW, USDA Nutrient Database) and niacin (0.42 mg/100g FW, USDA Nutrient Database). A high content of manganese (0.34 mg/100g FW, USDA Nutrient Database) makes blueberries an excellent source of this mineral (Bushway et al., 1983).

Table 1.1 Wild blueberry nutrition information

Compound	Content, per 100g of FW	Source
Moisture	84.65	USDA Nutrient Database
Proteins	0.74	USDA Nutrient Database
Lipids	0.42	USDA Nutrient Database
Sugars	9.96	USDA Nutrient Database
Dietary fiber	2.41	USDA Nutrient Database
Pectin	0.60	Proctor and Peng (1989)
Acids	0.51	Mazza (2005)
Vitamins/minerals	0.12	Bushway et al.(1983)
Polyphenolics	0.40	Kalt et al. (2001), Prior et al. (1998)
Other	0.29	Found by subtraction

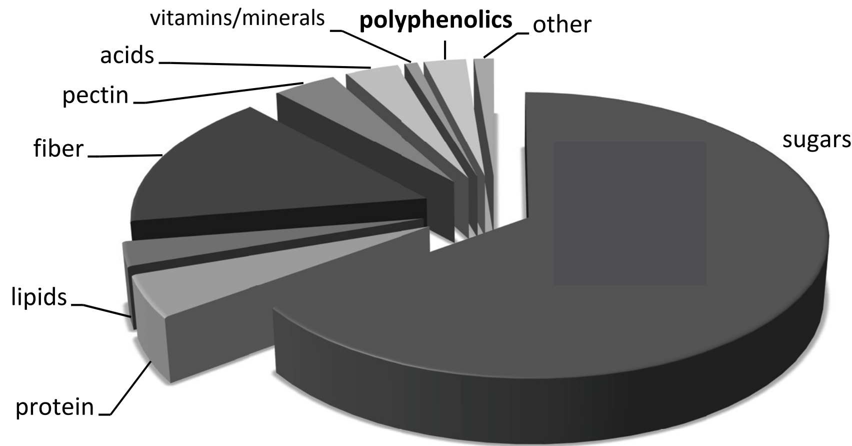
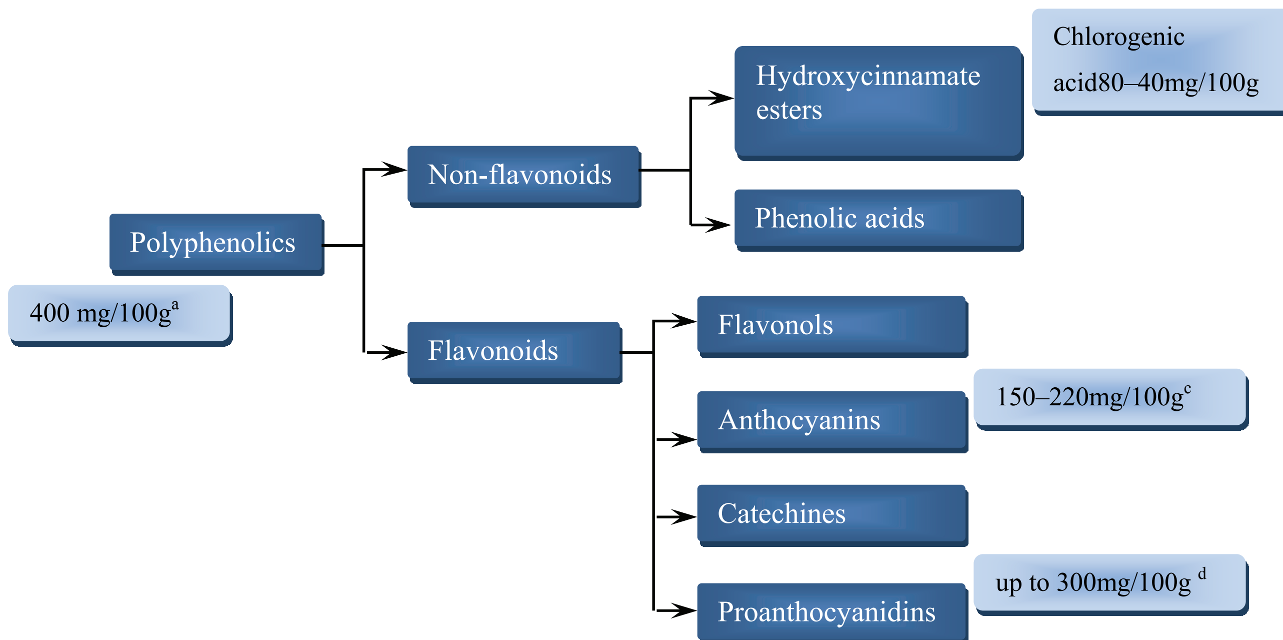


Figure 1.2 Wild blueberry dry matter

Wild (lowbush) blueberries *Vaccinium angustifolium* are well known as a rich source of polyphenolic substances. Fruit and vegetable polyphenolic have been of great interest to scientists for the past decades for their purported health benefits. Regular intake of fruit and vegetables is recommended by Health Canada in order to decrease the risk of development of certain diseases such as cancer and cardio-vascular diseases (Health Canada, 2009). Researchers attribute fruit and vegetables polyphenolics to be responsible for providing these potential benefits in human health.

Polyphenolics in small fruits species (including blueberries) can be divided into two major classes, namely flavonoids and non-flavonoids (Kalt et al., 2001) (Fig.1.3). The predominant non-flavonoid compound of blueberries is chlorogenic acid (group of hydroxycinnamate esters), with reported concentration of 80 to 140 mg/100g (Gao and Mazza, 1994; Tarusco et al., 2004). Other non-flavonoid compounds include hydroxycinnamic acids (for example, p-coumaric, caffeic, and ferulic acids) and simple phenolic acids (gallic acid) (Kalt and McDonald, 1996). The flavonoid compounds include several classes (Fig.1.3) with anthocyanins as one of the major sub-groups. Anthocyanins are well known as phytopigments and confer purple, red and blue coloration of fruit, vegetables and flowers, and they also have been reported as substances characterized by some biological activities in human health (For review see: Pascual-Teresa and Sanchez-Ballesta, 2008). Another significant group of flavonoids with potential biological activities is proanthocyanidins (condensed tannins). Their concentration has been reported by Gu et al. (2002). However, the major amount of total proanthocyanidin

concentrations in blueberries (330mg/100g FW) was due to the polymeric proanthocyanidins with a degree of polymerization more than 10 (up to 290 mg/100g FW), whose biological activity is questionable since they would be poorly absorbed (Gu et al., 2002; Crozier, 2009). Potential health benefits of the polyphenolics including anthocyanins will be reviewed in Section1.2.



[a] total phenolics, mg gallic acid equivalent per 100 g of FW, according to Prior et al. (1998) and Kalt et al. (2001)

[b] chlorogenic acid concentration according to Gao and Mazza (1994) and Tarusco et al. (2004)

[c] anthocyanin concentration, mg C3G/100g FW, according to Prior et al. (1998), Kalt et al. (2001) and Gao and Mazza (1994)

[c] proanthocyanidin concentration according to Gu et al. (2004)

Figure 1.3Polyphenolic compounds of wild blueberries

However, the polyphenolic substances in blueberry are very sensitive to different processing factors. It was reported, that traditional processing and storage technologies reduced anthocyanin concentration in blueberry-based products (Brownmiller et al., 2009; Kalt et al., 2000; Lee et al., 2002). Researchers have identified the need for the optimization of blueberry processing technology to prevent losses in food quality (Brownmiller et al., 2009; Kalt et al., 2001; Queiroz et al., 2009). More information on degradation of the polyphenolic substances will be provided in Section 1.3.

1.2Wild Blueberry Compounds and Their Purported Benefits in Human Health

Fruit and vegetables are the essential to proper human nutrition. Health Canada suggest regular intake of fruit and vegetables in order to decrease risk of development certain diseases such as some cancer and cardio-vascular diseases. There are numerous approaches to investigate the health-related bioactivity of plants foods like blueberries and their polyphenolic compounds.

Historically blueberries were first most intensively investigated in relation to their *in vitro* antioxidant activity which reflects the high redox capacity of their polyphenolics, including anthocyanins, and is based on chemical capturing different forms of free radicals. A high antioxidant capacity is implicated as the basis for potential positive effect of blueberries and other plant foods, on human health. Since polyphenolics differ in their antioxidant capacity (for review see: Rice-Evans et al., 1996; Prior and Wu, 2006; Cao et al., 1996), these simple redox methods allow for comparisons between compounds and even food types; however these tests do not reflect *in vivo* concentrations or conditions for these reactions. The next step of *in vitro* studies is modeling of physiological processes in specific use of specific cells or subcellular fractions to probe polyphenolic effects in more complex metabolic processes. These methods provide information on biological effects of the studied compounds but do not take into account bioavailability and hence do not insure that these reactions can take place intracellulary. In whole cell studies more complex multi-site processes can typically be proved. The further step of biological activity investigation is *in vitro* experiments with intact cells which allow one to collect more information on more complex cellular responses.

Studies conducted with animal are called *in vivo.* These studies are most often conducted using rodents. Compared to *in vitro* studies *in vivo* studies provide stronger evidence of food phytochemical benefits since positive result infer that bioactive compounds have been digestively absorbed in a quantity sufficient to achieve a benefit. Human clinical studies provide the strongest evidence of a health benefit of certain photochemical. Identifying a clinical benefit of a food or phytochemical in a free-living and genetically diverse human population suggests a strong effect and intervention. Clinical studies are typically expensive. However, human and animal studies are the most important part in any research on biological effects of phytochemicals and the results of these experiments will be reflected in this review.

1.2.1 Role of Polyphenolic Substances in Human Health

Numerous works were devoted to the antioxidant capacity of polyphenols as bioactive compounds in materials of plant origin. It was found that different groups of polyphenolics demonstrate no equal antioxidant capacity (Bors et al., 1997; Burda and Oleszek, 2001; Pietta, 2000), and anthocyanins are reported to have one of the highest ability against oxidative materials *in vitro*. Extensive research was carried out to study the relationship between molecular structure and antioxidant properties of the polyphenolics. For effective radical scavenging there were three criteria found important: the o-dihydroxy structure of B ring (provides higher stability for the radical form and participates in electron delocalization), the 2,3 double bond in configuration with a 4-oxo function in the C ring (is responsible for electron delocalization from the B ring), and 3- and 5-OH groups with 4-oxo function in A and C rings (are required for maximum radical scavenging potential (Rice-Evans et al., 1996).

The chemistry, nutritional significance, and health promotion properties of polyphenolic compounds in berry fruit phytochemicals are widely discussed (for review see Crozier et al., 2009; Del Rio et al., 2010; Tapiero et al., 2002; Stoclet and Schini-Kerth, 2011). Polyphenolics were reported to be involved in certain mechanisms of brain aging protection (Galli et al., 2002). The role of flavonoids on different aspects of the central neural system regulation and brain activity in particular was reviewed by Jager and Saaby (2011) and Spencer (2010).Some groups of polyphenolic substances were found to be preventive for breast cancer in an epidemiological study in Italy (Bosettii et al., 2005). Certain flavonoids demonstrated a high activity against cancer cell development, therefore their use as purified substances in anticancer medicine was considered by Liu et al (2010). A review of the biological mechanisms and effects of polyphenolic substances in cancer was done by Nichenametla et al. (2006).A five year epidemiological study demonstrated a strong correlation between daily polyphenolics intake and coronary heart disease (Hertog et al., 1993; Hollman et al., 1996). Activity of polyphenolics, and flavonoids in particular, in cardio-vascular diseases were summarized by Peluso (2006).

Activity of polyphenolic substances and its potential role in human health were studied and summarized by numerous authors (Crozier et al., 2009; Del Rio et al., 2010; Jager and Saaby, 2011; Spencer, 2010; Nichenametla et al., 2006). Some information on the biological activity of anthocyanins, as a group of polyphenolics, is provided in Section 1.2.2.

1.2.2 Role of Anthocyanins in Human Health

Anthocyanins are a major part of polyphenolic substances of wild blueberry and these compounds contribute significantly to the antioxidant properties of this fruit (Kalt et al., 2008; Wang et al., 2010). The anthocyanin aglycones (i.e. anthocyanidins) delphinidin and cyanidin have the highest antioxidant activity compared to other polyphenolics. Quercetin was reported to have the highest antioxidant capacity (Rice-Evans et al., 1996). The health-related bioactivity of anthocyanin extracts was studied using *in vitro* and *in vivo* models. The anthocyanins rich mixture of six edible berries including blueberries was studied for therapeutic effect determination by Zafra-Stone and co-workers. A significant inhibition of *Helicobacter pylori*, which is known to be a causative factor gastrointestinal diseases including gastric cancer, was observed for this mixture. Marked *in vivo* antiangiogenic properties (*in vivo* model of proliferating hemangioma) of edible berries anthocyanin extracts were also reported, which might be useful for cancer therapy. *In vivo* studies using hamsters showed a great effect of anthocyanin extracts against atherosclerosis (Zafra-Stone et al., 2007a, 2007b).*In vitro* results of anti-inflammatory properties of anthocyanin-rich berry blend were supported with the results of clinical trials (Jensen et al., 2008).

The chemistry, nutrition significance, and health promotion properties of polyphenolic compounds and anthocyanins in particular as berry fruit compounds are widely discussed. Anthocyanins are not involved in the mechanisms of protection directly as radical scavenging substances but participate indirectly in many metabolic reactions which results in blocking some pathways and hence positive effect in human health. For example, anthocyanins were reported to demonstrate certain activity against cardiovascular diseases (Mazza, 2007) and some forms of cancer (Nichenametla et al., 2011). Anthocyanins have demonstrated systematic activity in the human body such as antineoplastic, anticarcenogenic, antiviral and anti-inflammatory effects, inhibition of platelets aggregation and immune stimulation (for review see Stintzing and Carle, 2004). Antimicrobial effect of anthocyanins was reviewed by Cisowska et al. (2011). Chen and Luo (2010) demonstrated the effectiveness of anthocyanins application for the complex therapy of ethanol neurotoxicity.

Significant amount of works has been devoted to anticancer activity of anthocyanins (for review see Cooke et al., 2005; Fimognari et al., 2008; Hou, 2003; Thomasset et al., 2009; Wang and Stoner, 2008). Strong *in vitro* evidence supported with results of several clinical studies of

anthocyanins to play a role of cancer chemoprotective substances in human diet was reported by numerous authors (Fimognari et al., 2008; Thomasset et al., 2009; Wang and Stoner, 2008).Selective absorption and distribution of anthocyanins in organs and tissues was studied (Kalt et al., 2008; Talavera et al., 2005; Kalt et al., 2007); the ability of anthocyanins to be absorbed by gastric walls and rich target organs was supported by the results of these research works.

Potential biological activities of anthocyanins in human health and its bioavailability have been well investigated in many in vitro studies and some *in vivo* studies including experiments with human subjects and epidemiological studies (for review see McGhie and Walton, 2007; Galvano et al., 2007; Kong et al., 2003; Kowalczyk et al., 2003; Prior and Wu, 2006).

1.2.3 Role of Blueberry in Human Health

Benefits of plant origin products (fruits and vegetables) in decreasing the risk of different diseases has been demonstrated in numerous studies. The activity (as part of a complex therapy) against several diseases, such as certain cancers, cardiovascular diseases, and neurodegenerative diseases, has been referred to antioxidant compounds contained in these products (Schreiner and Huyskens-Keil, 2006). Polyphenolic compounds, a group of substances with antioxidant activities, are contained in different products of plant origin but usually attributed to berries.

Fruits and vegetables, as a source of different polyphenolic compounds, are known to be powerful antioxidant products recommended for consuming (Stintzing and Carle, 2004; Wang et al., 1996). Wild blueberries are reported as a product characterized by a high antioxidant capacity. Wild blueberries have the highest ORAC (oxygen radical absorbance capacity) value and total phenolics content among the twenty-five most commonly consumed USA fruits and berries (Wolfe et al., 2008) and high lipophilic and hydrophilic activities among common fruits in the US (Wu et al., 2004; Wu et al., 2006). The benefits of berry crops, blueberries in particular, as a source of antioxidants were shown in the works of Carbone and Zafra-Stone (Carbone et al., 2008; Zafra-Stone Shirley et al., 2007a; Zafra-Stone et al., 2007b).

Vaccinium species are known to be characterized by significant antioxidant activity due to high content of polyphenolic substances, but these properties can vary. Researchers agree that wild populations of *Vaccinium angustifolium* berries tend to have a higher antioxidant capacity

than cultivated ones (*Vaccinium corymbosum*) (Kalt et al., 2001; Kalt et al., 2008; Prior et al., 1998; Taruscio et al., 2004). Horticultural factors such as genotype variation, geographical variation (Häkkinen and Törrönen, 2000), weather conditions, maturity (for review see Kalt et al., 2001), organic production vs. conventional type of growing (Wang et al., 2008) significantly affect anthocyanin concentration of blueberries. There are contradictory information on changes of ORAC and polyphenolic content during ripening: Kalt and co-workers found no difference in ORAC value among five ripeness stages of highbush blueberry (Kalt et al., 2001), while Prior et al. (1998) reported higher ORAC value as a result of increased maturity and Castrejón et al. (2008) reported decreasing antioxidant capacity during ripening.

The antioxidant capacity of blueberries may be affected dramatically by different processing factors. The influence of cold storage on the ORAC value was studied (Connor et al., 2002; Kalt et al., 1999) and small changes during storage were reported. Heat treatment usually results in a significant decrease of the ORAC value of blueberry-based products (Brownmiller et al., 2009; Kalt et al., 2000; Schmidt et al., 2005). Antioxidant capacity of blueberry-based products also reduces during storage (Brownmiller et al., 2008; Srivastava et al., 2007). Intensification of antioxidant activity as a result of skin-contact fermentation of blueberry juices was reported for Rabbiteye blueberry (Su and Chien, 2007). A more complete analysis of processing factors, which have an influence on antioxidant properties of blueberries, is presented in Section1.3.2 of this document.

A regular intake of berries was reported to have a health promoting effect on the human body. A mixture of six edible berries extracts was studied for therapeutic effect determination by Zafra-Stone and co-workers (2007a, 2007b): a significant antimicrobial effect against *Helicobacter pylori*, which is known to be a causative factor for gastrointestinal diseases, including gastric cancer, and marked *in vivo* antiangiogenic properties were reported. Health-promoting properties of berry fruits including *Vaccinium* species, such as the improvement of blood vessel elasticity, urinary system infections prevention and treatment as part of a complex therapy; night vision improving was reviewed by Szajdek and Borowska (2008). The impact of berry fruit in a complex therapy of cardio-vascular diseases was reviewed by Basu et al. (2010).

Health promoting effects of wild blueberries on the human body are of particular interest. Numerous works were devoted to different aspects of wild blueberry consumption and their activity in preventing certain diseases (for review see Neto et al., 2007; Kalt et al., 2007; Shi et

al., 2002; Smith et al., 2000; Kalt and Dufour, 1997). More specific information on the role of blueberry supplementation in certain diseases prevention is summarized below in Sections1.2.3.1 to 1.2.3.7.

1.2.3.1 Blueberries and Cardiovascular Diseases

It has been thought that cardioprotective effect of blueberries may be partly explained by the inhibition of oxidation of low-density lipids (LDL) by berry phenolics and the ability of phenolics to reduce aggregation of blood platelets, which can lead to heart attack and stroke. Oxidation of LDL by free radicals initiates and promotes atherosclerosis, as well as blood platelets aggregation and adhesion to blood vessels resulting in thrombosis development (Hertog et al., 1993). For example, *in vitro* lipophilic antioxidative capacity of blueberry was proved by Wu and others (Wu et al., 2004; Wu et al., 2006). However the low plasma bioavailability of polyphenolics including anthocyanins (for review see Crozier, 2009) would suggest that direct *in vivo* antioxidant effects of polyphenolics on LDL are not be likely. No relationship between polyphenolics content and anti-platelet activity of blueberry was demonstrated by Kalt and co-workers (Kalt et al., 2007, 2008). At the same time, reduced total cholesterol in pig plasma was found as a result of feeding with blueberry when added to a plant-rich diet (Kalt et al., 2008). Another mechanism which prevents cardio-vascular diseases is an ability of polyphenols to increase vasodilation by inducing relaxation of vascular smooth muscles. Blueberry polyphenolic effects was studied *in vivo* and *ex vivo* (Kalea et al., 2006; Kalea et al., 2009a; Kalea et al., 2009b; Norton et al., 2005). It was found that feeding blueberries for up to three months reduced ischemic damage to the heart, in rats (Ahmet et al., 2009). Improved vasorelaxation was observed *ex vivo* after blueberry feeding (Kalea et al., 2009a). Wiseman et al. (2011) reported an anti-hypertensive effect in rats after 2 weeks of receiving a blueberry rich diet.

1.2.3.2 Blueberries and Cancer

Several reviews have been published with the conclusion that fruit and vegetables may reduce risk of some types of cancer (Block et al., 1992; Bomser et al., 1996). Results of studies conducted *in vitro* suggested berries polyphenolics (including *Vaccinium* species and wild blueberries in particular) were able to inhibit processes associated with tumor development (Schmidt et al., 2004; Seeram, 2008; Bomser et al., 1996). Schmidt and co-workers found strong *in vitro* antiproliferative activity of wild blueberry extracts (Schmidt et al., 2004; Schmidt et al.,

2005). Wild blueberry extracts demonstrated activity against cancer cells growth in *in vitro* studies by Bomser et al. (1996). Seeram and co-workers reported ability of berry bioactives to regulate carcinogen and xenobiotic metabolizing enzymes, various growing factors, inflammatory cytokines and signaling pathway of cancer cells proliferation and tumor angiogenesis *in vitro* (Seeram et al., 2006; Seeram, 2008). The possible protective effect of blueberries against cancer may be related to their ability to indirectly participate in some defensive mechanisms of the human body (Dulebohn et al., 2008; Seeram et al., 2006; Seeram, 2008). However, there is a lack of in vivo evidence of anti-cancerogenic effect of blueberry. Boateng et al. (2007) reported positive effect of blueberry extracts in colon tumor development in rats; however, Simmen et al. (2009) reported that results of in vivo studies of colon cancer in rats did not indicate robust effects of blueberry juice. Researchers agree that more in vivo and clinical studies are needed to prove effect of blueberry against tumor development (Moon et al., 2006; Neto, 2007).

1.2.3.3 Diabetes

Regular consumption of fruit and berries rich in polyphenolics is beneficial for prevention type II diabetes (for review see Hanhineva et al., 2010). Results of clinical study by Lankinen et al. (2011) suggested using bilberry (European wild blueberry) as a part of healthy diet (rich in whole grains and fish) for reducing glucose concentration in blood and improving glucose metabolism. Blueberry anthocyanins were reported as substances which decrease aldol reductase of diabetic patients (Ghosh and Konishi, 2007; Varma, 1986).Fruit juices fermented by *Serratia vaccinii* demonstrated significant anti-inflammatory, antiobesity and antidiabetic activity in *in vitro* and *in vivo* tests with rats (Vuong et al., 2006, 2007, 2009).

1.2.3.4 Vision

The European type of blueberry (bilberry, *Vaccinium myrtillus*) is well known historically used for benefits in vision and eye health. Bilberry was purported to be used in World War I and II by British Air Force pilots to improve night vision (see Canter and Ernst, 2004). However, there is not enough evidence from clinical studies to conclude that night vision and dark adaptation of healthy human subjects was affected significantly by bilberry anthocyanins consumption (for review see Canter and Ernst, 2004).There is a significant lack of

relevant information on activity of phytochemicals of wild blueberry on human vision, especially of human clinical placebo-controlled trials (Kalt et al., 2010).

1.2.3.5 Microbial Infections, Inflammation

The possibility of using *Vaccinium* berries for urinary system infection prevention was reported by Ofek (1991). Low bush blueberry had one of the highest anti-adhesion activities (within *Vaccinium* species and other fruit crops) *in vitro*, against *E.coli* which causes urinary tract infection (Kalt et al., 2007). Wild blueberry extract showed a significant inhibition of *Helicobacter pylori* growth in tests *in vitro* (Zafra-Stone et al., 2007). *In vitro* assays of anti-inflammatory activity were conducted (Gabor, 1986; Jensen et al., 2008) and demonstrated the participation of blueberry bioactive substances in different mechanisms of inflammatory reduction. The ability of blueberry to reduce chronic inflammation is emerging as an important bioactivity since chronic inflammation underlies disease, degeneration and aging. Evidence of *in vivo* benefits against chronic inflammation by blueberries is significant (for review see Giacalone et al., 2011; Chun et al., 2008). However, most of this research was carried out as *in vitro* experiments. There is a significant lack of *in vivo* data which prevents one from making clear statements on blueberry activity in anti-inflammatory processes in the human body.

1.2.3.6 Hepatoprotective Action

Significant and strong results of blueberry (bilberry) intake have been demonstrated in *in vivo* experiment with rats infected with provoked toxic hepatitis. Bilberry purée processed using hydrothermodynamic technology demonstrated almost complete prevention of most symptoms of hepatic pathology (Levitsky et al, 2009).). Significant effect on liver health in rats fed with blueberry diet was reported by Wang et al. (2010) and in hamster fed with blueberry pomace diet by Kim et al. (2010).

1.2.3.7 Brain Activity

Deterioration of brain functions such as short-term memory loss, information retrieval, balance and coordination is associated with aging. A blueberry rich diet was shown to reverse the decline in brain functions induced by aging (Joseph et al., 1999). Significant positive effects arising from blueberry supplementation diet was reported for the cognitive and motor behavior function of rats, as well effects neuronal signal transmission and protective mechanisms associated with brain-aging process (Joseph et al., 1999; Joseph et al., 2003; Joseph et al., 2009;

Shukitt-Hale et al., 2008). Studies of motor behavior, cognitive performance, learning, and memory functions, as influenced by a blueberry rich diet for aged rats demonstrated a strong positive effect of wild blueberry consumption on brain activity in Alzheimer disease model (Joseph et al., 1999; Papandreou et al., 2009). In an *in vivo* study, protective effect of blueberry on brain vessels decreased ischemia-induced damage of the brain hippocampus, in rats (Sweeney et al., 2002). Clinical studies on older humans with early memory changes who consumed blueberry juice for 12 weeks showed significant cognitive function performance improvement. It was recommended to use blueberry supplementation as a preventive approach in Alzheimer disease (Krikorian et al., 2010). For review see Joseph et al. (2009), Willis et al. (2009), Kalt et al. (2007).

To sum it up, wild blueberries are a food product with one of the highest antioxidant activity in *in vitro* tests. Antioxidant activity of blueberry is related to high polyphenolics content, particularly anthocyanins. Biological activities of polyphenolics and anthocyanins in particular were also demonstrated in *in vivo* experiments with both mammals and human subjects. Hence, wild blueberries are a promising raw material for health-promoting food products. Development of high quality blueberry-based products will allow producers to meet a demand from health-conscious consumers and to lunch more products on the local market. However, anthocyanins are sensitive to temperature and oxidation, so processing of blueberry results in the destruction of this substances and thus in the decrease of antioxidant activity. For saving antioxidant properties of blueberry-based products it is necessary to optimize blueberry processing (Kalt et al., 2001). More details on blueberry anthocyanin degradation are presented in Section 1.3.

1.3 Effect of Processing and Storage on Blueberry Polyphenolics

The most popular food products based on wild blueberries are clarified blueberry juices and blueberry-based beverages. Some traditional products such as jams, jellies, and purées are also presented on the market but not a wide variety. Also, blueberries are a very popular addition for different bakery products like muffins and pies (Villata, 1998). Both fresh and frozen berries can be used for pie filling. A quickly growing sector of the market of functional food includes concentrated and powdered blueberry juices as well as different products based on wild blueberry pure and blended extracts.

Processing and storage of blueberries can significantly affect their bioactive and antioxidant properties. Thermal treatment, necessary for enzyme inactivation and product pasteurization, results in significant degradation of anthocyanins (Brownmiller et al., 2008; Brownmiller et al., 2009; Kalt et al., 2001; Kalt et al., 2000; Schmidt et al., 2005). Oxidation of polyphenolics can be provoked by light (Carlsen and Stapelfeldt, 1997), oxygen (Jackman et al., 1987), enzymes (Kader et al., 1999) and products of sugars degradation (Queiroz et al., 2009).

1.3.1 Effect of Processing Methods

The effect of processing methods such as freezing, thawing, pressing and filtration, powdering and concentration, and pasteurization on blueberry polyphenolics was summarized and presented in this section. Most of processing methods negatively affect polyphenolics (anthocyanins in particular).

1.3.1.1 Freezing

Most of blueberries (95%) are frozen just after harvesting, so the largest source of various blueberry products are frozen blueberries. There are two different methods of freezing such as bulk freezing and individual quick freezing (IQF), and it is generally assumed that IQF retains better the quality of fresh berries (Schmidt et al., 2005). An advantage of using IQF berries for juice processing could be attributed to breakdown of cell walls by ice crystals, which helps in releasing of anthocyanins and other bioactive compounds from cells (Stewart, 1996).

1.3.1.2 Thawing

Frozen wild blueberries are the main raw material for blueberry-based products. Methods and regimes of thawing dramatically affect the amount of polyphenolic substances initially presented in fresh blueberries (Brownmiller et al., 2008; Brownmiller et al., 2009; Stewart, 1996). During thawing, polyphenolic substances become available as a substrate for the oxidative enzymes such as polyphenoloxidase (PPO) and peroxidase (PO) as a result of cell wall disruption during the process of freezing. Significantly superior quality of blueberry juice reported by Brownmiller and co-workers (Brownmiller et al., 2008) could be attributed, as suggested by the authors, to different methods of thawing. It was found that rapid thawing using a high-temperature (steam) treatment prevents enzymatic oxidation and results in significantly higher retention of anthocyanins: 80% of original monomeric anthocyanin concentration

(Brownmiller et al., 2008) as compared to 22% (Lee et al., 2002) and 32% (Skrede et al., 2000). These results were obtained for non-clarified juice (without filtration). For clarified juice the difference was less obvious but still significant: 46% (Brownmiller et al., 2008) vs. less than 22% (Lee et al., 2002). It follows that all additional technological operations such as crushing, pressing, depectinization, filtering etc. in blueberry processing results in significant losses of polyphenols (Brownmiller et al., 2009; Lee et al., 2002; Skrede et al., 2000), because of additional enzymatic and non-enzymatic oxidation.

1.3.1.3 Pressing and Filtration

Pressing and filtration during berry juice processing results in significant losses of bioactive substances. Lee et al. (2002) reported the amounts of bioactive substances found in the pomace (the pulp from a press-cake) as compared with the initial quantities found in frozen blueberries: 15 to 20 % of polyphenolics, 41 to 55% of anthocyanins, and 10 to 15 % of cinnamic acid. Some other studies reported contradictory results. The quantity of anthocyanins found in the pomace by Skrede et al. (2000) was 18%. Kalt et al. (2000) reported that the pomace by-product of juice processing had about 16% of the ORAC value of fresh wild blueberries and was correlated with anthocyanin concentration. Similar results were shown by Brownmiller and co-workers (2008, 2009): 15 % of anthocyanins were lost at the press-cake and another 25% was removed as sediment at the clarification step. Most researchers agree that a significant quantity of anthocyanins is lost in blueberry juice processing with pomace: from 15 % (Brownmiller et al., 2008) to 55% (Lee et al., 2002). Since anthocyanins are mostly located in skin tissues, filtration of pulp results in significant losses of antioxidant properties in the end product (Brownmiller et al., 2009).

Lee et al. (2002) reported the following amounts of bioactive substances retained in the juice: only 13 to 23% of anthocyanins and 36 to 39% of polyphenolics was present in the pasteurized juice compared to the frozen fruit, and 25 to 35% of anthocyanins degraded (not founded in the final product and a pomace). Skrede et al. (2000) reported recoveries in juice of only 32, 35, 43 and 53% of anthocyanins, flavonols, procyanidins and chlorogenic acid respectively. It was demonstrated that losses were mainly due to the berries' PPO and PO enzyme activity (Skrede et al., 2000). Brownmiller et al. (2008) reported only5% loss of anthocyanins in non-clarified juice, and explained this high level of retention as a result of the rapid thawing step and high-temperature enzyme inactivation.

Sulfur dioxide treatment with of blueberries during juice processing is sometimes used in juice manufacturing, but the data on the effects of SO_2 contradict each other. For example, Lee and others found that using SO_2in blueberry juice processing allows better enzyme inactivation and increased anthocyanins recovery (Lee et al., 2002). However, Wrolstad (2002) reported anthocyanins react with SO_2 to form colorless products.

Treatment with pectolitic enzymes is used for pectin destruction in blueberry tissues thus increasing the quantity of pressed juice, and it could be another reason for anthocyanin losses. Pectolitic enzymes used for juice processing may have glycosidase contaminating activity which deglycosylate and destabilize anthocyanins (Wightman and Wrolstad, 1996).

Wild blueberry juice is characterized by strong organoleptic properties (intensive flavor and color) and is deemed a healthy product, but traditional technologies of juice processing results in dramatic losses of health promoting phytochemicals in the end product. It follows that pressing and filtration steps result in losses of bioactives: only 80% and 46% of anthocyanins were found at non-clarified and clarified blueberry juices respectively (Brownmiller et al., 2008) as compared with frozen berries, which is higher than that obtained by Skrede and co-workers (32%) and Lee and co-workers (35 to 36%) for non-clarified juices (Skrede et al., 2000; Lee et al., 2002).

1.3.1.4 Extraction, Powdering, Juice Concentration

Different methods of moisture removal are used to concentrate blueberry juice and extracts such as drying (spray-drying, freeze-drying) and evaporation under normal pressure and lower pressure (and, thus, lower temperature). Increasing the solids content in juice should result in a higher concentration of bioactive substances in the end product. Concentrated or powdered juices can be used as an ingredient for beverage mixing, and powdered extracts are used as a compound for pharmaceuticals (more popularly for bilberry, *Vaccinium myrtillus* (Kalt and Dufour, 1996). Unfortunately, these processing methods might result in markedly lower content of antioxidants in the end product. It was found that antioxidant capacity (ORAC) of commercial juice concentrate (65 °Brix) was only 65% as compared to fresh fruit (12 °Brix) (Kalt et al., 2000). However, Skrede et al. (2000) and Lee et al.(2002) reported 82.0 to 98.5 % anthocyanin recovery after low pressure evaporation step.

Powders, obtained with extraction and following drying, are characterized by relatively high content of bioactives. Jackman and Smith (1996) reported spray-dried powders processed

from grape pomace can contain about 1 to 4% of anthocyanins. Sugar added as a carrier during drying process resulted in improved anthocyanin retention. However, some contradictory results on the usefulness of extract powdering were presented. It is known that there is a rather low level of anthocyanins recovery using extraction (about 20%) (Srivastava et al., 2007). Drying processes also significantly affect phenolics content: a 2-fold loss of total anthocyanins as a result of slow cabinet drying was reported (Lohachoompol et al., 2004). Powdered and concentrated blueberry juices used as colorants for extruded corn meal, showed noticeable degradation of anthocyanins as a result of a high temperature (130°C) treatment. Only 26% and 10% of anthocyanins were obtained for blueberry concentrate and blueberry powder respectively (Camire et al., 2002).

Moisture removal from blueberry juice and blueberry extracts can demonstrate good results and allows getting a product with high antioxidant content, but only when accompanied with reduced impact technologies such as low pressure evaporation or freeze-drying to prevent bioactive substances destruction (Skrede et al., 2000; Lee et al., 2002).

1.3.1.5 Thermal Treatment (Pasteurization)

Pasteurization is the method of heat treatment which is commonly used for increasing the shelf-life of blueberry products and prevents the microbial spoilage during storage. Heat treatment negatively affects the antioxidants content and is a factor for its destruction during the pasteurization process. From the other side, heat treatment is necessary for enzyme inactivation since enzyme activity is responsible for polyphenolic losses in blueberry processing. More detailed information about the effect of temperature on phenolics content is presented in Section1.3.2.1.

The effects of pasteurization have been well studied for blueberry juice processing, but results reported by different researchers contradict each other. First of all, Brownmiller and co-workers reported 8% and 5 % losses for non-clarified and clarified blueberry juices as compared with fresh berries at the pasteurization step (heating of bottled juice in a steam box till 90°C). On the other hand, some authors reported increased anthocyanin concentration after pasteurization: at 4 % (Skrede et al., 2000), high temperature short time (HTST) pasteurization at 90°Cfor 90 seconds, and at from 2.5% to 8% (Lee et al., 2002), HTST pasteurization at 90°Cfor 90 seconds. However, similar effect of pasteurization on total polyphenolic content was not found; Lee et al.

(2002) reported insignificant changes in the polyphenolics due to pasteurization. These results are difficult to compare because of different regimes and methods of treatment used.

Queiroz et al. (2009) reported losses of anthocyanins during handmade blueberry jam preparation. Pasteurization of jams was carried out during cooking at temperature 102 – 105 °C. A15 – 20 minute thermal treatment results in 70 – 80% of anthocyanins recovery, a 25 minute treatment retained of only 40% of the berry anthocyanins. Kalt and others reported a 2-fold drop in the ORAC level of pasteurized commercial jam as compared with fresh lowbush blueberry fruit (Kalt et al., 2000). Howard et al. (2010) reported 79% retention of anthocyanins in blueberry jams (made with sugar or sugar-free) cooked for 1 minute at 101 – 105°C. The ORAC value retention was found to be 90% and 79% for jam and sugar-free jam respectively.

1.3.1.6 Blueberry Juice Processing

The main stages of blueberry juice processing are presented at Figure 1.3 (Lee et al., 2002; Skrede et al., 2000).Table 1.2 shows that losses of anthocyanins during thawing, crushing, depectinization and following pressing of the blueberries can reach from 5 to 54 %. Quick thawing (using steam) allows minimal anthocyanin loss (Brownmiller et al., 2008). A significant part of anthocyanins is removed from the final product as a pomace byproduct, so the content of anthocyanins in the juice is only 12 to 41% of the initial content in frozen berries.

Blueberry skin and outer layer of the pulp contains a much higher amount of anthocyanins than the pulp of blueberries (Gao and Mazza, 1994). Riihinen et al. (2008) reported up to a 320-fold difference in anthocyanin concentration for berry skin and berry pulps in blueberries *Vaccinium angustifolium x Vaccinium corymbosum* (compare 622.3 mg/100g in skin and 1.9 mg/100g in pulp) and a 19-fold difference in bilberries *Vaccinium myrtillus* (compare anthocyanin concentration 2025.6 mg/100g in skin and 104.0 mg/100g in pulp). Blueberry juice processing involves separating skins from the final product; hence, a significant amount of potentially beneficial human health substances, particularly anthocyanins, is removed from the final product (juice). Processing blueberries into purée (whole blueberries with skin) allows one to produce more blueberry-rich products with higher amounts of anthocyanins and possibly with improved properties in some health and food applications.

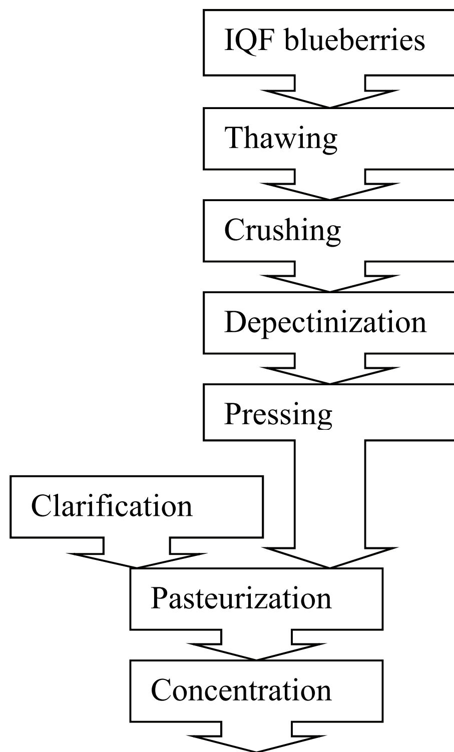


Figure 1.4 Blueberry juice processing stages
(Lee et al., 2002; Skrede et al., 2000)

For blueberry juice processing, the best result for anthocyanins retention (72% of initial anthocyanin concentration) was obtained for non-clarified blueberry juice processed using intensive thawing (Brownmiller et al., 2008). The worst results (5% of initial anthocyanin concentration) were found for clarified juice processed without blanching as an intermediate step (Lee et al., 2002).

Table 2.2 Anthocyanins retention in blueberry juice processing stages (Lee et al., 2002; Skrede et al., 2000; Brownmiller et al., 2008, 2009)

Process stage	Semi-product	% of anthocyanins retention
Thawing	Defrosted berries	n/a
Crushing	Blended berries	n/a
Depectinization	Depectinized berries	n/a
Pressing		
juice	Pressed juice	5 – 80
press-cake	Pomace	15 – 55
Clarification	Clarified juice	5 – 46
Pasteurization	Pasteurized juice	13 – 41 (72*)
Concentration	Concentrated juice (calculated for initial berries °Brix)	13 – 31

* - for non-clarified juice

Blueberry juice is one of the most convenient ways for customers to consume blueberries. Unfortunately, commercial technologies of blueberry processing as shown above do not provide high retention of anthocyanins. Since most of all polyphenolics are contained in solid material, a significant part of them is removed from the product as a waste product (pomace). Moreover, additional losses are caused by degradation of bioactives during process stages because of enzymes activity, oxygen, light, temperature, etc.

1.3.2 Effect of Processing Factors

Effects of processing on anthocyanins can be attributed to the effects of different processing factors of physical (temperature, oxygen, light), chemical (pH, sugars, pectins) and biochemical (enzymes) nature (Francis, 1989). Some of these factors affect anthocyanin stability negatively by increasing the rate of oxidative reactions. Other factors prevent polyphenolics from oxidation (low pH level) and there are some factors like temperature (which was reviewed in Section 1.3.1.5) and sugar content which can both increase or decrease degradation of polyphenolics depending on the value of influenced factor.

1.3.2.1 Effect of Temperature

Thermal methods are commonly used for blueberry preservation. Pasteurization is necessary for providing a prolonged shelf life of jams, juice, purée and other blueberry foods as a technique to reduce microbial load. Also blueberries are traditionally used as a filling for pastry baking and are therefore subjected to high temperature. But polyphenol substances, anthocyanins in particular, are characterized by a very high sensitivity to thermal treatment and storage temperature. From the other side, thermal treatment is necessary to inactivate enzymes which contribute to polyphenolic oxidation and condensation (Kader et al., 2002; Kader et al., 1997; Kader et al., 1998; Kader et al., 1999).

Different research shows a significant degradation of the polyphenolic substances and a decrease in antioxidant properties as a result of thermal treatment. For blackberry and other berry juices it was found that the rate of anthocyanin loss is strongly time and temperature dependent (Cisse et al., 2009; Wang and Xu, 2007).In these studies, anthocyanin loss was reported by authors to be explained by a first order exponential dependence; higher temperatures resulted in higher anthocyanin loss for the same period of time.

Brownmiller and co-workers reported perfect anthocyanin retention after 3 minutes at 95°C treatment in highbush blueberries (Brownmiller et al., 2008; Brownmiller et al., 2009). Exposure of canned blueberry jars in boiling water for 15 minutes resulted in 43%, 34% and 28% loss of anthocyanins for highbush blueberry purée, berries canned in water and berries canned in syrup respectively. Blanching resulted in a 26% loss of the antioxidant capacity of berries. The antioxidant capacity of purée, canned in water and canned in syrup berries decreased by 47%, 42% and 46% respectively as compared with fresh berries (Brownmiller et al., 2008; Brownmiller et al., 2009).

Anthocyanin loss during jam preparation was studied (Queiroz et al., 2009). Handmade blueberry jams characterized by different sugar content were processed at 102 – 105°C during 15, 20 and 25 minutes. Higher anthocyanin loss (30 – 45%) was in the samples prepared without sugar as compared with 64 – 76 °Brix samples (20 – 35% of anthocyanin loss) in case of 15 and 20 minute of heat treatment. Samples with high sugar content heated for 25 minutes demonstrated a significant loss of anthocyanins (approx. 60%) compared with similar anthocyanin concentration in no sugar samples (for 20 and 25 minute treatment) (Queiroz et al., 2009).

Kalt et al. (2000) reported the effect of extraction temperature on polyphenolics content and antioxidant activity of lowbush blueberry purée extracts as a result of extraction and during storage. Treatment at 60 °C resulted in a much higher extraction of total phenolics, anthocyanins and antioxidant capacity compared with 25 °C treatment but poorer retention after 2 weeks of storage at 20°C.The authors recommend optimizing time-temperature regimes during blueberry processing to maximize antioxidant capacity of a final product.

Health functional bioactivity of polyphenolics can be affected by processing. Studying the antioxidant capacity (ORAC) of different commercial lowbush blueberry products (such as purée, pie filling, canned fruit, jam, dried fruit, concentrated juice) showed significant losses compared with fresh fruits (Kalt et al., 2000). Unfortunately, regimes of heat treatment were not specified in this work. One of the most significant losses was observed for concentrated juice: the ORAC value of juice concentrate (65 °Brix) was only 65% of fresh blueberries (12°Brix). Kalt et al. (2000) reported 20% loss in the ORAC in commercial pasteurized blueberry purée compared with fresh berries (calculated for dry weight). There were some contradictory results from Brownmiller et al. (2008), which demonstrated that highbush blueberry purée processing led to a 43% loss of monomeric anthocyanins and a 47% loss of antioxidant capacity.

The baking of berries as part of pastry and other dishes resulted in loss of antioxidant properties similar to that for other types of heat treatment. Kalt et al. (2000) found about a 15% ORAC loss for fresh baked blueberries in pies compared with fresh wild blueberries. Berries from the pies prepared using commercial canned pie filling had only about one third of the fresh baked fruit ORAC level. It was reported by Queiroz et al. (2009) that the anthocyanin degradation of highbush whole blueberries cooked in stuffed fish ranged between 45 and 50%.

Schmidt and co-workers found that thermal treatment of fresh and IQF blueberries resulted in a loss of anti-proliferative activity in *in vitro* anti-cancer test (Schmidt et al., 2005). Regimes of heat treatment were not specified, however there was no significant anticancer activity found in heat processed blueberry products (jam, juice concentrate, canned whole fruit, and spray-dried powder).Despite some contradictory results studies agree that a significant amount of phenolic antioxidant loss during heat treatment was observed.

1.3.2.2 Effect of Oxygen

Oxygen has a negative effect on the polyphenolic compounds. Kalt et al. (2000) found a 76%, 30% and 46% decrease in monomeric anthocyanins, total phenolics and the antioxidant capacity respectively during 6 hours of intensive oxygen influence on lowbush blueberry juice. Anthocyanins stored under vacuum or in nitrogen media had higher stability than anthocyanins exposed to oxygen (Jackman et al., 1987). Oxygen oxidizes anthocyanins directly, and the products of this reaction do not have antioxidant activity (Jackman et al., 1987). Also some anthocyanins (with o-diphenolic B ring) can be oxidized by o-quinones formed as a result of the oxidation of chlorogenic acid by PPO (Kader et al., 1997). For the prevention of polyphenolic compound losses as a result of oxidation authors suggest using special types of packaging with a high oxygen barrier and minimizing headspace and packaging spare volume.

1.3.2.3 Effect of Light

Visible and UV light can contribute to losses in anthocyanins. It was reported that light is one of the major causes of anthocyanin degradation during storage of elderberry extracts and depended on the wavelength of irradiation (Carlsen and Stapelfeldt, 1997). Shorter wavelengths were more deteriorating, thus excluding ultraviolet light would improve the stability of anthocyanins. Selecting proper packaging materials with a high light barrier can improve significantly the stability of bioactive substances during shelf-life.

1.3.2.4 Effect of pH Level

Anthocyanins demonstrate higher stability at low pH (Francis, 1989). Cabrita et al. (2000) found that after 60 days of storage at 10 °C in acidic conditions (pH levels 1 – 3) more than 70% of the initial anthocyanins remained, while more than 90%was lost after 8 days at a pH level of 5 – 6 at the same temperature. Additional acidification may provide stability of anthocyanins. As the antioxidant capacity is correlated with the anthocyanin concentration, eventually it results in maximum antioxidant activity of the final products (Kalt et al., 2000).

1.3.2.5 Effect of Sugars

It was reported (Queiroz et al., 2009) that adding sugar (64 – 76 °Brix) to blueberry jams led to 20 to 30% loss of anthocyanins, and higher sugar content (80°Brix) resulted in50 to 60% loss. The authors connected anthocyanins loss with sugar degradation in the Maillard reaction.

Other studies on blackberry anthocyanin loss during processing agree that its retention is influenced by sugar content (Rubinskiene et al., 2005; Wang and Xu, 2007).

1.3.2.1 Effect of Enzymes

Blueberries contain enzymes such as PPO which damage polyphenolics and reduce antioxidant activity (Skrede et al., 2000). Anthocyanins are not the main substrate for PPO, but they degrade in the reaction with oxidation products of PPO such as hydroxycinnamate esters, phenolic acids, flavones and flavonols (Kader et al., 1997; Kader et al., 1998; Kader et al., 1999). Peroxidase can also lead to in anthocyanin degradation (Kader et al., 2002). In the intact fruits, PPO and peroxidase are located in the cytoplasm, while polyphenols are located in vacuoles of plant cells. Once tissues are disrupted during processing, it creates conditions for enzymatic oxidation of phenolic substances (Lee et al., 2002). Glycosidases may deglycosylate and destabilize anthocyanins but berries are not a source of this type of enzymes. Anthocyanins glycosides (i.e. anthocyanidin glycosides) are much more stable than anthocyanidin aglycones. Potential sources of glycosidase activity can be from moldy fruits and commercial enzyme systems which are used for depectinization in juice processing (Wightman and Wrolstad, 1996). Heating of berries for enzyme inactivation is necessary for retaining maximum antioxidant capacity in the finished product and during shelf-life (Brownmiller et al., 2008). Adding sulfur dioxide may also decrease enzyme activity (Lee et al., 2002) but SO_2 can react with anthocyanins and form colorless components (Wrolstad, 2000).

1.3.3 Effect of Storage

1.3.3.1 Fresh Blueberry Storage

Fresh wild blueberries are only available for a short period after harvesting and cold storage. Polyphenolic content may increase during cold storage of some fruits and berries (Kalt, 2005). Increasing anthocyanin concentration was observed in underripe wild blueberries after harvest in cold storage (Kalt and McDonald, 1996), fully blue fruit did not do so. In the study by Kalt et al. (1999) anthocyanin concentration and ORAC did not change during 8 days of storage at 0, 10, 20, and 30°C. Higher (Connor et al., 2002; Kalt et al., 1999) and lower (Lohachoompol et al., 2004) anthocyanin concentration was observed during cold storage in highbush

blueberries. It may be conclude that short cold storage does not affect significantly the antioxidant properties of blueberries.

1.3.3.2Frozen Blueberries Storage

Most wild blueberries (95%) are frozen just after harvesting, so the general source for various blueberry products are frozen blueberries. Information about the changes of bioactive substance concentration during freezing and low temperature storage is quite limited. There was a report about the decreasing antioxidant capacity of IQF samples of blueberries compared with fresh samples (Kalt et al., 2000) but it was not specified whether these blueberries originated from the same composite and what the time of storage was. Lohachoompol et al. (2004) reported the increasing anthocyanin concentration in blueberries stored for 1 and 3 months in a frozen condition but any explanation of this finding was not suggested. The quality of IQF blueberries is considered to be stable during all periods of storage and a lot of researchers use it as a raw material and/or control for experiments (Brownmiller et al., 2009; Lee et al., 2002; Queiroz et al., 2009). However, information on quality changes during storage of blueberries in frozen conditions is limited; also there is no information on berry quality IQF vs. bulk freezing. The kinetic of polyphenolics change and loss during low-temperature storage has not been studied.

1.3.3.3Blueberry Based Product Storage

Researchers have reported significant degradation of polyphenolics during product storage (Gil et al., 1999; Larrauri et al., 1997; Srivastava et al., 2007) and this process is described as time and temperature dependent. Srivastava et al. (2007) found a very high stability of polyphenols and antioxidant activity of blueberry extracts stored in glass bottles at -20°C for 30 days. At the same time, after 30 days of storage at 35°C anthocyanins were not detected.

Brownmiller and co-workers studied the effect of storage on the polyphenol composition of highbush blueberry juices, purée and canned berries (Brownmiller et al., 2008; Brownmiller et al., 2009). It was reported that there were significant losses of bioactive substances after 6 months of storage at 25°C: the level of the anthocyanins retained in the products before and after 6 months of storage when compared with fresh fruit changed from 80% to 23%, from 46% to 15%, from 50% to 20%, from 72% to 29%, and from 66% to 38% for blueberry non-clarified and clarified juices, purée, canned in sugar syrup and canned in water berries respectively. The decrease in ORAC levels was not so dramatic and showed from a 5% to 1% loss for different

products after 6 months storage period. After 6 months of storage, non-clarified and clarified blueberry juices contained only 23% and 15% of berry anthocyanins respectively (Brownmiller et al., 2008).

The temperature during storage significantly affects polyphenols stability. It was found that anthocyanin degradation in pasteurized blueberry jams is significantly higher at 25°C than at 4°C (Howard et al., 2010). All authors agree that temperature is a key factor of the shelf-life quality degradation, so it's necessary to provide a low temperature of the product to retain polyphenolic compounds and antioxidant properties.

To sum it up, anthocyanins are very sensitive to different processing factors. Conventional technologies result in great losses of anthocyanins and the antioxidant properties of processed blueberry products. In order to reduce these losses and to improve the product quality, minimization of the number of technological steps (in order to reduce oxidation), milder heat treatment regimes, and processing whole blueberries with skin in the form of paste or purée can be suggested. In order to insure the quality of the product, different methods of blueberry-based product quality control can be applied (for more information see Section 1.4 of this document). Modification of conventional technologies or applying new technologies for blueberry-based product processing is needed to develop new high quality blueberry-based products. One of the potential technologies for blueberry processing, namely hydrothermodynamic technology, is discussed in Section 1.6 of this document.

1.4 Methods of Blueberry-Based Products Quality Control

For wild blueberry quality parameter determination, there is quite a variety of analytical methods used. The attention of researcher has been focused on bioactive substance content and the antioxidant properties of wild blueberries (Gu et al., 2002; Kalt et al., 2000; Kalt and McDonald, 1996). Some studies were devoted to texture property determination and sensory panel evaluation of fresh wild blueberries (Donahue and Work, 1998; Donahue et al., 1999; Silva et al., 2005; Carbobe et al., 2008), of blueberry juices (Main et al., 2001) and of products prepared with blueberry purée (Leheska et al., 2006). In this study, spectrophotometric method of anthocyanin determination was used due to its relative simplicity and high accuracy.

1.4.1 Extraction

The extraction of phenolic compounds is a very important part of the analytical quantification. Different solvents are used for phenolics extraction from wild blueberries as there is no standardized method of extraction. Aqueous acetone, ethanol, methanol and their combinations are all used by different researchers; acidified media for anthocyanins stabilizing is provided by hydrochloric, formic and other acids. The acidified aqueous solution of methanol (88% methanol, 12% water, 0.1% formic acid) suggested by Kalt et al. (2001) as an extraction method, resulted in extracts with the highest anthocyanin and total phenolics concentrations and provided the most consistent results. Careful homogenization of the blueberry samples in the solvent media is necessary for more complete extraction (Khanal et al., 2009; Nicoue et al., 2007; Shi et al., 2002).

1.4.2 Spectrophotometry

1.4.2.1 Measurements of Total Polyphenolics Content

The most common spectrophotometric method utilized for measuring of the total polyphenols content is based on Folin-Ciocalteau reagent with gallic acid as a reference standard (other phenolic acids equivalents also can be used: tannic, chlorogenic, caffeic, ferulic etc.) (Prior et al., 2005). This method measures the quantity of phenolic groups; the number of phenolics group per molecule varies depending on the structure therefore results of this measurement cannot be correlated with actual molecular weight of polyphenolics (Singleton and Rossi, 1965). Also it is a redox assay hence redox active substances (such as ascorbic acid and some sugars) might interfere with assay and affect the results. Different phenolic reference standards differ in their sensitivity to the assay and may affect phenol results (Prior et al., 2005).It is generally found that total polyphenolics is positively correlated with the antioxidant capacity and can be used for its estimation (Brownmiller et al., 2008; Connor et al., 2002; Kalt et al., 1999; Kalt, 2005).

1.4.2.2 Measurements of Anthocyanin Concentration

The spectrophotometric pH differential method is based on the ability of the monomeric anthocyanins to change the color depending on the pH level. In contrast, the polymeric anthocyanins retain the color within this range of pH, so the difference in absorbance between

the two standard buffer solutions (pH=1 and pH=2) is due to the monomeric anthocyanins pigments (Wrolstad, 1976; Guisti and Wrolstad, 2001). For calculations, polymerized anthocyanin pigments (also known as derived tannins, or polymeric color) are excluded because they do not show reversible behavior with the pH and are not bleached by metabisulfite. The total monomeric anthocyanin concentration is calculated using the molecular weight and extinction coefficient of standard anthocyanin (usually cyanidin-3-glucoside).There was a very good agreement between results in a collaborative study with 11 laboratories which tests independently 7 different products (Wrolstad et al., 2005). Anthocyanin measures obtained by HPLC and pH differential methods showed very good agreement (Nicoue et al., 2007).

In conclusion, spectrophotometric methods for total phenolic, monomeric anthocyanins and polymeric color can be used for easy and fast monitoring of blueberry product quality. Reported high correlation between polyphenolic or anthocyanin concentration and the ORAC value (see Sections 1.2 and 1.3) allows one to infer about the antioxidant properties of the blueberry-based foods using results of the spectrophotometric analysis. Wrolstad's pH differential method can be used as a simple and precise analysis for blueberry products quality quantification.

1.5 Statistical Methodology for Optimization Problem

In order to find the best levels of processing factors for optimized response some statistical methods can be applied. First of all, it is necessary to analyze that factors can be potentially significant. Usually the list of measurable and controllable factors can be done by an analysis of the system. Then the chosen factors should be screened for the significance. One of the well-known methods for factors screening is 2^k factorial design. However, since 2 or 3 replications are needed for each run, the total number of runs increases dramatically with increasing the number of analyzed factors. For example, in case of three replicates for 2^4 factorial designs the total number of runs is 48. Such a big number of experimental runs makes the experiment labor intensive, time consuming and expensive.

There is an alternative method of factor screening which allows a researcher to decrease the number of runs markedly. Unreplicated 2^k factorial design is practically useful if the significance of many factors should be investigated. Due to the lack of replication there is no

pure error in unreplicated design so classic ANOVA analysis is impossible. One of the approaches to conduct ANOVA analysis, in this case, is to assume that the highest order interaction effect is non-significant so that mean squares of this interaction effect can be used to estimate the error. The second approach is to collapse the model. It is possible if one of the factors (main effect and all its interaction effects) is not significant. In this case two levels of this factor can be considered as replicates and the ANOVA analysis might be applied. Another approach of analyzing data from unreplicated designs that doesn't require error degrees of freedom for ANOVA analysis is based on the normal probability plot of the estimates of the effects (Daniel, 1959; Astatkie et al., 2006; Lenth, 1989) and can be applied by using statistical software.

This further investigation involves determination of the optimal level for the factors chosen through the screening process. For this purpose response surface methodology (RSM) is commonly used. RSM enables optimization of the response, affected by several process variables, by using a set of mathematical and statistical techniques. In the case of multiple responses, individual optimums are not of great interest and overlapping response surfaces is used to identify optimal processing regimes.

To investigate the kinetics of anthocyanin degradation during storage, repeated measurements analysis should be used. Since the measurements in a shelf-life experiment are taken on the same experimental unit over time and the consequence could not be randomized, classic ANOVA analysis cannot be applied. Since every measure depends on the previous one, the time trends should be taken into account. Repeated measurements analysis allows a researcher to analyze this kind of data using developed methodology (Littell et al. 1998) and statistical software applications.

Nonlinear regression analysis is used to analyze the data, which cannot be described by linear models. Nonlinear modeling gives more flexibility in choosing the model and usually is based on the theory about the mechanism of the analyzed process. For example, kinetics of anthocyanin loss may be expressed by first-order exponential model (Wang, 2007; Kechinski et al., 2010) so anthocyanin loss studied in this work was expected to be described by the same model. A non-linear analysis is a complex and time consuming method but the parameters involved in non-linear models are physically meaningful and help in understanding the nature of the process, moreover, non-linear models can be used as predictive models which is important

for shelf-life studies (Bates and Watts, 1988).More detailed information on statistical methods is provided in Sections 2.2.3, 3.2.3 and 4.2.3.

1.6 Hydrothermodynamic Technology

Hydrothermodynamic technology (HTDT) is a new technology that can be successfully used for blueberry processing (Osipenko, 2009). HTDT uses the physical phenomenon of cavitation to provide quick heating and blending/grinding of materials at the same time. An illustration of the principles of HTDT is presented in Fig. 1.5, where moving fluid induces local turbulence and multi-phase cavitation bubbles while going through the obstacle (i.e. cavitator) with disturbing streams of the liquid. Cavitation bubbles are unstable and collapse right after going through the cavitator (active area). Collapsing of cavitation bubbles creates local shear forces and high local pressure. Energy released as a result of bubbles collapsing leads to an increase of the temperature of the moving fluid and results in crushing and homogenizing of the product (www.tekmash.ca).

HTDT was developed for the processing of different types of food (fruits, berries, vegetables, beans) to particles 10 to 1000 nm with minimum quality degradation. Simultaneous grinding, mixing and volumetric heating in an anaerobic environment prevents destruction of bioactive compounds.

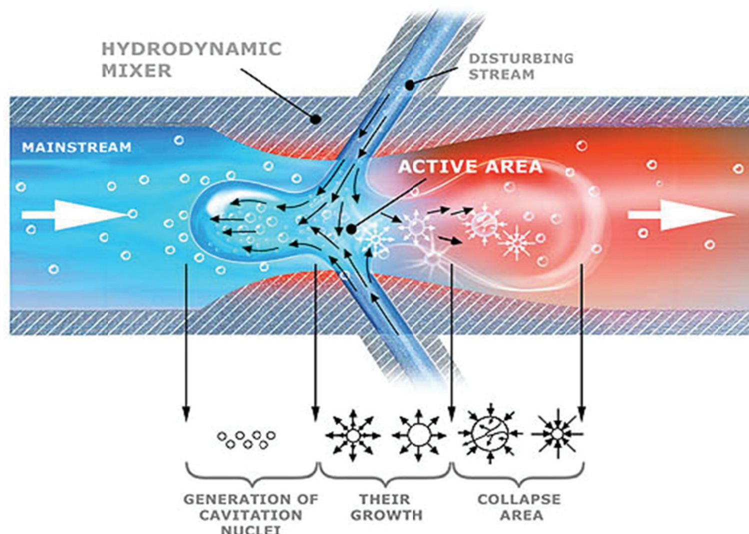


Figure 1.5 Principles of HTD technology

The potential advantages of HTD technology includes uniform volumetric heating of the product without scorching (no contact heaters are used in the HTDT apparatus), reduced high-temperature oxidation due to no contact with oxygen during processing, and a prolonged product shelf-life due to pasteurization (Osipenko, 2008 and 2009).It was reported that the HTDT sealed batch process prevents products from oxidation. Simultaneous unit operations (crushing, heating, homogenization, and pasteurization) take place in the same tank (Osipenko, 2008) excluding additional oxidation due to multiple processing steps.

The most detailed information on the effect of the HTDT on processed food product quality is available for soy beans. Soybeans were processed into homogeneous paste after soaking in water. Complete retention of vitamins and unsaturated fat acids of soybeans as a result of HTDT processing was reported (Osipenko, 2008; www.tekmash.ca). Moreover, an increase in some bioactive compounds content was obtained (Osipenko, 2008; www.tekmash.ca). The following increase of nutrient content for HTDT processed soy beans as compared with raw beans was reported: 1.3-fold, 5.0-fold, 3.3-fold, 2.7-fold, 1.5-fold, and 2.4-fold increases for vitamins B_1, B_2, niacin, B_6, folic acid, and carotenoids respectively (Osipenko, 2008; www.tekmash.ca). Also, partial converting of polysaccharides to mono- and disaccharides was reported. The improved nutrient concentration may occur due to the fine homogenization and hence improved release of nutrients from the seed matrix.

Using HTDT technology, wild bilberries (*Vaccinium myrtillus*) can be processed in a uniform suspension without removing skins and seeds (“whole fruit”, “liquid berry”) (Osipenko, 2009). The author reported complete retention of total polyphenolics and assumed the higher bioavailability of phenolic substances due to fine product homogenization (Osipenko, 2009).

Resources available for this technology are very limited and basically includes information from the corporate web-site (www.tekmash.ca) and patents (Osipenko, 2008 and 2009). There is an article (in Ukrainian) devoted to the hepatoprotective properties of HTDT bilberry paste in an experiment with mice (Levitskiy, 2009). In spite of limited information, all the above mentioned leads to a hypothesis that the use of THDT processing for blueberry might result in a high quality product with good retention of health beneficial components.

Conclusions

1. Wild blueberries are a delicious food and food ingredient and an important crop in Atlantic Canada. They are characterized by the highest polyphenolics concentration among cultivated crops that may benefit health.
2. At the moment, the selection of commercially available wild blueberry-based food products is very limited. Consumers are interested in getting healthier and more natural foods but these kinds of products are absent on the market. Blueberry production in Atlantic Canada is increasing significantly, so producers and processors are interested in new blueberry-based product development in order to increase blueberry markets.
3. Processing of wild blueberry generally has a negative effect on polyphenolics and, by extension, on their health promoting properties. Information on the impact of processing factors on blueberry polyphenolic losses suggests that process optimization is warranted to minimize polyphenolic degradation.
4. The spectrophotometric method for determination of anthocyanin concentration and tannin content is relatively simple and precise, allowing one to infer both phenolic concentration and antioxidant capacity of blueberry-based products. Anthocyanin concentration can be used as a response in process optimization.
5. Effect of storage on quality degradation of blueberry products requires further research.
6. HTD technology is a promising method for blueberry processing; its effectiveness should be further investigated.

GOAL AND OBJECTIVES

The goal of this research is optimization of blueberry purée processing using the HTD technology to maximize the content of bioactive substances (anthocyanins) with respect to food safety.

Objectives of the research:

1. Compare HTD technology and conventional blueberry processing technology. Study the difference between HTDT blueberry purée and commercial blueberry-based products in order to prove the efficiency of HTDT to process blueberries into a high quality pasteurized product;
2. Study and model kinetics of quality degradation during HTDT blueberry purée storage in order to develop shelf-life recommendations;
3. Study the effects of processing factors (temperature, time, heating rate, cavitation mode) on food quality and safety and optimize time-temperature regimes for HTDT blueberry processing.

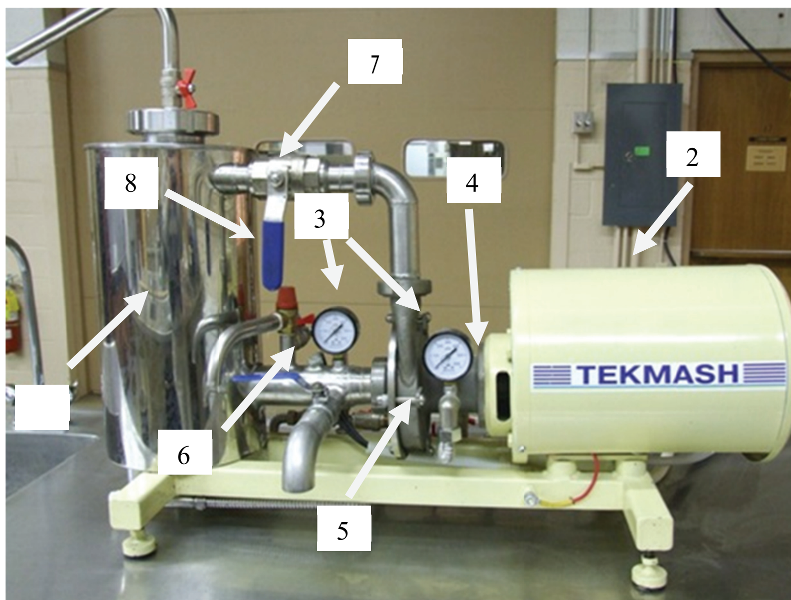
2. PRODUCT CHARACTERIZATION

2.1 Introduction

This chapter is devoted to the comparative analyses of HTDT blueberry purée versus other blueberry products (frozen blueberries, blueberry purée processed using conventional technology and some commercial blueberry products) in order to characterize the product obtained using HTD technology. The experiment was conducted to check the hypothesis that THDT processing of blueberries provides the superior quality of the final product as compared with conventional technology. Also it was hypothesized that HTDT blueberry purée may have a higher anthocyanin concentration than some commercial blueberry based products presented on the market.

2.2 Materials and Methods

2.2.1 Experimental Apparatus



1 – the tank; 2 – the motor; 3 – the pressure gauges; 4 – the gasket;
5 – the pump; 6 – the thermocouple; 7 – the cavitator; 8 – the switcher.

Figure 2.1 General view of pilot-scale HTDT food processor TEK-1

Experimental work on blueberry processing was done on the pilot-scale experimental apparatus TEK-1 with 4.5L capacity (Fig.2.1). Pilot-scale unit TEK-1 provided pasteurization and homogenization of 4.5 L of blueberry purée in a batch process. Thawed blueberries were

loaded manually into the tank (1), and the motor (2) was started using a pushbutton from the control panel (not shown in the picture). Two pressure gauges (3) were used to keep the same pressure on both sides of the gasket (4), between the motor parts and the moving liquid, using a water stream though the system. Continuous movement of the liquid (blueberry purée) was provided by the pump (5). For temperature control, the thermocouple, (6) immersed into thermo conductive purée in the well, incorporated to the pipe was used. Heating of the liquid was provided due to its moving through the cavitator (7) which had two possible working positions: the valve regulator down was assigned as cavitation mode A, and the valve regulator up was assigned as cavitation mode B. These two cavitation modes are provided by the different shape of the cavitator which can be changed by the switcher (8). Cavitation mode A provided the highest heating rate of the product, cavitation mode B was used for providing the highest homogenization of the product at lower heating rates.

2.2.2 Materials

Raw materials for this study were provided by PEI Organic Fruit and Berries Ltd. (frozen berries, harvested in September 2009). Frozen berries were stored in 10 kg plastic buckets at –18°C before processing. Berries in buckets were thawed prior to processing during 12 to 14h at room temperature. HTDT blueberry purée was processed in September 2009 at the Engineering Department of the NSAC using the following regimes: temperature of pasteurization at 95°C, no holding time; cavitation mode A up to 70°C, then changed on cavitation mode B up to 95°C, heating rate 1.9°C/min. In order to simulate the conventional process of blueberry pasteurization, the following technique was used: 2 kg blueberry was blended for 5 minutes, and then heated using the same heating rate in an open contact heater (roaster) up to 95°C. In both cases the pasteurized products were filled into 250 ml jars and the jars were sealed, then jars were left for cooling down in upside down position self-pasteurization of the lids. Other methods of conventional blueberry pasteurization should be applied in order to compare the effectiveness of HTDT with state-of-the-art pasteurization techniques (such as heat exchanger or high temperature sort time (HTST) pasteurization; however, these methods requires using a specific equipment which was not available at NSAC.

Some commercial products covering a variety of blueberry-based products including fresh blueberries, frozen blueberry purée, juices (clarified, non-clarified, organic, and from concentrate), syrup and jams (blueberry spread, organic blueberry spread, and homemade jam)

were compared (Table 2.1). Commercial products were purchased in stores (products from shelves); three replications for each product were used. Fresh blueberries were provided by “PEI Organic Fruit and Berries Ltd” (harvested in September 2009). A sample of commercial frozen blueberry purée (“Jasper Wyman and Son”) was provided by “PEI Organic Fruit and Berries Ltd” in April 2010.

Table 2.1 Blueberry products used for comparison analysis

#	Product type	Sample name	Sample description	Producer	Code name
1	Raw / semi-product	Frozen blueberries	Fresh wild blueberries(harvested September 2009)	PEI Organic Fruit and Berries Ltd.	FB
2		Frozen blueberry purée	Frozen wild blueberry purée	Jasper Wyman and Son	FP
3	Ready to be consumed	HTDT blueberry purée	Wild blueberry paste produced using HTDT technology	NSAC	HTDT
4		Van Dyk’s Wild Blueberry juice	Pasteurized clarified blueberry juice, not from concentrate	Van Dyk’s Health Juice Products Ltd.	juice1
5		Acadian Maple Wild Blueberry juice	Pasteurized non-clarified blueberry juice, not from concentrate	Acadian Maple Products Ltd.	juice2
6		“Just Jus”	Wild blueberry organic juice (non-clarified, not from concentrate)	Pure Source Inc.	juice3
7		“Just Blueberry”R.W.Knudsen	Blueberry juice (from concentrate)	Smucker Quality Beverages Inc.	juice4
8		Acadian Maple Wild blueberry syrup	Wild blueberry syrup, 52% sugars content	Acadian Maple Products Ltd.	Syrup
9		Pure Labrador Wild blueberry spread	Wild blueberry spread, more than 80% sugars content	Labrador Preserves Co	jam1
10		“Organics” PC Wild blueberry spread	Organic wild blueberry spread, more than 50% sugars content	Loblaw’s Inc.	jam2
11		Homemade blueberry jam	Homemade blueberry jam (organic blueberries, more than 60% sugar content)	Provided by PEI Organic Fruit and Berries Ltd.	jam3

2.2.3Analytical Methods

2.2.3.1 Measurement of Anthocyanin Concentration and Polymeric color Content (Tannins)

An approximately 15 g subsample of the product or raw material was ground in two volumes in a cold acidified (0.1% formic acid) mixture of methanol, acetone and distilled water (40:40:20 v/v) for 2 minutes followed by filtering the suspensions (glass fiber filter, #6) in order to obtain extracts (Kalt et al., 2008). Extracts (50 mL) were stored at a temperature of –18°C prior to the analysis; the storage temperature of the extracts was reached by placing them into a freezer.

Total monomeric anthocyanin concentration and the percent contribution of polymeric color were determined by a pH differential spectrophotometric method (Wrolstad, 1976; Guisti and Wrolstad, 2001). This method uses the color change of monomeric anthocyanins depending on pH. Since polymeric anthocyanins also contribute to color, bleaching analysis is to be applied, where 20% potassium metabisulfite bleaches monomeric anthocyanins so that only polymeric compounds are measured. This determines the percentage contribution by tannins to color. Anthocyanin concentration was determined in three replicates and calculated using the extinction coefficient of cyanidin-3-glucoside (26,900). Anthocyanin concentration was specified per both dry and fresh weights of the product. The analyses were conducted at the facilities of NSAC and AAFC (Kentville, NS).

Reagents used for the analysis:

i) The acidified solution for extraction was prepared by the mixing of acetone, methanol, distilled water and formic acid (40:40:20:0.1 by volumes).
ii) The pH 4.5 buffer was prepared as follows: 400 ml of 1M solution of sodium acetate (136 g of CH_3COONa per 1 L of distilled water) was combined with 240 ml 1N solution of HCl (83,0 ml of concentrated HCL per 1 L of distilled water) and 350 ml of distilled water. The buffer was adjusted to required pH using 1N HCl or 1N NaOH, the final volume was made to 1 L.
iii) The pH 1.0 buffer was prepared as follow: 125 ml of 0.2N KCl solution (14,6 g of KCl per 1 L of distilled water) was combined with 385 ml of 0.2N HCl solution (100 ml of 1N HCl was diluted up to 500 ml with distilled water). The buffer was adjusted to required pH using 1N HCl.

iv) The metabisulfite solution must be prepared fresh daily since it becomes yellow over time and this will contribute to absorbance reading. To prepare a 20% solution, 2 g of potassium metabisulfite was mixed in 10 ml of 0.1% solution of trifluoroacetic acid (0.1% TFA).

Measurement of anthocyanin concentration was carried out on a microplate reader (Spectra Max 190, Molecular devices, Menlo Park Calif., U.S.A.). The procedure and the calculation are adopted from Guisti and Wrolstad (2001).

Procedure for anthocyanin concentration and tannin content analyses:

Anthocyanin analysis:

i) The final volume of microplate wells for anthocyanin analysis was 300 µl. Two plates were used for analysis: samples in pH 1 buffer and samples in pH 4.5 buffer.
ii) For each analysis, two plates were filled with 290 µl of buffers first (to avoid contamination of tips) and then 10 µl of samples were added. A multichannel pipettor with appropriate tips was used. Each sample was analyzed in triplicate.
iii) When not being read, plates were stored in the dark.
iv) Absorbance was read at 520 and 700 nm. The absorbance readings of samples should be between 0.1 and 1.0. Every plate was read 2 to 3 times to provide good mixing and therefore more accurate reading.
v) "Softmax Pro" software was used for reading and collecting data, which was then exported as a text file into Excel spreadsheet.

Polymeric color (bleaching analysis):

i) The final volume of microplate wells for polymeric color analysis was 225 µl. For each analysis two plates were used: samples in 0.1% TFA ("unbleached") and samples in 0.1% TFA with metabisulfite ("bleached"). Sample dilution and layout should be the same for both plates.
ii) "Unbleached" plates were filled with 215 µl of 0.1% TFA and "bleached" plates were filled with 190 µl of that (TFA solution was added first to prevent tips contamination). Then 10µl samples were added in both plates. Finally, 25µl of 20% potassium bisulfate were added to "bleached" plates. When not being read, plates were stored in the dark.

iii) The absorbance was read at 405, 520 and 700 nm. The absorbance readings of samples should be between 0.1 and 1.0. Every plate was read 2 to 3 times to provide good mixing and therefore a more accurate reading.

iv) "Softmax Pro" software was used for reading and collecting data, which was exported as a text file into Excel program file.

Calculations for anthocyanin concentration and tannin content analysis:

Monomeric anthocyanin concentration was calculated as follow:

$$A_{MA} = (A_{520nm\ pH\ 1.0} - A_{700nm\ pH1.0}) - (A_{520nm\ pH\ 4.5} - A_{700nm\ pH\ 4.5}),$$

where A_{MA} – is monomeric anthocyanins absorbance; $A_{520nm\ pH\ 1.0}$, $A_{700nm\ pH1.0}$ – is absorbance of the sample in pH 1.0 buffer at 520 and 700 nm wavelength; $A_{520nm\ pH\ 4.5}$, $A_{700nm\ pH\ 4.5}$ – is absorbance of the sample in pH 4.5.0 buffer at 520 and 700 nm wavelength.

$$C = \frac{A_{MA}}{\varepsilon \cdot l} \cdot 10^3 \cdot MW \cdot DF,$$

where *C* is the concentration of monomeric anthocyanins, calculated as cyanidin-3-glucoside (C3G), mg/L; ε – is extinction coefficient, ε = 26900 for C3G; l – is a pathlength, calculated as follow: $l = \sqrt[3]{\frac{12\cdot(1.2274+V_{assay})}{8.846\cdot 10^{-3}}} - 11.90$, V_{assay} was 0.3 mL which gave a pathway equal to 0.8486 cm; *MW* – is a molecular weight, *MW* = 449.2 for C3G; *DF* – is a dilution factor, *DF* = 30.

Polymeric color (derived tannins) was calculated as follow:

$$T = \frac{CD}{CD_{PC}} \cdot 100\%,$$

where *CD* – is color density, calculated as $CD = [(A_{520nm} - A_{700nm}) + (A_{405nm} - A_{700nm})] \cdot DF$; A_{520nm}, A_{700nm}, and A_{405nm} – are absorbance measurements from "unbleached" plate (without potassium metabisulfite); *DF* – is a dilution factor, *DF* = 22.5; CD_{PC} – is polymeric color density, $CD_{PC} = [(A'_{520nm} - A'_{700nm}) + (A'_{405nm} - A'_{700nm})]\cdot DF$; A'_{520nm}, A'_{700nm}, A'_{405nm} are absorbance measurements from "bleached" plate (with potassium metabisulfite); *DF* – is a dilution factor, *DF* = 22.5.

Polymeric color content represents percentage of derived tanning in total anthocyanin concentration. This analysis accounts only soluble tannins; insoluble fraction is removed from the analysis as a result of filtration.

2.2.3.2 Particle Size Distribution

Particle size distribution analysis was provided by Agat Laboratories (Calgary, Alberta). Analysis was done in three replications for blueberry purée pasteurized using HTDT pilot scale machine and blueberry purée produced using conventional technology (blending followed by open contact heating up to 95°C).A random sampling of blueberry purée (in 3 replications for each sample) was used for the particle size analysis.

2.2.4Statistical Methods

The analysis of variance (ANOVA) was applied to analyze the obtained data using SAS and MiniTab software.

The basic single factor ANOVA model is:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij}, \begin{cases} i = 1, 2, \dots, a \\ j = 1, 2, \dots, n \end{cases} \quad (1)$$

Where y_{ij} is a response of ith treatment j^{th} replication; μ is an overall mean; τ_i is the ith treatment effect; ε_{ij} is the experimental error, assumed to be distributed normally $\varepsilon_{ij} \sim$ NID $(0, \sigma^2)$; a is the number of treatments; n is the number of replications.

The statement of hypothesis for ANOVA is:

H_0: $\mu_1 = \mu_2 = \dots = \mu_a$

H_a: at least one μis different.

For performing a ANOVA test three assumptions were checked:

i) Normality.

For the normality assumption to be valid, the error term must be normally distributed. The normal probability plot of the residuals was generated by MiniTab while running the ANOVA test. If NID $(0, \sigma^2)$ assumption of error terms is satisfied, the normal probability plot of residuals looks like a sample from a normal distribution centered at zero. Estimation of normality is rather subjective; generally the error terms are assumed to be normally distributed if dots on the plot can be covered by a pen. In case the normality assumption is violated, the ANOVA F-test is invalid. To induce normality, different types of data transformation may be applied.

ii) Constant variance.

A violation of the constant variance assumption results in inflation of mean square error, as a result F-value is deflated and a chance to accept the null hypothesis wrongly (type II error) is

increased. To check this assumption, a plot of the residuals versus fitted values was used. Constant variance was assumed in case the plot of residuals versus fits gave the impression of horizontal band across the top and the bottom of the plot. Data transformation (e.g. log transformation) can also be used to achieve constant variance.

iii) Independence.

Complete randomization of experiment is supposed to provide independence of collected data. Violation of this assumption also results in a deflation of the F-value and increasing the probability of making a type II error. For this study independence assumption was assumed to be valid through proper randomization.

A significance level for this study was chosen $\alpha = 0.05$. Results with a p-value less than 0.05 was reported as significantly different (the null hypothesis was rejected, which means that at least one mean was different). For the multiple means comparison Tukey's method in SAS was used.

2.3 Results and Discussion

2.3.1 HTDT Blueberry Purée versus Frozen Blueberry

Blueberry purée processed using the HTDT pilot scale machine TEK-1 was compared with initial raw material (frozen blueberries). Anthocyanin concentration and tannin content were measured in order to estimate the changes resulted from blueberry pasteurization and homogenization. Results of analysis are presented in Table 2.2. Assumptions of normality and constant variance were met, independence of data is assumed through randomization.

Table 2.2 Results of ANOVA analysis of HTDT blueberry purée vs. frozen berries

Product	Dry weight, %		Anthocyanin concentration, mg C3G/g				Tannins, %	
			per dry weight		per fresh weight			
	average	std dev	average	std dev	average	std dev	average	std dev
HTDT purée	11.26 (a)	0.37	13.98 (b)	1.18	1.57 (b)	0.09	14.97 (a)	1.73
Frozen berries	12.00 (a)	0.90	17.31 (a)	1.69	2.02 (a)	0.06	9.03 (b)	0.50

There was no significant difference in dry matter content which was expected since no water was added or evaporated during the process. Anthocyanin concentration was significantly higher in frozen blueberries as compared with THDT blueberry purée. Anthocyanin losses were 19.2% or 22.3% as calculated per dry weight and fresh weight respectively. Tannin content was significantly higher in the HTDT blueberry purée as compared with fresh blueberries. Tannin content increased65.8% as a result of heat treatment during HTDT blueberry processing.

2.3.2 HTDT versus Conventional Processing

Blueberry purée processed using the HTDT pilot scale machine was compared with the purée produced using conventional technology (with the same 1.9^{o}C/min heating rate and 95°C pasteurization temperature). Anthocyanin concentration and tannin content results are presented in Table 2.3.Assumptions of normality and constant variance were met, independence of data is assumed through randomization.

Table 2.3Results of ANOVA analysis of purée produced using HTDT vs. conventional technology

Purée	Dry weight, g/100g		Anthocyanin concentration, mg C3G/g				Tannins, %	
			per dry weight		per fresh weight			
	average	std dev	average	std dev	average	std dev	average	std dev
HTDT	11.26 (b)	0.37	13.98 (a)	1.18	1.57 (a)	0.09	14.97 (b)	1.73
Conventional	16.58 (a)	0.52	7.49 (b)	0.37	1.24 (b)	0.02	18.03 (a)	0.21

Significant difference in dry weight was found for blueberry purée prepared using HTDT and conventional technologies (11.3g/100g and 16.6g/100g respectively), likely due to water loss from the open heater in conventional purée heating to 95°C. Anthocyanin concentration in conventional blueberry purée was significantly lower than that in HTDT blueberry purée. Tannins were significantly higher in blueberries processed using conventional technology. Taking into consideration the results in Table 2.3, compared with conventional technology HTDT of blueberry processing provides better anthocyanin retention. However, the conventional method used (contact heating) does not reflect modern techniques of fruit purée pasteurization

(such as HTST method); different results might be obtained in case of using other conventional pasteurization method for the comparison.

Results of the particle size distribution in blueberry purée for HTDT versus conventional technology is presented on Fig. 2.2 (error bars indicate standard deviation).

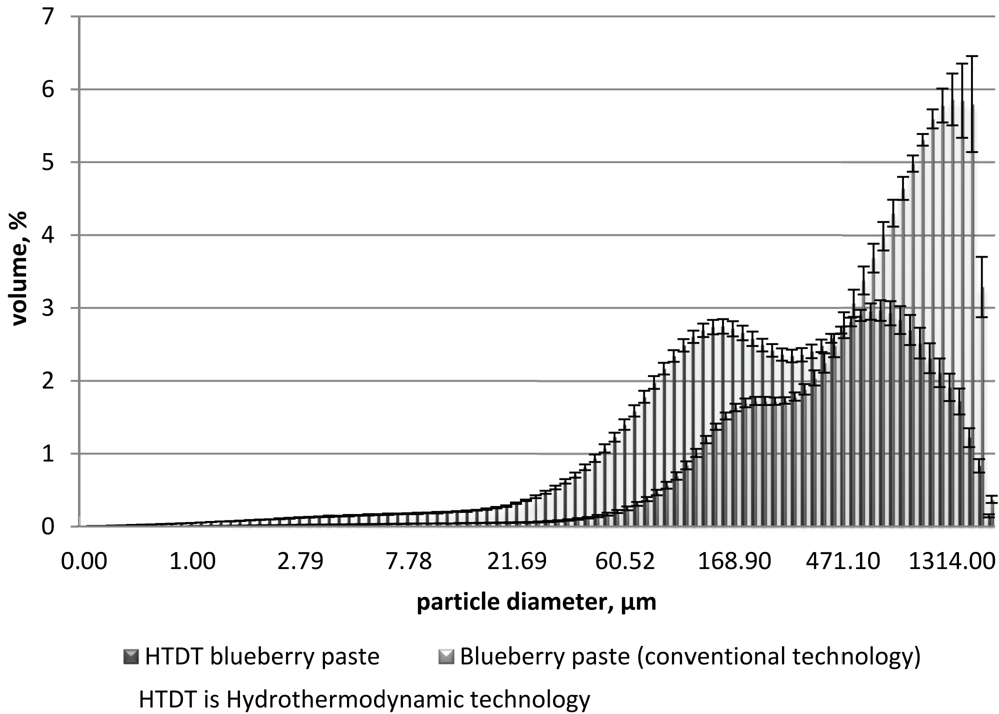


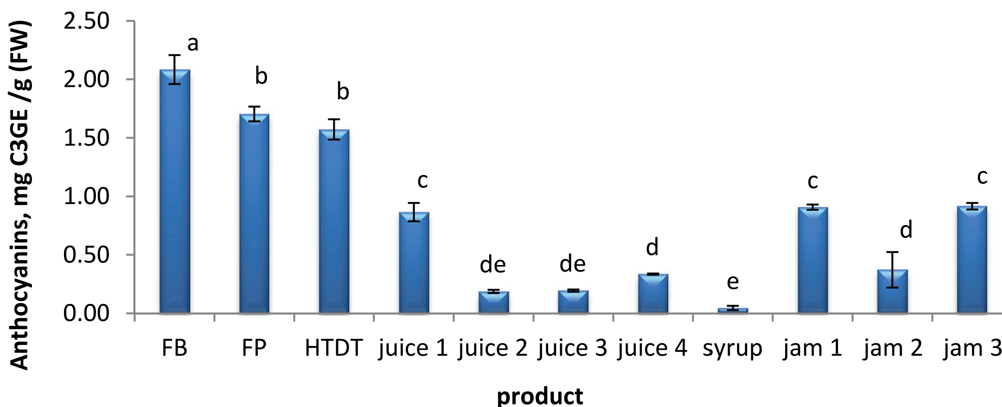
Figure 2.2 Particle size distribution in blueberry purée processed by HTDT vs. conventional technology

Purée prepared using conventional technology contained larger particles than the HTDT product (Fig. 2.2). In conventional purée, 75% of particles were larger than 0.39 mm, while the HTDT product contained only 26% particles larger than 0.39 mm (seeds and seed particles). HTDT also provided finer crushing to a size less than 0.12mm (25% of particles), while in purée prepared using conventional technology, the amount of particles smaller than 0.12mm was only 5.6%. In the HTDT purée, 50% of the particles were less than 0.27mm, and for the purée prepared using conventional technology this median characteristic size was 0.82 mm. Information provided by the particle size distribution analysis has shown that HTDT resulted in better crushing of raw blueberries to homogeneous suspension. The size of the particles after HTDT was more consistent as compared with conventional technology that might affect

nutritional and nutraceutical value of the product (Parada and Aguliera, 2007). Smaller and more uniform particle size of HTDT purée may be advantageous in various applications.

2.3.3 HTDT Blueberry Purée versus Some Commercial Products

Triplicate samples of HTDT blueberry purée was compared with some commercial blueberry products (Table 2.1) (purchased at local stores, Truro, Nova Scotia, Canada).Difference in anthocyanin concentration and tannin content between commercial products and the HTDT processed blueberry purée are illustrated in Figures 2.3 and 2.4. Assumptions of normality and constant variance were met, independence of data is assumed through randomization.



HTDT is Hydrothermodynamic technology

Figure 2.3 Anthocyanins concentration in HTDT blueberry purée and some commercial blueberry products

The highest anthocyanin concentration was detected in raw materials: frozen blueberries (FB, PEI Organic Fruits and Berries Ltd.) and frozen blueberry purée (FP, "Jasper Wyman and Son"). No significant difference in anthocyanin concentration was found for frozen blueberry purée (FP) and HTDT blueberry purée. Most of the blueberry juices (juice 2, juice 3 and juice 4) had markedly lower anthocyanin concentration (0.19, 0.19, and 0.33 mg C3G/g FW respectively) as compared with frozen blueberries (2.08 mg C3G/g FW). However, Van Dyk's blueberry juice (juice 1) had the highest anthocyanin concentration among analyzed juices (0.86 mg C3G/g FW).Anthocyanin concentration in jams (jam 1, jam 2, and jam 3) was at least two times lower compared to raw materials (FB and FP): 0.91, 0.37, and 0.91 mg C3G/g FW versus 2.08 and 1.70

mg C3G/g FW respectively. The lowest anthocyanin concentration and the highest polymeric substances content were found in Acadian Maple blueberry syrup (syrup): anthocyanin concentration in this product (0.04mg C3G/g FW) was 50 times less than that in frozen berries. Significantly higher concentration of anthocyanins was detected in HTDT blueberry purée as compared with studied commercial blueberry products.

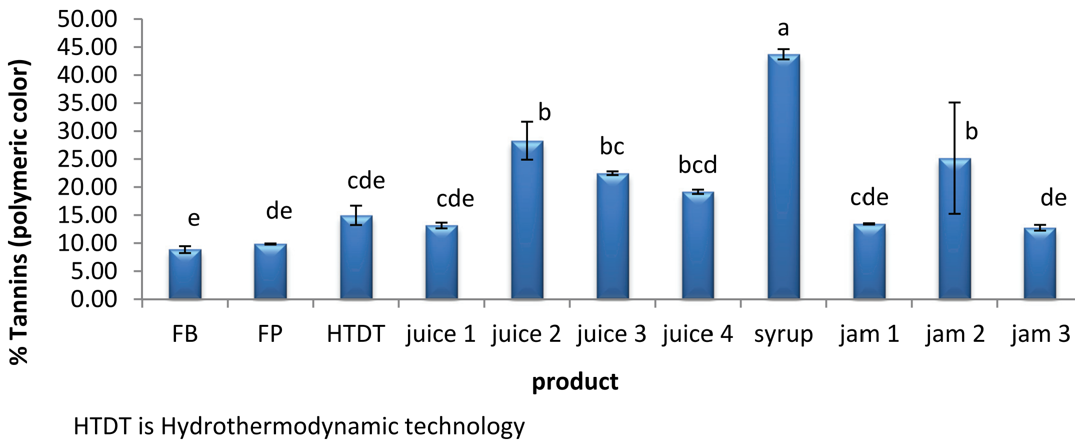


Figure 2.4 Tannin content in HTDT blueberry purée and some commercial products

Polymeric color (tannin) content (Fig. 2.4) which reflects oxidation products of anthocyanins was lower in raw materials (FB and FP, 8.9% and 9.9% respectively) than that in the commercial products and the HTDT blueberry purée. Higher amount of tannins in the products was considered as an indicator of anthocyanin oxidation as a result of processing and storage.

Among all ready-to-eat processed blueberry products, the HTDT blueberry purée (HTDT) was characterized by the highest anthocyanin concentration (1.57 mg C3G/g FW). Anthocyanin concentration in the HTDT blueberry purée was comparable with raw materials: there was no significant difference (at 5% significance level) between frozen blueberry purée (FP) processed without thermal treatment and blueberry purée (HTDT) processed using the HTDT pilot-scale machine. Also, no difference was found in the tannin content of the HTDT blueberry purée and that of the raw materials (FB and FP). Information on the previous processing and storage of commercial products was unavailable. The processing factors and storage conditions likely affected anthocyanin concentration and tannin content in these products.

2.4 Conclusion and Recommendations

The results illustrate that HTDT can be used to produce pasteurized blueberry purée with minimal loss in anthocyanin concentration. The quality of the HTDT blueberry purée (in terms of anthocyanin concentration, tannin content, and particle size and uniformity) is higher than that of most commercially available blueberry products. For a better understanding of the benefits of HTDT for blueberry processing, HTDT purée was also studied in a product shelf-life study which is presented in Chapter 3. Also results of optimization studies of HTDT blueberry processing are presented in Chapter 4.

3. ANTHOCYANINS AND TANNINS CHANGES DURING STORAGE OF BLUEBERRY PURÉE

3.1 Introduction

In Chapter 2 it was shown that anthocyanin concentration in the HTDT blueberry purée was lower as compared with frozen blueberries, likely as a result of thermal processing. However, significant losses of anthocyanins and increasing of tannin content usually occurs not only as a result of processing but also during storage of blueberry based products. In this chapter, the kinetics of anthocyanin degradation and tannin formation is explored in order to develop recommendations of temperature regimes for storage and to determine shelf-life of the HTDT blueberry purée.

3.2 Materials and Methods

3.2.1 Materials

Raw materials for this study were provided by PEI Organic Fruit and Berries Ltd. (frozen berries, harvested in September 2009). Frozen berries were stored in buckets at – 18°C before processing. The HTDT blueberry purée was processed in September 2009 at the Engineering Department of the NSAC using the following regimes: temperature of pasteurization 95°C, no holding time, cavitation mode A up to 70°C, then changed on cavitation mode B up to 95°C, heating rate 1.9°C/min. Pasteurized product was filled into 250 ml jars and the jars were sealed (self-pasteurization).Jars with blueberry purée were marked (number of batch, order of filling) and divided into two groups. One group was stored at +4°C (cold storage facilities, Haley Institute, NSAC; the temperature was controlled automatically). The second group was stored at room temperature (20 to 25°C; the temperature was logged every week throughout the experiment) in the food laboratory (Engineering Department, NSAC). Three replicates were used in the experiment.

3.2.2. Analytical Methods

Extract preparation, measurement of anthocyanin concentration and polymeric color was carried out as described in Section 2.2.2.1. The analysis was conducted at the facilities of the NSAC and the Agriculture and Agri-Food Center (AAFC, Kentville, NS).

3.2.3 Statistical Analysis

Repeated measures analysis and non-linear regression analysis were applied for anthocyanin concentration and tannin content of the blueberry purée stored for 18 months at two different storage temperatures. Non-linear regression analysis was applied in order to model anthocyanin degradation during storage and to determine whether or not different storage temperatures differentially affected the rate of anthocyanin degradation. Repeated measures analysis was applied to determine if the changes in anthocyanin concentration and tannin content were significant for the HTDT purée stored for different periods of time.

3.2.3.1 Repeated Measures Analysis

Repeated measures analysis was used as a statistical method to analyze a response taken on the same experimental unit repeatedly. Experimental design included two response variables: anthocyanin concentration and tannin content, with storage temperature as a factor. The advantage of repeated measures analysis is the possibility to analyze the evolution of temperature effect on the responses over time. Hence, the trends of responses over time are taken into account.

In the present study, state-of-the-art mixed model methodology was used(Littell et al. 1998).This is the most sophisticated and latest method to analyze repeated measures data, which addresses directly the covariance structure and uses the MIXED procedure in the SAS system. Mixed model methodology provides valid standard errors and efficient statistical tests. Moreover, in contrast to GLM as the older procedure, one missing value does not lead to rejecting the whole block of data from that experimental unit of the analysis.

There are two basic steps in the mixed model methodology. First is the identification of the most appropriate covariance structure. Second is the analysis of time trends for treatments by estimating and comparing means. In modeling the covariance structure, covariance within the same experimental unit measurement is a concern, since measures on different units are independent due to randomization. In using PROC MIXED as a repeated measures methodology, the REPEATED statement is applied to specify the covariance structure.

There are numerous covariance structures available in the PROC MIXED procedure. In this study the two most common covariance structures were used: Compound Symmetry (CS), and Unstructured (UN). Covariance structures can be compared by using the goodness of fit criteria (Akaike information criterion (AIC)) and was used for this study (Littell et al. 1998).

Structure with the closest to zero value should be picked. Different covariance structures result in different standard errors of estimates calculated. The covariance structure with the best fit should be used; otherwise standard errors calculated might not be the smallest. Also, normality and constant variance of the error terms should be adhered to. Estimates and significance of the factors provided by PROC MIXED procedure were manually analyzed further in order to report letter grouping of means (significance level for letter grouping was chosen α=0.01).Validity of assumptions (normality of error terms through normal probability plot of residuals, and constant variance by plotting residuals versus fits) was checked.

3.2.3.2 Non-Linear Regression Analysis

Nonlinear regression analysis was used to analyze the data, which could not be described by linear models. Nonlinear modeling gives more flexibility in choosing the model and is usually based on the theory about the mechanism of the analyzed process. That is why the parameters involved in non-linear models are very often physically meaningful. However, disadvantages of nonlinear regression analysis are the complexity of the method.

A non-linear regression model for each response y_i (i=1, 2, …, n) can be specified as

$$y_i = f(x_i, \theta) + \varepsilon_i \qquad (2)$$

Where f is an expectation function; ε_i is disturbance (error term), $\varepsilon_i \sim N(0, \sigma^2)$.

When analyzing a set of data it is considered that the vectors x_i, i=1, 2, …, n are fixed and concentrated on the dependence of the expected response of θ. Using the N-dimensional prediction vector $\eta(\theta)$ with components $\eta_i(\theta) = f(x_i, \theta)$,nonlinear regression model can be written as $y = f(x, \theta) + \varepsilon$. The model is considered to be nonlinear if at least one of the derivatives, with respect to the parameters θ, involves one or more parameters.

Parameters included in the non-linear model cannot be calculated exactly and directly. Finding parameter estimates requires an iterative algorithm using the non-linear least squares (NLS) method. The problem of finding the least square estimates can be stated geometrically as a data vector y, an expectation function $f(x, \theta)$ and a set of design vectors x. The first step of NLS is to find the point $\hat{\eta}$on the expectation function which is closest to y and then, the second step is to determine the parameter vector $\hat{\theta}$corresponding to the point $\hat{\eta}$. Both of these steps are very complicated in the case of non-linear modeling, so an iterative method is used to determine NLS estimates. The most commonly used algorithm for the NLS parameter estimation is the Gauss-

Newton method. This method involves linear approximation to improve iteratively the initial value of parameter θ^0 for θ and continues improving the estimates until there is no change (change is less than the minimal set difference).

The non-linear model should meet the following assumptions: (i) additive noise (error terms in the model are additive), (ii) normal distribution of error terms (can be checked by using normal probability plot of residuals), (iii) constant variance (can be checked by plotting residuals versus fitted values; in case of constant variance the impression of horizontal band across the top and the bottom should occur), (iv) independence of successive error terms (can be checked by plotting residuals versus time order), (v) adequacy of model function (by plotting residuals versus independent variable) (Bates and Watts, 1988).

To start the iteration process, starting values of parameter estimates should be calculated and specified. In some cases starting values can be obtained from behavior of the expectation function in terms of parameters (graphically or analytically). In the case of transformably-linear models (nonlinear models which can be expressed in a linear form by transforming independent or response variables, or both) starting values can be found by running a linear regression analysis. Combination of these two methods can also be used successfully.

According to Ratkowsky (1993), nonlinear modeling should satisfy the following principles:

i) Parsimony (model should contain as few parameters as possible in order to follow Ockham's razor);
ii) Parameterization is used to find the set of parameters which has the best estimation properties. A correlation matrix can help to see if a model might be potentially simplified and which set of parameters has the smallest cross correlations. High correlation coefficients show hidden dependencies since the orthogonality of derivatives cannot be induce. Correlations above 0.98 reflect redundancy of model parameters and should be investigated further;
iii) Range of applicability (the data set to which the model is fitted should cover the full range for which the model applies);
iv) Stochastic specification (the error term needs to be modeled, too. In this report additive error terms assumption was used for modeling);
v) Interpretability (parameters should be meaningful as much as possible).

The principles mentioned above should be taken into account when analyzing applicability of different models for the same data set. Bates and Watts (1988) suggest that the scientific reasons should be the first priority in preferring one model over others, and the second reason is the best fit. If there is no evidence that one of the models describes the mechanism of the expectation function behavior, then the model with the smallest mean square error and the most random-looking residuals should be chosen. Minitab statistical software was useful for graphical presentation of data obtained from the SAS output.

3.3 Results and Discussion

3.3.1 Results of Repeated Measures Analysis

Samples for the analysis were picked in random order. HTDT blueberry purée were analyzed at day 8, 30, 58, 122, 189, 285, 376, and 546 of storage. Results of the analysis are presented in Table 3.1.

For the repeated measures analysis holding time during pasteurization for each sample was used as a covariate factor. In the batch process, it took approximately 50 seconds to fill every jar, so the total holding time was different from jar to jar. Since every jar with a product had its unique number throughout the experiment, it was possible to estimate the pasteurization holding time of the product for each jar and use it in the analysis of covariance (ANCOVA).

Table 3.1 Anthocyanin concentration and tannin content during shelf-life of blueberry purée

Time, day	Anthocyanins, mg C3G/g								Tannins, %			
	Fresh weight				Dry weight				Replication			average
	Replication			average	Replication			average				
	#1	#2	#3		#1	#2	#3		#1	#2	#3	
Cold storage												
0	1.51	1.67	1.54	1.57	13.37	15.34	13.22	13.97	15.02	13.22	16.68	14.97
8	1.42	1.63	1.39	1.48	11.57	13.49	10.84	11.96	15.21	14.01	13.16	14.13
30	1.46	1.38	1.31	1.38	12.08	10.88	10.49	11.15	10.75	14.12	14.15	13.01
58	1.41	1.54	1.52	1.49	11.01	12.33	12.06	11.80	13.15	15.21	15.76	14.71
122	1.28	1.34	1.31	1.31	11.96	12.13	11.75	11.95	13.44	13.40	13.96	13.60
189	1.29	1.34	1.24	1.29	11.79	12.55	11.01	11.78	13.49	13.18	13.29	13.32
285	0.93	1.01	1.19	1.04	7.80	8.35	9.27	8.47	13.34	14.13	13.83	13.77
376	0.58	0.69	0.55	0.61	5.16	6.47	4.93	5.52	19.67	21.07	23.28	21.34
546	0.76	0.86	0.82	0.81	7.00	7.91	7.32	7.41	18.65	22.31	17.85	19.60
Room temperature												
0	1.51	1.67	1.54	1.57	13.37	15.34	13.22	13.97	15.02	13.22	16.68	14.97
8	1.41	1.54	1.15	1.36	11.52	12.37	8.76	10.88	14.17	13.55	14.43	14.05
30	1.30	1.24	1.46	1.33	10.69	9.72	11.55	10.65	14.36	14.43	14.43	14.41
58	1.15	1.18	1.04	1.12	9.10	9.47	8.05	8.87	17.02	18.72	15.09	16.94
122	1.02	1.00	1.12	1.05	9.35	9.08	10.11	9.51	16.37	17.93	16.71	17.00
189	0.78	0.84	0.83	0.82	7.11	7.73	7.37	7.40	19.61	16.40	18.12	18.04
285	0.63	0.58	0.69	0.63	5.08	4.70	5.34	5.04	22.01	24.27	22.21	22.83
376	0.33	0.36	0.28	0.32	3.09	3.18	2.55	2.94	37.60	35.75	40.64	38.00
546	0.30	0.26	0.30	0.29	2.80	2.33	2.72	2.62	38.18	39.61	37.62	38.47

For the repeated measures analysis holding time for each sample was used as a covariate factor. During the experiment, it took approximately 50 seconds to fill every jar, so the total holding time was changed from jar to jar in the batch process. Since every jar with a product had its unique number throughout the experiment, it was possible to estimate the holding time of the product for each jar and use it in the analysis of covariance (ANCOVA).

For choosing the best covariance structure results of goodness of fit analysis using Akaike information criterion (AIC) were compared as presented in Table 3.2. Covariance structures were chosen based on the value of AIC (the closest to zero is preferable).

Table 3.2 Results of Akaike information criterion

	Anthocyanins		Tannins
	per fresh weight	per dry weight	
Unstructured (UN)	15.1	188.7	225.9
Compound symmetry (CS)	– 54.9	120.8	149.0

The analysis of the results presented in Table 3.2 resulted in a decision to use unstructured covariance for the repeated analysis of anthocyanin concentration calculated per fresh weight and compound symmetry covariate structure for the analysis of tannins and anthocyanin concentration calculated per dry weight.

The assumption of normality was met for all response variables. The assumption of constant variance was met for tannins and partially violated for both anthocyanins responses so different types of transformation (square root, cubic root, reciprocal, and logarithmic) were applied to the data in order to achieve constant variance. However, it was not possible to induce constant variance and satisfy normality assumption. Non-transformed data was analyzed using the PROC MIXED procedure in SAS statistical application (Table 3.3).

Table 3.3 Significance of the factors for anthocyanins and tannins repeated analysis

Effect	p-value		
	Anthocyanins		Tannins
	per fresh weight	per dry weight	
Storage temperature	<0.0001	<0.0001	0.0002
Storage time	<0.0001	<0.0001	<0.0001
Storage temperature*Storage time	<0.0001	<0.0001	0.0056
Pasteurization holding time (covariate)	<0.0001	0.0045	<0.0001

Both storage temperature and storage time significantly affects the levels of anthocyanins and tannins in the HTDT blueberry purée. Holding time used for ANCOVA was determined to be significant, affecting anthocyanin concentration and tannin content. A significant interaction effect of time and storage temperature was also detected; multiple means comparison procedure was done for the interaction effect. Mean estimates with letter grouping are listed in Table 3.4.

Table 3.4 Mean estimates and letter grouping for anthocyanins and tannins data

Temperature regime	Time, day	Anthocyanins, mgC3G/g per fresh weight Estimate	Letter grouping	Anthocyanins, mgC3G/g per dry weight Estimate	Letter grouping	Tannins, % Estimate	Letter grouping
Cold storage	0	1.58	a	14.07	a	14.84	ef
Cold storage	8	1.47	ab	11.90	ac	14.22	ef
Cold storage	30	1.42	b	11.46	bc	12.55	f
Cold storage	58	1.45	ab	13.08	ab	14.29	ef
Cold storage	122	1.30	b	11.89	bc	13.69	f
Cold storage	189	1.29	c	11.76	bc	13.35	f
Cold storage	285	1.01	c	8.24	ef	14.12	f
Cold storage	376	0.63	e	5.16	g	21.11	bc
Cold storage	546	0.81	d	7.22	f	19.63	cd
Room temperature	0	1.58	a	14.07	a	14.83	ef
Room temperature	8	1.37	b	10.89	cd	14.04	f
Room temperature	30	1.37	b	10.94	cd	13.97	f
Room temperature	58	1.11	c	8.77	ef	17.10	de
Room temperature	122	1.03	c	9.43	de	17.13	de
Room temperature	189	0.81	d	7.32	f	18.17	d
Room temperature	285	0.61	e	4.87	gh	23.08	b
Room temperature	376	0.35	f	3.12	hi	37.73	a
Room temperature	546	0.29	f	2.62	i	38.46	a

The results of the analysis are presented graphically in Figures 3.1 to 3.3.It follows that storage time negatively affects anthocyanin concentration. There is a significant drop in anthocyanin concentration after 16 months of storage at both temperatures. Cold storage at 4°C provided more favorable conditions for anthocyanin retention with no significant difference after 2 months. However, storage up to 6 months resulted in moderate losses in anthocyanins (18.4% as calculated per fresh weight; 16.4% as calculated per dry weight). At the same time, storage at

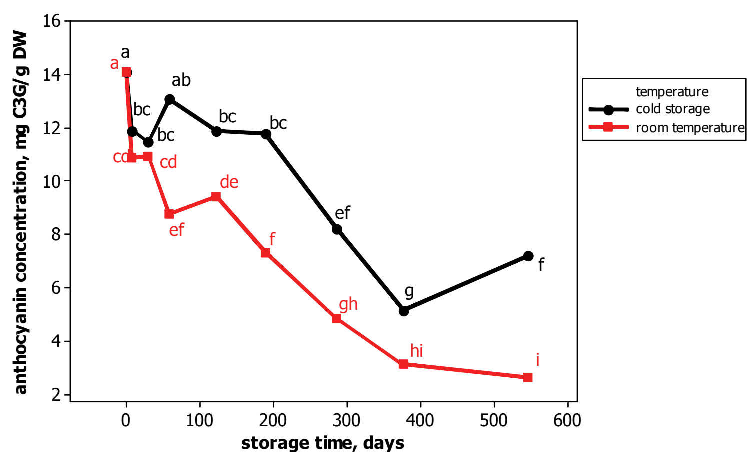


Figure 3.1 Interaction plot of anthocyanins, mgC3G/gDW versus time, days

room temperature resulted in significant losses of anthocyanins even in a short period of time. In two months of room temperature storage product lost 29.7% of initial amount of anthocyanins as calculated per fresh weight or 37.7% as calculated per dry weight.

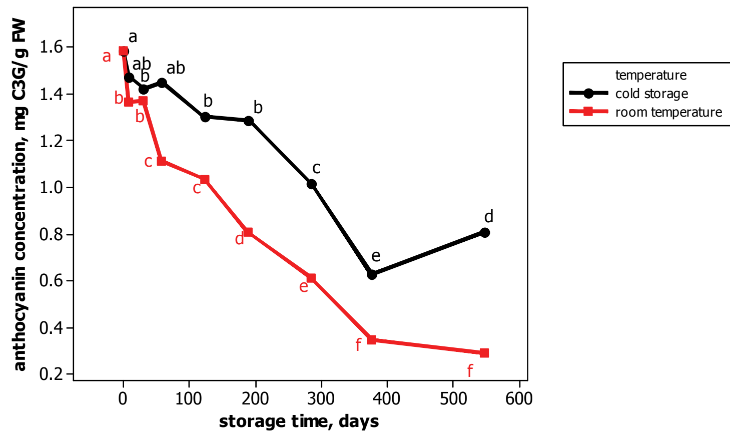


Figure 3.2 Interaction plot of anthocyanins, mgC3G/g FW versus time, days

The amount of tannins during cold storage (4°C) did not change significantly during 9 months of product storage. This fact supports initial assumption that color and taste (grainy texture) of the product will not be significantly affected during 9 months of cold storage. There were no significant changes in tannin content within four months of storage at room temperature; however after four months, noticeable increase in tannins was observed.

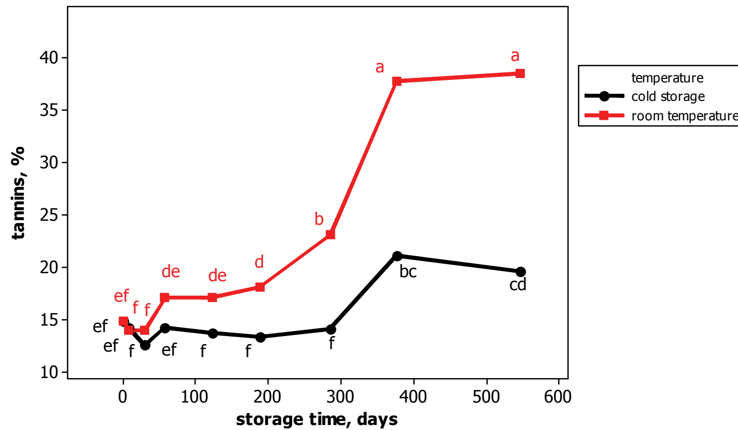


Figure 3.3 Interaction plot of tannins, % versus time, days

3.3.2 Results of Non-Linear Regression Analysis

For the non-linear regression analysis, the estimates of anthocyanin concentration and tannin content obtained in the repeated measures analysis were used.

3.3.2.1 Anthocyanins

Kinetics of anthocyanin degradation may be expressed using first-order exponential model (Equation3) (Wang, 2007):

$$C(t) = C_0 \cdot e^{-kt} \tag{3}$$

Where C_0 is the initial anthocyanin concentration, k is the temperature-dependent rate constant, t is time (days).

Accordingly, the following expectation function for modeling the process of anthocyanin degradation was used:

$$f(x, \theta) = \theta_1 \cdot e^{-\theta_2 \cdot x} \quad (4)$$

Where the response $f(x, \theta)$ is anthocyanin concentration; θ_1 is C_0, the initial anthocyanin concentration; θ_2 is k, the temperature-dependent rate constant, x is t, time (days).

This model was used for both anthocyanin concentration calculated per fresh and dry weight. The starting values were obtained through the interpretation of the expectation function behavior (θ_1) and following the transformation of the expectation function to obtain linear behavior (θ_2). Based on the analysis of the Fig.3.1 and 3.2, a starting value of θ_1= 1.6 mgC3G/g FW and θ_1=14.5mgC3G/g DW was determined for cold storage and room temperature storage respectively. The following transformation of the expectation function $y = \theta_1 \cdot e^{-\theta_2 \cdot x}$ (Equations 5a – 5c) was used to obtain linearity:

$$\frac{y}{\theta_1} = exp(-\theta_2 x), \quad (5a)$$

$$ln(\frac{y}{\theta_1}) = -\theta_2 x, \quad (5b)$$

where $y^* = ln\left(\frac{y}{\theta_1}\right)$; $\beta_1^* = -\theta_2$ (5c)

Coefficients β_1^* for anthocyanin concentration for both cold storage and room temperature storage were obtained by the linear regression analysis in MiniTab. That allowed us to come up with the starting values for and$\theta_2$for both room temperature and cold storage (Table 3.5).

Table3.5 Starting values for anthocyanin concentration non-linear modeling

	Cold storage		Room temperature	
	θ_1	θ_2	θ_1	θ_2
Anthocyanins, per fresh weight	1.6 mgC3G/g FW	–0.00148	1.6 mgC3G/g FW	–0.00304
Anthocyanins, per dry weight	14.5mgC3G/g DW	– 0.00149	14.5mgC3G/g DW	–0.00321

Obtained starting values were used in the PROC NLIN procedure (SAS) (the results are presented in Table 3.6.) The convergence criterion for both temperatures was met.

Table 3.6 Parameter estimates for anthocyanin degradation during HTDT purée storage

Kinetic model coefficient	Parameter of the expectation function	Parameter estimates			
		Anthocyanins, mgC3G/g DW		Anthocyanins, mgC3G/g FW	
		Cold storage	Room temperature	Cold storage	Room temperature
C_0	θ_1	13.26	12.31	1.544	1.4826
k	θ_2	$1.46 \cdot 10^{-3}$	$3.14 \cdot 10^{-3}$	$1.51 \cdot 10^{-3}$	$3.33 \cdot 10^{-3}$

The results are presented graphically in figures 3.4 which show measured values and fitted lines.

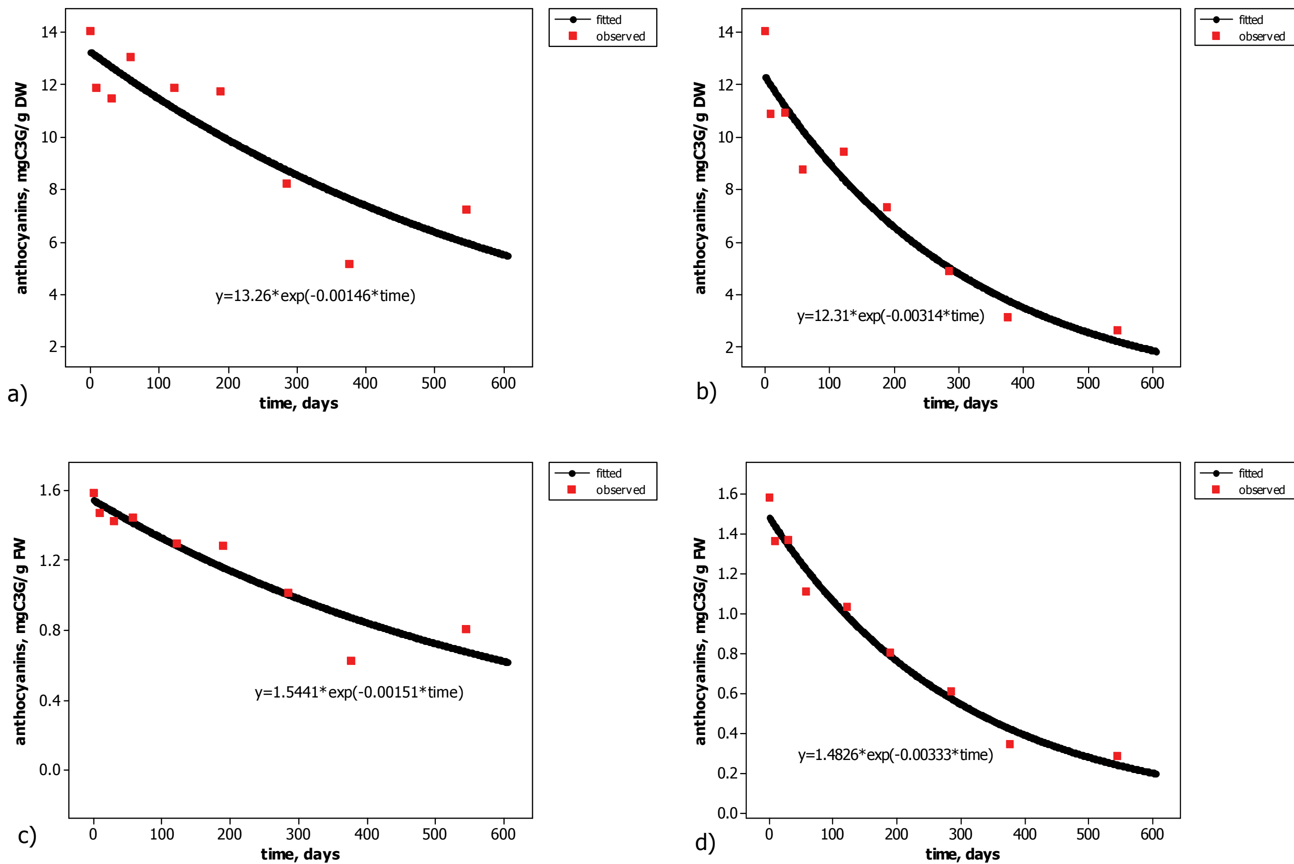


Figure 3.4 a) Kinetics of anthocyanin degradation (per DW, cold storage); b) Kinetics of anthocyanin degradation (per DW, room temperature); c) Kinetics of anthocyanin degradation (per FW, cold storage); d) Kinetics of anthocyanindegradation (per FW, room temperature)

In order to find if the parameters in the two models (for anthocyanin degradation at room temperature (rt) and during cold storage (cs)) are significantly different, the incremental parameters method was used.

The following model was fitted using the PROC NLIN procedure in SAS:

$$f(\theta,\varphi,x) = (\theta_1 + \varphi_1 x_1) \cdot e^{-(\theta_2+\varphi_2 x_1)\cdot x} \qquad (6)$$

Where x_1=0 for cold storage, x_2=1 for room temperature; φ_1 is an incremental parameter forθ_1 due to the change in treatment (cold storage compared to room temperature); φ_2 is an incremental parameter forθ_2 due to the change in treatment.

It means that coefficient θ_1 for cold storage is $\theta_1^{cs} = \theta_1 + \varphi_1 x_1 = \theta_1$, for room temperature is $\theta_1^{rt} = \theta_1 + \varphi_1 x_1 = \theta_1 + \varphi_1$. The same relationship is used for the rate constant coefficient: coefficient θ_2 for cold storage is $\theta_2^{cs} = \theta_2 + \varphi_2 x_1 = \theta_2$, for room temperature is $\theta_2^{rt} = \theta_2 + \varphi_2 x_1 = \theta_2 + \varphi_2$. If the null hypothesis is accepted and $\varphi = 0$, there is no significant difference between θ^{cs} and θ^{rt}. Since the PROC NLIN procedure does not allow one to estimate the parameters exactly and hence does not provide the results of t-test, an approximate 95% confidence interval of parameter estimates (φ_1 and φ_2) is used to find whether or not the differences between the parameters in the models for two different storage regimes are significant. If the 95% confidence interval contains 0, the difference is not significant. Results of this analysis are presented in Table 3.7.

Table 3.7 Incremental parameters estimates and significance of the difference for the model parameters (anthocyanin concentration)

Kinetic model coefficient	Parameter of the expectation function	Parameter estimates		Approximate 95% CI for the incremental parameter φ	Significance
		Cold storage	Room temperature		
Anthocyanin concentration, mg C3G/g of dry weight					
C_0	θ_1	13.26	12.31	(-3.142; 1.238)	not significant
k	θ_2	$1.46\cdot10^{-3}$	$3.14\cdot10^{-3}$	(0.00035; 0.00301)	significant
Anthocyanin concentration, mg C3G/g of fresh weight					
C_0	θ_1	1.544	1.4826	(-0.2307; 0.1077)	not significant
k	θ_2	$1.51\cdot10^{-3}$	$3.33\cdot10^{-3}$	(0.00093; 0.00272)	significant

The difference between the rate constants (θ_2^{cs} and θ_2^{rt} parameters) is significant. The parameter estimates of θ_1^{cs} and θ_1^{rt} (initial anthocyanin concentration) do not differ significantly from each other for both anthocyanin concentration calculated per dry weight and fresh weight.

The normality and constant variance assumptions were checked and found to be valid for both models. The plots of residuals versus the independent variable (time) allowed to conclude that the model is adequate (information is not presented in this study).

The analysis of Figures 3.4 – 3.7 and the comparison of the rate constant show that the rate of anthocyanin loss during cold storage is less as compared with storage at room temperature. At room temperature, the kinetic constant of anthocyanin degradation is almost twice as high as during the cold storage ($3.14 \cdot 10^{-3}$vs. $1.46 \cdot 10^{-3}$ for anthocyanin concentration per DW and $3.33 \cdot 10^{-3}$vs. $1.51 \cdot 10^{-3}$ for anthocyanin concentration per FW respectively). Fitted models of anthocyanin loss can be used to predict anthocyanin concentration for the particular period of storage for any specified temperature.

3.3.2.2 Tannins

There is no background information which describes the kinetics of changes in tannin content during the storage of blueberry based products. However, it is known that anthocyanins are a substrate for the condensation reaction which results in tannin formation (Brownmiller, 2008). Analysis of the graphs (Fig.3.3) allows one to suggest using the logistic growth model as a prediction function for describing kinetics of tannin formation during blueberry purée storage:

$$C(t) = \theta_1 + \frac{\theta_2}{1 + \exp(-\frac{x-\theta_3}{\theta_4})} \qquad (7)$$

Where C is an amount of tannins, % ; t is time, days; θ_1 is an initial amount of tannins; θ_2 is the difference between the asymptote and the initial amount of tannins, θ_3 is the time to half the asymptote, θ_4 is the time interval between about ¾ the asymptote and half the asymptote.

The expectation function has the following form:

$$f(\theta, x) = \theta_1 + \frac{\theta_2}{1 + \exp(-\frac{x-\theta_3}{\theta_4})} \qquad (8)$$

The starting values were obtained through interpretation of the expectation function behavior (θ_1 and θ_2) and following transformation of the expectation function to obtain linear behavior (θ_3 and θ_4). Based on the analysis of Fig.3.3, starting values of θ_1= 12% were

determined for both cold storage and room temperature storage, while θ_2 = 10% and θ_2=28% were obtained for cold storage and room temperature storage respectively.

The following transformation of the expectation function $y = \theta_1 + \frac{\theta_2}{1+\exp(-\frac{x-\theta_3}{\theta_4})}$ was used to obtain linearity:

$$\frac{\theta_2}{y-\theta_1} - 1 = exp\left(-\frac{x-\theta_3}{\theta_4}\right) \quad (9a)$$

$$ln\left(\frac{\theta_2}{y-\theta_1} - 1\right) = \frac{\theta_3}{\theta_4} - \frac{x}{\theta_4} \quad (9b)$$

Where $y^* = ln\left(\frac{\theta_2}{y-\theta_1} - 1\right)$; $\beta_0^* = \frac{\theta_3}{\theta_4}$; $\beta_1^* = -\frac{1}{\theta_4}$.

Coefficients β_0^* and β_1^* were obtained by the linear regression analysis in MiniTab, which allowed us to estimate the starting values for parameters θ_3 and θ_4 using the ratios specified above. Results of the calculations are presented in Table 3.8.

Table 3.8 Starting values of parameters in non-linear modeling of tannin changes

Storage regime	Starting values					
	obtained graphically				obtained from the expectation function transformation	
	θ_1	θ_2	θ_3	θ_4	θ_3	θ_4
Cold storage	12%	10%	350 days	20 days	315 days	161 days
Room temperature	12%	28%	300 days	10 days	252 days	97 days

The obtained starting values were used in the NLIN procedure in SAS statistical software in order to calculate parameter estimates (Table 3.9). For both temperature regimes the convergence criterion was met.

Table 3.9 Parameter estimates for the increase in tannin content during HTDT purée storage

Storage regime	Parameter estimates			
	θ_1	θ_2	θ_3	θ_4
Cold storage	13.82%	6.55%	330.6 days	15.276 days
Room temperature	15.77%	23.13%	304.9days	28.17 days

Starting values for the parameter estimates obtained by graphical analysis of data behavior was more accurate than that from the expectation function transformation (Tables 3.8 and 3.9).

Fig.3.5a illustrates the kinetics of tannin increase in blueberry HTDT purée during cold storage. Fig. 3.5b represents the kinetics of tannin increase in blueberry HTDT purée during storage at room temperature.

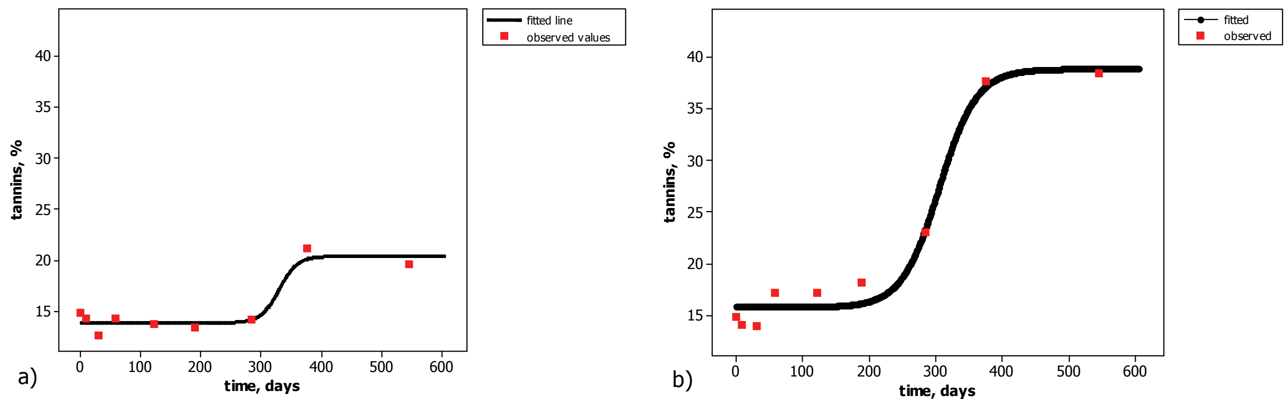


Figure 3.5 a) Kinetics of tannins (cold storage); b)Kinetics of tannins (at room temperature)

To determine the differences between kinetics parameters for tannin development at room temperature (rt) and cold storage (cs), the incremental parameters method was used.

The following model was used for fitting (NLIN in SAS):

$$f(\theta,\varphi,x) = (\theta_1+\varphi_1 x_1) + \frac{(\theta_2+\varphi_2 x_2)}{1+\exp(-\frac{x-(\theta_3+\varphi_3 x_3)}{(\theta_4+\varphi_4 x_4)})} \qquad (10)$$

Where x_1=0 for cold storage, x_2=1 for room temperature; φ_1 is an increment in the parameter θ_1 due to the change in treatment (cold storage compared to room temperature); φ_2, φ_3, φ_4 are increments in the parameters $\theta_2, \theta_3, \theta_4$ due to the change in treatment respectively.

The coefficient θ_1 for cold storage is $\theta_1^{cs} = \theta_1 + \varphi_1 x_1 = \theta_1$ and coefficient θ_1 for room temperature is $\theta_1^{rt} = \theta_1 + \varphi_1 x_1 = \theta_1 + \varphi_1$. If the null hypothesis is accepted and $\varphi_1 = 0$, there is no significant difference between $\theta_1^{cs}$and θ_1^{rt}. The same relationship was used for the other parameters. Since the NLIN procedure does not allow one to estimate the parameters exactly and hence does not provide the results of the t-test, approximate 95% confidence intervals of parameter estimates were used to find whether or not the difference between the parameters is significant. If the 95% confidence interval contains 0, the difference is assumed to not be significant (results of the analysis are presented in Table 3.10).

Table 3.10 Incremental parameters estimates and significance of the difference for the model parameters (tannins)

Parameter of the expectation function		Parameter estimates		Approximate 95% CI for the incremental parameter φ	Significance
		Cold storage	Room temperatu re		
θ_1	initial tannins	13.82%	15.77%	(-0.388; 3.716)	not significant
θ_2	difference between asymptote and initial tannins	6.55%	23.13%	(12.348; 19.004)	significant
θ_3	time to half asymptote	331 days	305 days	(0.627; 0.983)	significant
θ_4	interval between ¾ and half asymptote	15 days	28 days	(0.0241; 0.1963)	significant

It follows that the parameter estimates of θ_1^{cs} and θ_1^{rt} (initial tannin content) did not differ significantly from each other. Other parameters were found significantly different for the two storage regimes (storage at room temperature and at 4°C). Total tannin content (θ_2) was significantly higher at room temperature (6.6% versus 23.1%), and half the total tannin content (θ_3) was reached significantly faster (304 days versus 330 days) at room temperature storage as compared to cold storage. The normality and constant variance assumptions were checked and found to be valid for both temperature regimes. The general impression from plots of residuals

versus independent variables (time) allowed one to conclude that the model was adequate (data not shown).The models for tannin formation can be used to predict tannin content for the particular period of storage at room temperature or in cold storage conditions.

3.4 Conclusion and Recommendations

Cold storage at 4°C is preferable in terms of better preserve of anthocyanins and should be recommended for the HTDT blueberry purée storage. The rate of anthocyanin degradation and tannin formation at room temperature was twice as high as that at 4°C (Tables 3.7 and 3.10, Fig. 3.4 – 3.9). For anthocyanin concentration, storage up to 6 months at 4°Cis equivalent to 2 months at room temperature and can be recommended as a shelf-life period for HTDT blueberry purée. Fitted models, described in this work, can be used to predict anthocyanin concentration and tannin content for different periods of storage at room temperature and at 4°C.For better understanding of the HTDT processing factors effect on blueberry anthocyanins, tannins and microbial load, optimization studies was conducted (results are presented in Chapter 4).

4. PROCESS OPTIMIZATION

4.1 Introduction

This chapter is devoted to the process of optimization of HTDT blueberry processing in order to maximize anthocyanin concentration, minimize tannin content and ensure food safety. The first step in the optimization process is screening of the most significant process factors. Four potentially significant parameters were chosen for this study: (i) temperature of pasteurization, (ii) holding time, (iii) rate of product heating, and (iv) cavitation mode of the HTDT pilot-scale machine. The second step of the optimization process involved response surface methodology (multiple response analysis) to determine the set of processing parameters to provide a high quality of the product.

4.2 Materials and Methods

4.2.1 Materials

Raw materials for this study were provided by PEI Organic Fruit and Berries Ltd. (frozen berries, harvested in September 2009). Frozen berries were stored in buckets at – 18°C before processing. The HTDT blueberry purée was produced in September 2009 at the Engineering Department of the NSAC. The pasteurized product was filled into 250 ml jars and the jars were sealed (self-pasteurization).Jars containing HTDT blueberry purée were labeled (number of batch, order of filling) and stored at +4°C (cold room, Haley Institute) prior to the analysis. Only the first 5 jars from each batch were used for the analysis (to exclude the effect of prolonged holding time for the jars filled at the end of the filling process).

4.2.2. Analytical Methods

4.2.2.1 Measurement of Anthocyanin Concentration and Polymeric color Content (Tannins)

The measurement of anthocyanin concentration and polymeric color content (derived tannins) was done as described in Section 2.2.2.1. The analysis was conducted at the facilities of the NSAC.

4.2.2.2 Microbial Analysis

The aerobic plate count analysis was performed for the pasteurized blueberry purée and the raw material (frozen blueberries after thawing). Subsamples of the blueberry product/

material (approximately 10g) were aseptically transferred into sterile plastic bags with inner filters, diluted with buffered peptone water in a 1:10 ratio, and mixed for 2 minutes in a stomacher. The series of decimal dilutions were performed by transferring 10 ml of inoculate into 90 ml of sterile buffered peptone water. All dilution samples (1 ml) were aseptically inoculated into labeled Petrifilm™ plates and were incubated for 48 hours at 30°C. Duplicate plates were used for each dilution. If no colonies were detected at a plate, the result was reported as "less than one per plate".

4.2.3 Statistical Analysis and Experimental Design

The unreplicated 2^k factorial design is practical and useful if the significance of two or more factors needs to be investigated. This type of experimental design suits well to the sequential experimental approach and can be used in the early stages because it provides the smallest number of runs for studying k factors in full factorial design. In 2^k factorial design it is assumed that (i) all factors are fixed; (ii) the design is completely randomized to provide independence, and (iii) normality and constant variance assumptions are satisfied. To achieve independence, the sequence of runs was completely randomized using the Random Data feature by MiniTab software.

4.2.3.1 Screening of Processing Factors

For the determination of the significance of the processing factors a 2^4 factorial design with four factors on two levels each: (i) temperature of pasteurization, °C, (ii) holding time, min, (iii) heating rate, °C/min, and (iv) cavitation mode was used (Table 4.1).

Table 4.1 Levels of factors for 2^4 factorial design

Factor	Level	
	–	+
Temperature of pasteurization	85°C	95°C
Holding time	0 min	5 min
Heating rate	0.9 °C/min	1.7 °C/min
Cavitation mode	A	A+B

The temperature was controlled automatically by two thermocouples and the results were analyzed in real-time by the LabView 8.5 software (National Instruments). The holding time of pasteurization was provided by the start-stop regime of the motor, keeping the temperature of pasteurization at ± 0.2°C. The heating rate was regulated by cooling with cold water through the cooling jacket of the TEK-1processor. The cavitation mode was kept at A for the low level and was changed to B for the period of the last 10°C of heating for the high level (A+B).

Each treatment combination was used in a single replication. The responses were: (i) anthocyanin concentration, (ii) polymeric color (tannins) content and (iii) aerobic plate count (APC).

The statistical model for a replicated 2^4 factorial design is:

$$\left.\begin{aligned} y_{ijklm} &= \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \delta_l + (\alpha\delta)_{il} \\ &+ (\beta\delta)_{jl} + (\alpha\beta\delta)_{ijl} + +(\gamma\delta)_{kl} + (\alpha\gamma\delta)_{ikl} + (\beta\gamma\delta)_{jkl} + (\alpha\beta\gamma\delta)_{ijkl} + \varepsilon_{ijklm} \end{aligned}\right\} \begin{aligned} & i = 1, 2 \\ & j = 1, 2 \\ & k = 1, 2 \\ & l = 1, 2 \\ & m = 1, 2, \ldots n \end{aligned} \quad (11)$$

Where y_{ijklm} is a total response from the ith, j^{th}, k^{th}, l^{th} factors and the mth replication; μ is the overall mean; $\alpha_i, \beta_j, \gamma_k, \delta_l$ are the main effects; $(\alpha\beta)_{ij}, (\alpha\gamma)_{ik}, (\beta\gamma)_{jk}, (\alpha\delta)_{il}, (\beta\delta)_{jl}, (\gamma\delta)_{kl}$ represent 2-way interaction effects; $(\alpha\beta\gamma)_{ijk}, (\alpha\beta\delta)_{ijl}, (\alpha\gamma\delta)_{ikl}, (\beta\gamma\delta)_{jkl}$ represents 3-way interaction effects; $(\alpha\beta\gamma\delta)_{ijkl}$ represents 4-way interaction effects; ε_{ijklm} represents experimental error terms.

In the unreplicated factorial design, with only one replicate, there is no estimate of error ε_{ijklm}. Therefore, the statistical model of the 2^4unreplicated factorial design is:

$$\left.\begin{aligned} y_{ijklm} &= \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \delta_l + \\ & (\alpha\delta)_{il} + (\beta\delta)_{jl} + (\alpha\beta\delta)_{ijl} + +(\gamma\delta)_{kl} + (\alpha\gamma\delta)_{ikl} + (\beta\gamma\delta)_{jkl} + (\alpha\beta\gamma\delta)_{ijkl} \end{aligned}\right\} \begin{aligned} & i = 1, 2 \\ & j = 1, 2 \\ & k = 1, 2 \\ & l = 1, 2 \end{aligned} \quad (12)$$

Where y_{ijklm} is a total response from the ith, j^{th}, k^{th}, l^{th} factors; μ is the overall mean; α_i is an effect of the ith level of the temperature of pasteurization; β_j is an effect of the jth level of the holding time; γ_k is an effect of the kth level of the heating rate; δ_l is an effect of the lth level of the

cavitation mode; $(\alpha\beta)_{ij}$ represents an interaction effect of the interaction between the temperature and the holding time; $(\alpha\gamma)_{ik}$ represents an interaction effect between the temperature and the heating rate; $(\beta\gamma)_{jk}$ represents an interaction effect between the holding time and the heating rate; $(\alpha\delta)_{il}$ represents an interaction effect between the temperature and the cavitation mode; $(\beta\delta)_{jl}$ represents an interaction effect between the holding time and the cavitation mode; $(\gamma\delta)_{kl}$ represents an interaction effect between the heating rate and the cavitation mode; $(\alpha\beta\gamma)_{ijk}$ represents a 3-way interaction effect between the temperature, the holding time and the heating rate; $(\alpha\beta\delta)_{ijl}$ represents a 3-way interaction effect between the temperature, the holding time and the cavitation mode; $(\alpha\gamma\delta)_{ikl}$ represents a 3-way interaction effect between the temperature, the heating rate and the cavitation mode; $(\beta\gamma\delta)_{jkl}$ represents a 3-way interaction effect between the holding time, the heating rate and the cavitation mode; $(\alpha\beta\gamma\delta)_{ijkl}$ represents a 4-way interaction effect between the temperature, the holding time, the heating rate and the cavitation mode.

Hypotheses were stated as follow (Table 4.2):

Table 4.2 Statement of the hypotheses for a 4 factor unreplicated factorial design

Main effects	Two-way interaction	Three way interaction	Four-way
H_0: $\alpha_1 = \alpha_2 = 0$ H_a: at least one $\alpha \neq 0$	H_0: $(\alpha\beta)_{ij} = 0$ for all i, j H_a: at least one $(\alpha\beta)_{ij} \neq 0$	H_0: $(\alpha\beta\gamma)_{ijk} = 0$ for all i, j, k H_a: at least one $(\alpha\beta\gamma)_{ijk} \neq 0$	H_0: $(\alpha\beta\gamma\delta)_{ijkl} = 0$ for all i, j, k, l H_a: at least one $(\alpha\beta\gamma\delta)_{ijkl} \neq 0$
H_0: $\beta_1 = \beta_2 = 0$ H_a: at least one $\beta \neq 0$	H_0: $(\alpha\gamma)_{ik} = 0$ for all i, k H_a: at least one $(\alpha\gamma)_{ik} \neq 0$	H_0: $(\alpha\beta\delta)_{ijl} = 0$ for all i, j, l H_a: at least one $(\alpha\beta\delta)_{ijl} \neq 0$	
H_0: $\gamma_1 = \gamma_2 = 0$ H_a: at least one $\gamma \neq 0$	H_0: $(\beta\gamma)_{jk} = 0$ for all j, k H_a: at least one $(\beta\gamma)_{jk} \neq 0$	H_0: $(\alpha\gamma\delta)_{ikl} = 0$ for all i, k, l H_a: at least one $(\alpha\gamma\delta)_{ikl} \neq 0$	
H_0: $\delta_1 = \delta_2 = 0$ H_a: at least one $\delta \neq 0$	H_0: $(\alpha\delta)_{il} = 0$ for all i, l H_a: at least one $(\alpha\delta)_{il} \neq 0$	H_0: $(\beta\gamma\delta)_{jkl} = 0$ for all j, k, l H_a: at least one $(\beta\gamma\delta)_{jkl} \neq 0$	
	H_0: $(\beta\delta)_{jl} = 0$ for all j, l H_a: at least one $(\beta\delta)_{jl} \neq 0$		
	H_0: $(\gamma\delta)_{kl} = 0$ for all k, l H_a: at least one $(\gamma\delta)_{kl} \neq 0$		

Due to the lack of replication there is no pure error in the unreplicated design. Hence, one of the approaches to conduct ANOVA is to assume that the highest order interaction is negligible and to use its mean squares to estimate the error. This reduction of the model is often possible due to the "sparsity of effects principle"; that is, that most systems are dominated by the main effects and the low-order interaction effects, and in most cases the high-order interaction effects are negligible. The second approach is the collapsing of the model, which is possible in case one of the factors (the main effect and all interaction effects) is not significant. In this case, two levels of this factor can be considered as replicates and the ANOVA analysis might be applied.

For performing the ANOVA test, the assumptions of normality, the constant variance and independence were checked. To check the normal distribution of error terms, normal probability plots of residuals were generated and analyzed. In order to check the assumption of constant variance, plots of residuals versus fitted values were analyzed. Independence was assumed due to complete randomization and checked by using the autocorrelation function incorporated into the MiniTab software. Another approach of analyzing data from unreplicated designs that doesn't require error degrees of freedom for ANOVA is based on the normal probability plot of the estimates of the effects. This approach was initially developed by Daniel (1959) to detect active effects. MiniTab software allowed performing Daniel's plot with incorporated Lenth's test (Astatkie et al., 2006; Lenth, 1989) which used the examination of pseudo standard errors to present the significance of effects.

4.2.3.2 Process Optimization

For further investigation on the effect of processing factors on the HTDT blueberry purée quality and for determining the optimal level for the factors, the response surface methodology (RSM) was used. RSM enables optimization of the response, affected by several process variables, by using a set of mathematical and statistical techniques. The problem of optimization assumes that there is a curvature in the system which can be represented as a second order model:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}\sum \beta_{ij} x_i x_j + \epsilon \qquad (13)$$

Where y is a response, β_0, β_i are model parameters, x_i represent process variables.

In this experiment, the results of the factor screening from the 2^4 unreplicated factorial design were used. Since the cavitation mode did not affect the response variables, and taking into account the fact that using a maximum heating rate provides the best energy efficiency of the process, only two process variables were included into the optimization problem: (i) the temperature of pasteurization, °C and (ii) the holding time, min. In this particular case the second-order polynomial model is:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \qquad (14)$$

where $\beta_0, \beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12}$ are model parameters, x_1 represents the holding time,x_2 representsthe temperature of pasteurization, x_1^2 and x_2^2 represent the quadratic components of the model, $x_1 x_2$ represents the interaction between the temperature and the holding time.

A central composite design (CCD) with 2 variables at 5 levels each (Table 4.1) was used. The response variables studied were: (i) anthocyanin concentration, mg C3G/g of dry weight; (ii) polymeric color (tannins, %); and microbial load (aerobic plate count, cfu/g). Optimization for these multiple responses included the maximizing anthocyanin concentration and minimizing polymeric color (tannins). At the same time, the microbial load was taken into account as a constraint. For data analysis, the RSREG procedure of the SAS software, as well as the Minitab software were used to obtain predictive models. Optimization of the independent variables was conducted by employing canonical analysis (the SAS software). The assumptions of the normality and constant variance were checked. When the results showed a saddle point in response surfaces, the ridge analysis of the SAS RSREG procedure was used to compute the estimated ridge of the optimum response. The contour plots for three response variables, as well as overlaid contour plots, were generated using the Minitab software.

4.3 Results and Discussion

4.3.1 Processing Factors Screening

The unreplicated experimental design for the different combinations of treatments and the values of response variables are shown in the Table 4.3.

Table 4.3 Results of the unreplicated 2^4 experimental design

Number	Factor								Anthocyanins, mgC3G/gDW	Tannins, %	APC, CFU/g
	Level (coded)				Level (uncoded)						
	Temperature	Holding time	Heating rate	Cavitation mode	Temperature, °C	Holding time, min	Heating rate, °C/min	Cavitation mode			
1	–	–	–	–	85	0	0.9	A	10.53	15.04	20
2	+	–	–	–	95	0	0.9	A	9.69	15.04	<10
3	–	+	–	–	85	5	0.9	A	9.76	15.57	25
4	+	+	–	–	95	5	0.9	A	8.86	16.83	<10
5	–	–	+	–	85	0	1.7	A	11.05	14.14	35
6	+	–	+	–	95	0	1.7	A	10.57	15.01	<10
7	–	+	+	–	85	5	1.7	A	10.88	14.64	<10
8	+	+	+	–	95	5	1.7	A	10.14	16.46	<10
9	–	–	–	+	85	0	0.9	A+B	10.89	14.23	15
10	+	–	–	+	95	0	0.9	A+B	9.55	16.14	<10
11	–	+	–	+	85	5	0.9	A+B	10.59	15.37	<10
12	+	+	–	+	95	5	0.9	A+B	8.58	18.97	<10
13	–	–	+	+	85	0	1.7	A+B	11.37	13.85	307.5
14	+	–	+	+	95	0	1.7	A+B	11.34	14.14	5
15	–	+	+	+	85	5	1.7	A+B	10.82	15.75	40
16	+	+	+	+	95	5	1.7	A+B	9.52	15.18	<10

4.3.1.1 Anthocyanins

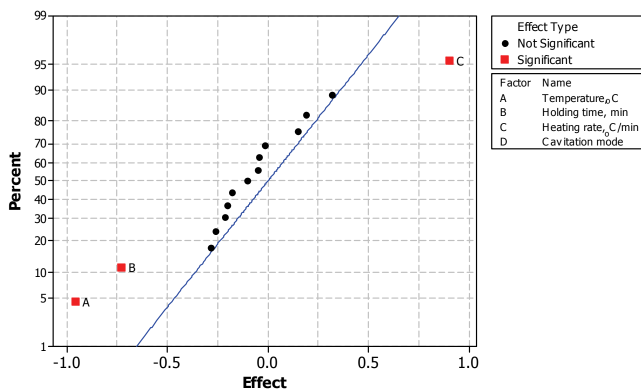


Figure 4.1 Normal plot of the effects (response is anthocyanin concentration, mg C3G/g DW, α = 0.05)

To determine the effect of the processing factors (temperature of pasteurization, holding time, heating rate and cavitation mode) on anthocyanin concentration (estimated as mg C3G/g DW), Daniel's plot (normal plot of the effects) with incorporated Lenth's test was performed (Figure 4.1).

The analysis of Daniel's plot shows that the temperature of

pasteurization, the holding time and the heating rate affects anthocyanin concentration significantly. No interaction effects were observed nor was there an effect of the cavitation mode. This fact allows one to collapse the model and consider two levels of factor D (cavitation mode) to be replicates. In this case, the design can be analyzed as a three factor factorial with two replications using ANOVA. The normality and constant variance assumptions were checked and found to be valid. The model was found to be adequate based on the general impression from the plots of residuals versus the independent variable (time).The complete randomization and the results of the autocorrelation function analysis provided enough evidence to assume independence. The results of the ANOVA test for anthocyanins with two levels of factor D (cavitation mode) used as replicates are shown as table 4.4.

Table 4.4 Results of ANOVA test for anthocyanins

Treatment combination	p-value
Temperature of pasteurization, °C (A)	0.001
Holding time, min (B)	0.003
Heating rate, °C/min (C)	0.001
Temperature * Holding time (A*B)	0.149
Temperature * Heating rate (A*C)	0.110
Holding time * Heating rate (B*C)	0.945
Temperature * Holding time * Heating rate (A*B*C)	0.587

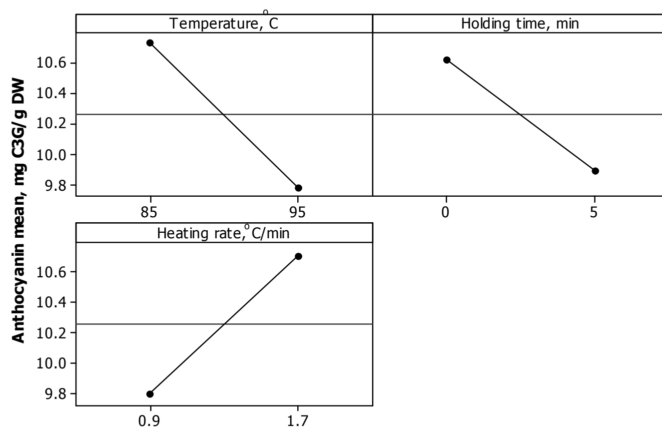


Figure 4.2 Main effects plot for anthocyanin concentration, mg C3G/g DW

The adjusted R^2 is 82.67%, which means that 82.67% of the total variability of the system is explained by the factors chosen for the experiment (temperature of pasteurization, holding time and heating rate).

The main effects of the temperature of pasteurization, the holding time and the heating rate are presented in Figure 4.2.Taking into account the results of the ANOVA test it was possible to conclude that the

temperature of pasteurization, the holding time and the heating rate affected the anthocyanin concentration in the pasteurized HTDT blueberry purée. The cavitation mode as well as all two- and three- way interaction effects did not affect anthocyanin concentration. To conclude which level of these three factors is preferable to achieve higher response (concentration of anthocyanins in the product) main effect plots were analyzed. A low level of factor A (temperature of pasteurization is 85 °C), a low level of factor B (holding time is 0 min) and a high level of factor C (heating rate is 1.7 °C/min) provided a higher anthocyanin concentration in the HTDT blueberry purée.

4.3.1.2 Tannins

Daniel's plot for the effect of the processing factors on the polymeric color content (tannins) is presented in Figure 4.3.

The analysis of Daniel's plot shows that the temperature of pasteurization affects the anthocyanin concentration significantly. No interaction effects, neither the effect of the holding time, the temperature, and the cavitation mode, show up as a significant effect at the significance level α=0.05. However, some effects are deviated from the straight line and might be marginally significant. Therefore, the effects were also explored at α=0.10. At significance level α=0.10 both factors A and B (temperature and holding time) significantly affected the polymeric color content (Figure 4.4).

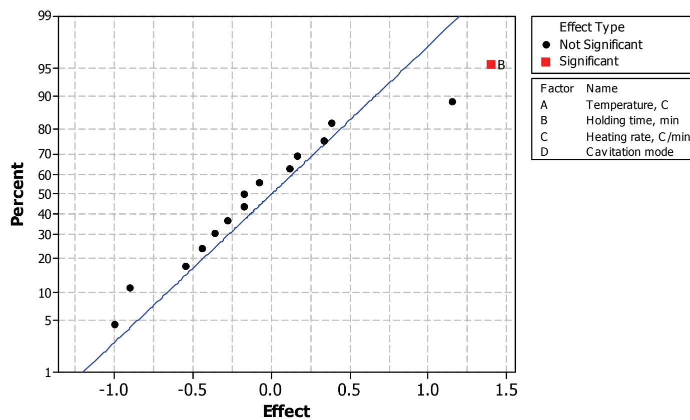


Figure 4.3 Normal plot of the effects(response is tannins, %, α= 0.05)

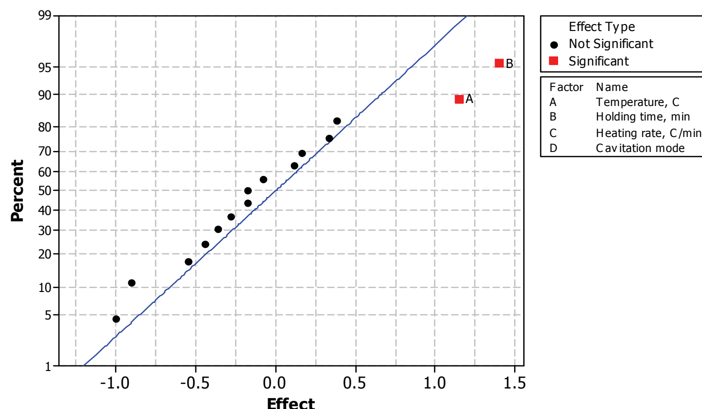


Fig. 4.4 Normal plot of the effects(response is tannins, %, α = 0.10)

Then the design was collapsed to and the data was analyzed as a three factor factorial with two replications.

The assumption of normality was checked and found to be valid; however, the constant variance assumption was violated. To induce constant variance, the logarithmic transformation (log based 10) was used (the normality assumption for transformed data was valid too).The complete randomization and results of the autocorrelation function analysis for transformed data provided enough evidence to assume independence. The following ANOVA results were obtained using the transformed response values. The results of the ANOVA test for tannins for the transformed data are shown in Table 4.5.

Table 4.5 Results of the ANOVA test for processing factors and tannin content

Treatment combination	p-value
Temperature of pasteurization, °C (A)	0.019
Holding time, min (B)	0.007
Heating rate, °C/min (C)	0.033
Temperature * Holding time (A*B)	0.432
Temperature * Heating rate (A*C)	0.232
Holding time * Heating rate (B*C)	0.770
Temperature * Holding time * Heating rate (A*B*C)	0.431

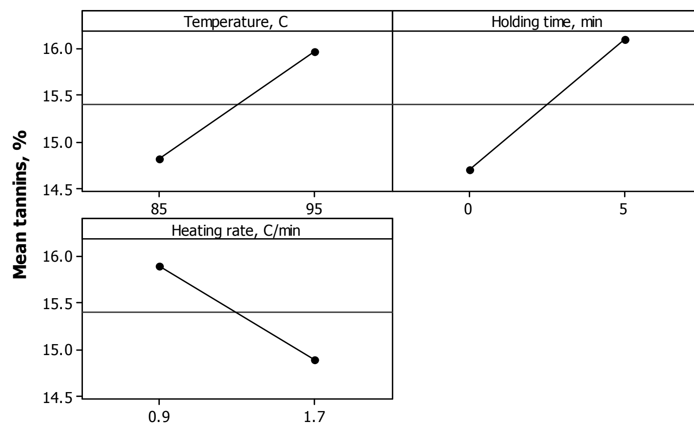


Figure 4.5 Main effects plot for tannins, %

The adjusted R^2 of 62.10% means that 62.10% of the total variability of the system was explained by the factors chosen for the experiment (temperature of pasteurization, holding time and heating rate).

The main effects of the temperature of pasteurization, the holding time and the heating rate

on tannins are presented in Figure 4.5. For easier comprehension, the main effects plot was performed using non-transformed data (tannins, %).

Taking into account the results of the ANOVA test, one can conclude that the temperature of pasteurization, the holding time and the heating rate significantly affected the polymeric color content (tannins, %) of the product. The cavitation mode as well as all two- and three- way interaction effects did not affect significantly the tannin content. To conclude to which level of these three factors is preferable to achieve the lowest response (tannin content) the main effect plots were analyzed. It follows that low levels of factor A (temperature of pasteurization is 85 °C), low levels of factor B (holding time is 0 min) and high levels of factor C (heating rate is 1.9 °C/min) are desirable to achieve a low polymeric color content in the product.

4.3.1.3 Microbial Load

The direct analysis of the microbial load data (Aerobic plate count, cfu/g) did not provide meaningful results because the initial microbial load of the raw material within the batch varied. To normalize the effect of unequal microbial load in the raw material, log reduction was calculated as a response variable. Log reduction for each treatment combination was calculated as a difference between logarithms of cfu/g for the raw material and the pasteurized product.

Table 4.6 shows the results of the microbial tests taking into account the microbial load of the initial raw material for each batch. or a treatment combination number 13, the initial microbial load was noticeably higher than that for the other batches (the period of thawing of the frozen blueberries for this batch was much longer as compared with the other batches, so the process of fermentation probably started which might provoke some microbial growth in the raw material). It resulted in a markedly higher microbial load of the pasteurized product.

Table 4.6 Results of the unreplicated 2^4 design for microbial load

	Factor								Microbial load				
	Level (coded)				Level (uncoded)				Raw material		Pasteurized product		
Number	Temperature	Holding time	Heating rate	Cavitation mode	Temperature, °C	Holding time, min	Heating rate, °C/min	Cavitation mode	cfu, g	Log number	cfu, g	Log number	Log reduction
1	–	–	–	–	85	0	0.9	A	$2.6 \cdot 10^4$	4.41	20	1.30	3.11
2	+	–	–	–	95	0	0.9	A	$1.4 \cdot 10^4$	4.15	<10	<1.00	>3.15
3	–	+	–	–	85	5	0.9	A	$3.5 \cdot 10^4$	4.55	25	1.40	3.15
4	+	+	–	–	95	5	0.9	A	$2.0 \cdot 10^4$	4.29	<10	<1.00	>3.29
5	–	–	+	–	85	0	1.7	A	$1.5 \cdot 10^4$	4.18	35	1.54	2.64
6	+	–	+	–	95	0	1.7	A	$1.7 \cdot 10^4$	4.23	<10	<1.00	>3.23
7	–	+	+	–	85	5	1.7	A	$0.9 \cdot 10^4$	3.96	<10	<1.00	>2.96
8	+	+	+	–	95	5	1.7	A	$2.2 \cdot 10^4$	4.35	<10	<1.00	>3.35
9	–	–	–	+	85	0	0.9	A+B	$1.6 \cdot 10^4$	4.19	15	1.18	3.02
10	+	–	–	+	95	0	0.9	A+B	$2.4 \cdot 10^4$	4.38	<10	<1.00	>3.38
11	–	+	–	+	85	5	0.9	A+B	$1.9 \cdot 10^4$	4.27	<10	<1.00	>3.27
12	+	+	–	+	95	5	0.9	A+B	$2.8 \cdot 10^4$	4.44	<10	<1.00	>3.44
13	–	–	+	+	85	0	1.7	A+B	$1.8 \cdot 10^5$	5.26	307.5	2.49	2.78
14	+	–	+	+	95	0	1.7	A+B	$1.2 \cdot 10^4$	4.08	5	0.70	3.38
15	–	+	+	+	85	5	1.7	A+B	$7.0 \cdot 10^4$	4.84	40	1.60	3.24
16	+	+	+	+	95	5	1.7	A+B	$1.6 \cdot 10^4$	4.21	<10	<1.00	>3.21

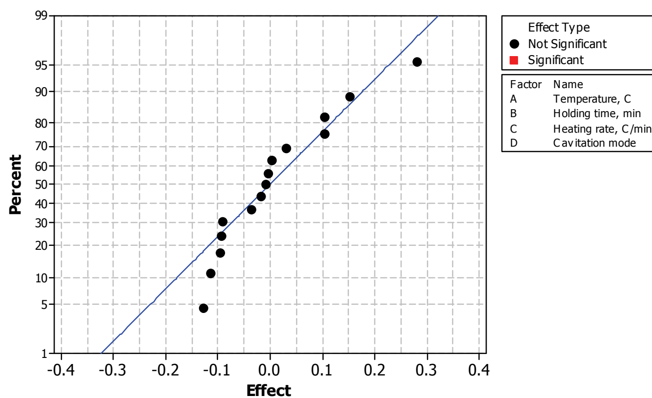


Figure 4.6 Normal plot of the effects(response is log reduction, α = 0.05)

The effect of the processing factors on log reduction was estimated by the analysis of Daniel's plot (Figure 4.6), which showed that there was no significant effect on log reduction at significance level α=0.05. No effect of the holding time, the temperature, the heating rate, the cavitation mode, or any interaction effects showed up as significant. However, some effects

deviated from the straight line and might be marginally significant. When the significance at α=0.10 was checked, factor A (temperature of pasteurization) showed up as significantly affecting log reduction (Fig. 4.7).

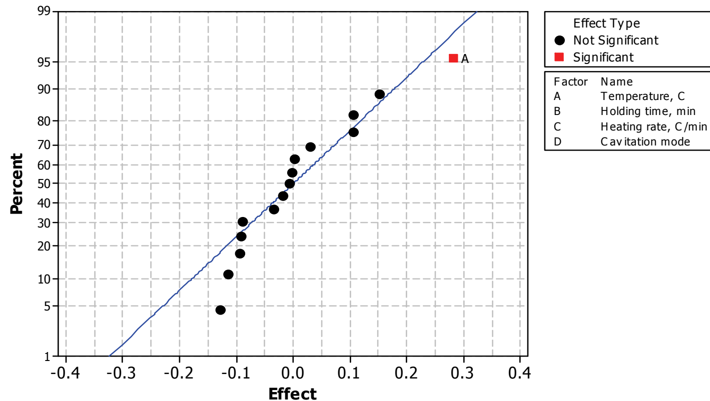


Fig.4.7 Normal plot of the effects(response is log reduction, α = 0.10)

As the next step in analysis, the experimental design was collapsed and the data was analyzed as a three factor factorial with two replications using ANOVA.As a result of the analysis, it was found that the error terms are not distributed normally. The power transformation (reciprocal) was applied to induce the normality of the data and then the assumptions of the constant variance and independence were found to be valid for the transformed data. The results of the ANOVA test for the transformed log reduction response are shown in Table 4.7.

Table 4.7 Results of ANOVA test for log reduction

Treatment combination	p-value
Temperature of pasteurization, °C (A)	0.001
Holding time, min (B)	0.025
Heating rate, °C/min (C)	0.046
Temperature * Holding time (A*B)	0.062
Temperature * Heating rate (A*C)	0.074
Holding time * Heating rate (B*C)	0.449
Temperature * Holding time * Heating rate (A*B*C)	0.121

The adjusted R^2 accounted for 73.55% of the total variability was explained by the factors chosen for the experiment (temperature of pasteurization, holding time and heating rate).

The plots for significant interaction effects are presented in figure 4.8 for non-transformed data. The letter grouping is done using the SAS statistical software (using the lsmeans statement of PROC GLM, α=0.01).

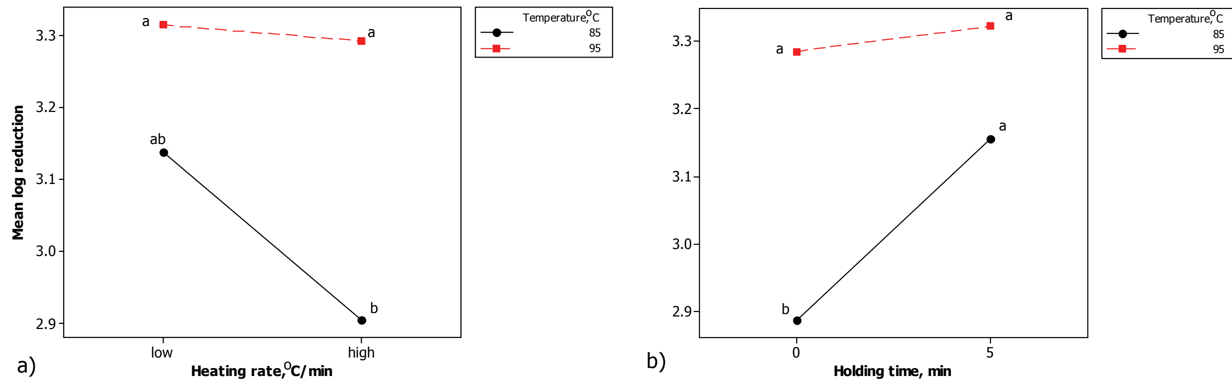


Figure 4.8 a) Interaction plot for log reduction (temperature*heating rate); b) Interaction plot for log reduction (temperature*holding time)

Taking into account the results of the ANOVA test, it was concluded that the temperature of pasteurization, the holding time and the heating rate significantly affected the log reduction of the product. A high level of factor A (temperature of pasteurization is 95 °C),a high level of factor B (longer holding time, i.e. 5 min) and a low level of factor C (heating rate is 0.9 °C/min) provided a higher log reduction during the process of pasteurization.

The interaction plot for the temperature and the heating rate interaction effect shows that at a high level of temperature (95°C) heating rate did not affect log reduction. But at a lower level of temperature (85°C), log reduction at a lower heating rate was significantly higher than at a higher heating rate. This may be because cumulative thermal treatment was higher if the product heated slower, which resulted in higher log reduction. The interaction plot for the temperature and the holding time provided similar results. There was no significant effect of the holding time on log reduction at 95°C. A significantly higher log reduction at 85°C temperature level was observed at a higher level of holding time (5 minutes) as compared with a lower level (0 minutes). A longer holding time resulted in a higher cumulative heat treatment and hence, in a higher log reduction.

In both cases, the effect of the heating rate and the holding time at a high temperature level (95°C) was not significant. The reason for this phenomena is that the microbial load of the raw material has a low variability from batch to batch (with some exception), and the heat treatment combinations at a high temperature level provides commercial sterility (when no

colonies were detected, the results were reported as less than 10 cfu/g), so log reduction was reported as “more than” specific number. In order to increase the sensitivity of the system to detect the impact of factors on log reduction, early fermentation of blueberries may be useful to increase the initial microbial load of the starting material. The cavitation mode as well as all three- way interaction effects did not affect log reduction significantly.

4.3.2 Process Optimization

As it was determined in the previous experiment, the cavitation mode did not affect the anthocyanin concentration, tannin content or log reduction significantly, so it was not included in the process of optimization. Also, since the heating rate was hard to control (physically) and since the heating rate was lowered by running cold water through the cooling jacket (with reduced energy efficiency), it was decided to keep the heating rate at the initial level (1.9°C/min) without cooling. The combined effects of the temperature and the heating rate on the response variables were investigated and the results of CCD design are presented in Table 4.8.

Table 4.8 Process variables and response variables for central composite design

#	Levels of operating parameters				Response		
	Coded		Uncoded				
	Time	Temperature	Time, min	Temperature, °C	Anthocyanins, mgC3G/g DW	Tannins, %	APC, cfu/g
1	–1	–1	2.2	85.0	12.07	14.13	25
2	1	–1	12.8	85.0	11.90	13.64	<10
3	–1	1	2.2	95.0	9.81	15.07	<10
4	1	1	12.8	95.0	9.26	16.60	<10
5	–1.41	0	0.0	90.0	12.07	12.15	12.5
6	1.41	0	15.0	90.0	9.19	15.23	<10
7	0	–1.41	7.5	83.0	10.98	13.16	25
8	0	1.41	7.5	97.0	9.13	17.12	<10
9	0	0	7.5	90.0	9.86	15.17	<10
10	0	0	7.5	90.0	10.02	14.79	<10
11	0	0	7.5	90.0	9.72	14.69	<10
12	0	0	7.5	90.0	11.17	15.50	<10

4.3.2.1 Anthocyanins

The statistical software (SAS and Minitab) was used to fit a response surface and develop the contour plots for anthocyanin concentration. Testing model adequacy: the analysis of the variance and regression coefficients are shown in Table 4.9. The assumptions of normality, independence and the constant variance were checked and found to be valid.

Table 4.9 Regression coefficients and ANOVA results for anthocyanin concentration

Parameter	Factor		Coefficient estimate		p-value	Significance
			Coded	Uncoded		
β_0		intercept	10.192	37.722	<0.0001	significant
β_1	x_1	holding time	- 0.599	0.037	0.0750	significant (marginally)
β_2	x_2	temperature	- 0.940	- 0.418	0.0150	significant
β_{11}	x_1^2	holding time* holding time	0.323	0.012	0.3395	not significant
β_{22}	x_2^2	temperature* temperature	- 0.095	0.001	0.8175	not significant
β_{12}	x_1 x_2	holding time* temperature	0.036	- 0.004	0.9127	not significant
Model					p-value	
Linear					0.0203	significant
Quadratic					0.6072	not significant
Cross product					0.8175	not significant
Total model					0.0828	marginally significant
Lack of fit (quadratic model)					0.3169	no lack of fit

The analysis of table 4.9 showed that there is no significant effect of the quadratic components and cross products, which corresponds to the fact that the linear model is appropriate. In coded variables the fitted model for anthocyanin concentration is:

$$\hat{y} = 10.19 - 0.60x_1 - 0.94x_2 \quad (15a)$$

In uncoded variables:

$$\hat{y} = 37.7 + 0.04\xi_1 - 0.42\xi_2 \quad (15b)$$

The equations above were used to generate the contour plot and the response surface for anthocyanin concentration (Fig. 4.9 and 4.10). The adjusted R^2 for the fitted model was 52.47%, which means that 52.5% of the total variability of the system was explained by the chosen factors. The canonical analysis indicated that the stationary point was a minimum (Eigen values are 0.662 and 0.056), and the results of the canonical analysis for the anthocyanin concentration are presented in Table 4.10.

Table 4.10 Results of the canonical analysis for optimization of anthocyanin concentration

		Holding time	Temperature
Critical values	Coded (x)	3.56	17.94
	Uncoded (ξ)	26.4 min	180°C
Predicted response		0.70 mg C3G/g DW	

The canonical analysis showed the minimum anthocyanin concentration at a temperature set at 180°C and the holding time of more than 26 minutes. These temperature regimes are not useful for food product pasteurization. However, this result as well as the analysis of the contour plot (Fig. 4.9a) and the surface plot (Fig. 4.9b) allows one to conclude that higher temperatures of pasteurization and longer holding times leads to greater loss of anthocyanins. Anthocyanins were more sensitive to high temperature than to holding time in these specific ranges (Fig. 4.9b). As a preliminary conclusion, to maximize the anthocyanin concentration in this time and

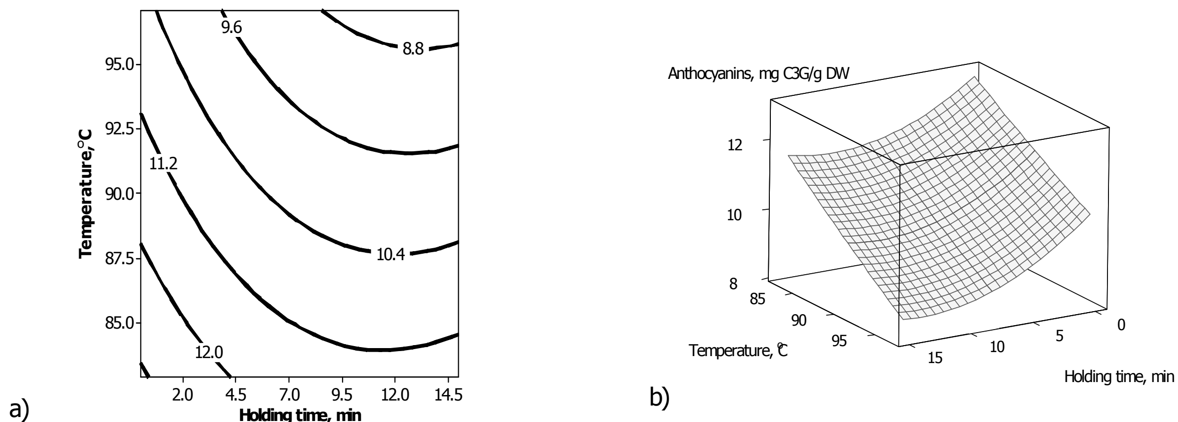


Figure 4.9a) Contour plot of anthocyanin concentration, mg C3G/g DW; b) Surface plot of anthocyanins, mg C3G/g DW

temperature range it is better to use mild regimes of pasteurization (temperature of pasteurization 83 to 87°C, holding time 0 to 5 min).

4.3.2.2 Tannins

The statistical software (SAS and Minitab) was used to fit a response surface and to construct the contour plots for tannin content. The assumptions of normality, independence and the constant variance were checked and found to be valid.

Table 4.11 Regression coefficients and ANOVA results for tannins

Parameter	Factor		Coefficient estimate		p-value	Significance
			coded	uncoded		
β_0		intercept	15.038	57.094	<0.0001	significant
β_1	x_1	holding time	0.675	- 1.287	0.0021	significant
β_2	x_2	temperature	1.188	- 1.075	0.0263	significant
β_{11}	x_1^2	holding time* holding time	- 0.563	- 0.020	0.5512	not significant
β_{22}	x_2^2	temperature* temperature	0.163	0.007	0.1719	not significant
β_{12}	$x_1\ x_2$	holding time* temperature	0.505	0.019	0.0716	marginally significant
Model					p-value	
Linear					0.0031	significant
Quadratic					0.1262	not significant
Cross product					0.1719	not significant
Total model					0.0101	significant
Lack of fit (quadratic model)					0.1056	no lack of fit

The analysis of Table 4.11 showed that there is no significant effect of the quadratic components, which confirms the proper choice of the linear model. In coded variables the fitted model for anthocyanin concentration is:

$$\hat{y} = 15.04 + 0.68x_1 + 1.19x_2 + 0.51x_1x_2 \qquad (16a)$$

In uncoded variables:

$$\hat{y} = 57.09 - 1.29\xi_1 - 1.08\xi_2 + 0.02\xi_1\xi_2 \qquad (16b)$$

The equations above were used to generate the contour plot and the response surface for tannin content (Fig. 4.10b and 4.10c). The adjusted R^2 for the fitted model is 77.80%, which means that 77.8% of the total variability of the system was explained by the chosen factors.

The canonical analysis indicated that the predicted response surface is shaped like a saddle (Eigen values are 0.484 and –1.284). Because the canonical analysis resulted in a saddle point, the estimated surface did not have a unique optimum. A ridge analysis of tannin response was conducted and the results are presented in Figure 4.10a. The ridge plot (Fig.4.10a) indicated that minimum tannin content in this specific range occurred at relatively low temperatures and short holding times. The contour plot of the predicted response surface (Fig.4.10b) and the surface plot (Fig.4.10c) confirm this conclusion.

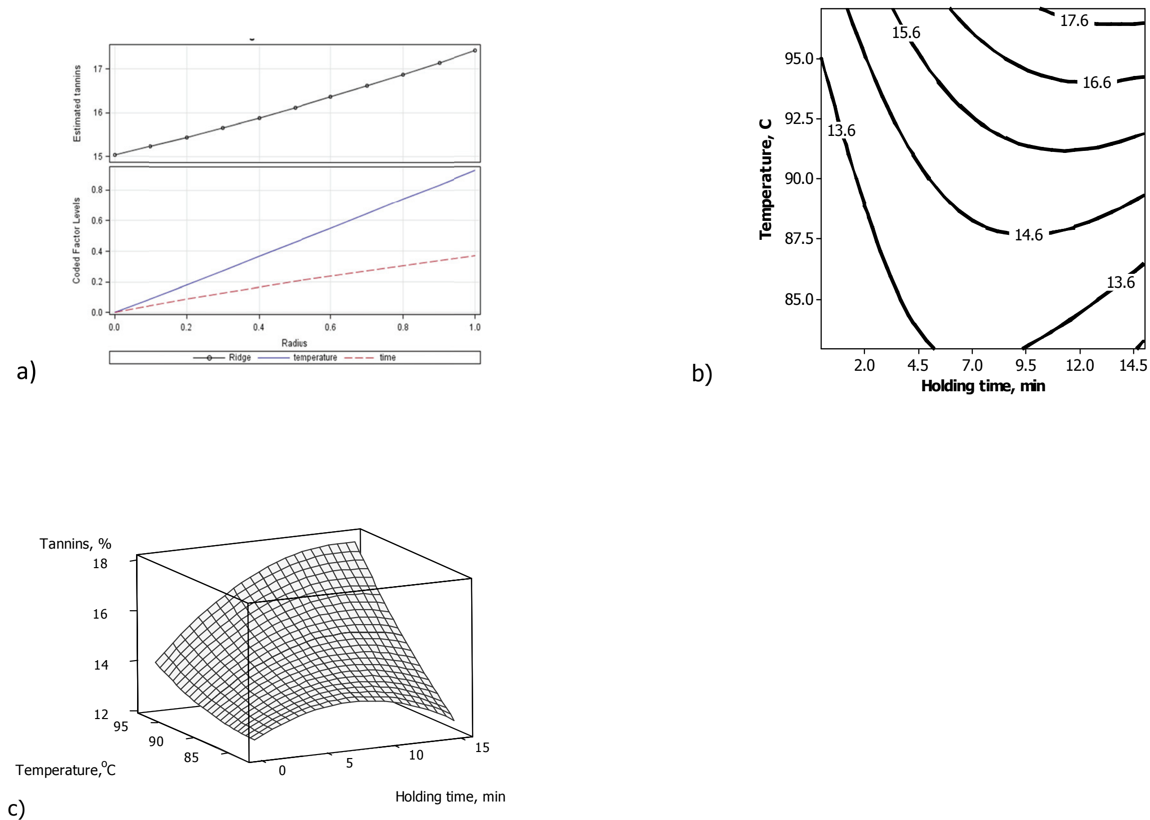


Figure 4.10 a) Ridge of maximum tannins; b) Contour plot of tannins, %; c) Surface plot of tannins, %

The analysis of the contour plot and the surface plot also indicates that at lower temperature (83 to 85 °C) effect of holding time was not so noticeable (small change in tannin content). At higher temperatures (95 to 97°C), the content of polymeric color increased markedly with longer holding times. It can be explained by the fact that increasing cumulative thermal treatment at higher temperatures results in acceleration of the reactions of anthocyanin condensation. To minimize tannin content, the same combination of temperatures (83 to 87°C) and holding times (0 to 5 min) can be suggested.

4.3.2.3 Microbial Load

The direct statistical analysis of the microbial load data (Table 4.8, Aerobic plate count, cfu/g) did not provide meaningful results because the microbial load of the raw material within the batch was different and because a significant amount of treatment combinations provided industrial sterility of the product (no bacterial colony growth was observed) which was reported as “less than 10 cfu/g”. Table 4.12 shows the results of the microbial tests taking into consideration the microbial load of the initial raw material for each batch. Log reduction for each treatment combination was calculated as a difference between logarithms of cfu per gram for the raw material and the pasteurized product. The statistical software (SAS and Minitab) was used to fit a response surface and to construct the contour plots for log reduction. Testing of the model adequacy, i.e. analysis of the variance and regression coefficients are shown in Table 4.13. The assumptions of normality, independence and the constant variance were checked and found to be valid.

Table 4.12 Microbial load of raw material and final product for optimization experiment

#	Levels of operating parameters				Microbial load				
	Coded		Uncoded		Raw material		Pasteurized product		Log reduction
	Time	Temperature	Time, min	Temperature, °C	cfu/g	Log number	cfu/g	Log number	
1	–1	–1	2.2	85.0	$2.2 \cdot 10^4$	4.34	25	1.40	2.94
2	1	–1	12.8	85.0	$2.1 \cdot 10^4$	4.33	<10	<1.00	>3.33
3	–1	1	2.2	95.0	$7.6 \cdot 10^4$	4.88	<10	<1.00	>3.88
4	1	1	12.8	95.0	$1.1 \cdot 10^5$	5.04	<10	<1.00	>4.04
5	–1.41	0	0.0	90.0	$6.8 \cdot 10^4$	4.82	12.5	1.09	3.73
6	1.41	0	15.0	90.0	$7.4 \cdot 10^4$	4.87	<10	<1.00	>3.87
7	0	–1.41	7.5	83.0	$3.5 \cdot 10^4$	4.54	25	1.40	3.14
8	0	1.41	7.5	97.0	$1.0 \cdot 10^5$	5.00	<10	<1.00	>4.00
9	0	0	7.5	90.0	$7.6 \cdot 10^4$	4.88	<10	<1.00	>3.88
10	0	0	7.5	90.0	$9.1 \cdot 10^4$	4.96	<10	<1.00	>3.96
11	0	0	7.5	90.0	$7.6 \cdot 10^4$	4.88	<10	<1.00	>3.88
12	0	0	7.5	90.0	$5.8 \cdot 10^4$	4.76	<10	<1.00	>3.76

Table 4.13 Regression coefficients and ANOVA results for log reduction

Parameter	Factor		Coefficient estimate		p-value	Significance
			Coded	uncoded		
β_0		intercept	3.870	- 64.052	<0.0001	significant
β_1	x_1	holding time	0.358	0.250	0.0002	significant
β_2	x_2	temperature	0.093	1.420	0.0830	marginally significant
β_{11}	x_1^2	holding time* holding time	- 0.184	- 0.002	0.0105	significant
β_{22}	x_2^2	temperature* temperature	- 0.058	- 0.007	0.4011	not significant
β_{12}	$x_1 x_2$	holding time* temperature	- 0.069	- 0.002	0.2172	not significant
Model					p-value	
Linear					0.0005	significant
Quadratic					0.0276	significant
Cross product					0.4011	not significant
Total model					0.0019	significant
Lack of fit (quadratic model)					0.1525	no lack of fit

The analysis of Table 4.13 indicates that there is a significant effect of one of the quadratic components. The linear model is specified as highly significant, and the quadratic model has significance too. In coded variables the fitted model for anthocyanin concentration looks like:

$$\hat{y} = 3.87 + 0.36x_1 + 0.09x_2 - 0.18x_1^2 \quad (17a)$$

In uncoded variables:

$$\hat{y} = -64.05 + 0.25\xi_1 + 1.42\xi_2 - 0.002\xi_1^2 \quad (17b)$$

The equations above were used to generate the contour plot and the response surface for log reduction (Fig. 4.15 and 4.16).The adjusted $R^2$for the fitted model is 87.55%, which means that 87.6% of the total variability of the system was explained by the chosen factors. The canonical analysis indicated that the stationary point was a maximum (Eigen values are –0.125 and – 0.382). The results of the canonical analysis for log reduction are presented in Table 4.14.

Table 4.14 Results of canonical analysis for optimization of log reduction

		Holding time	Temperature
Critical values	Coded (x)	0.926	0.290
	Uncoded (ξ)	12.4 min	91.5°C
Predicted response		4.05	

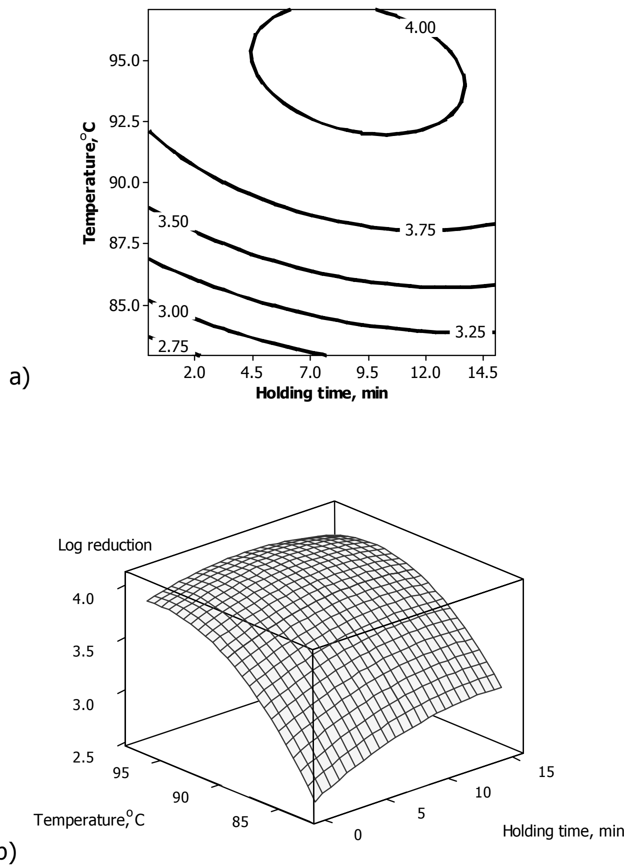


Figure 4.11 a) Contour plot of log reduction;
b) Surface plot of log reduction

The canonical analysis shows maximum log reduction at 91.5°C and at a holding time of 12 minutes. The contour plot (Fig. 4.11a) and the surface plot (Fig. 4.11b) demonstrate that there was an optimum for log reduction in the chosen range of time and temperature. It was not expected to find an optimum for log reduction since increasing the temperature of pasteurization and the prolonged holding time results in the higher ability to destroy the microbial cells. These results can be explained by the fact that many treatment combinations did not show any microbial growth in the product (Table 4.8). In this case, log numbers were reported as “less than 1”. For log reduction, for most of the samples treated at the temperatures higher than 90°C and holding time longer than 5 minutes, the results were reported as “more than log number”, however, for the analysis these data were used as fixed numbers. However, taking into account the above considerations, it’s possible to conclude that increasing the temperature of pasteurization and the holding time within specified ranges results in increasing the log reduction

of the microbial load. Higher levels of times and temperatures are preferable to provide safety of the pasteurized blueberry purée.

4.3.2.4 Multiple Responses Optimization

To optimize multiple responses an overlaid contour plot of anthocyanin concentration, tannin content and log reduction was generated in the Minitab software (Fig.4.17). Since desirable results in terms of product quality (minimum tannin content and maximum anthocyanin concentration) required lower levels of holding time and temperature, and in terms of product safety (maximum log reduction) it is required to apply higher levels of time and temperature, overlaying of the three response surfaces provided visual output useful for selecting processing regimes. In maximizing log reduction and anthocyanin concentration, the target values were set higher than was obtained from the experiment. A low level for anthocyanin concentration was set at 10 mg C3G/g of dry weight, for log reduction: 3.8 log number. In minimizing tannin content, the target value was set lower than was obtained during the experiment, the high level was specified as 14%. The three response surface overlay shows the area (white color at the contour plot) which provides optimum results and allows one to suggest possible treatment combinations, for example: 2 minutes at 92°C, 1 min at 93°C, or heating till 95°C with no holding time. The final decision on the regimes of pasteurization can be made based on technological convenience, energy efficiency and other considerations in the manufacturing process.

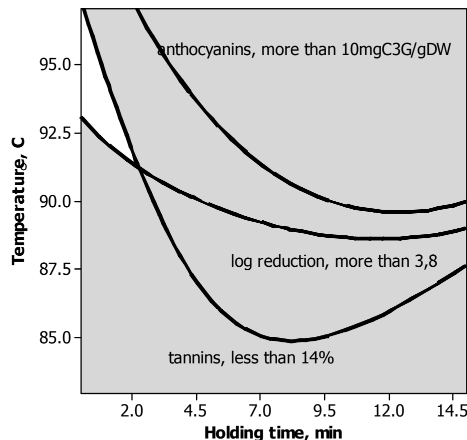


Figure 4.12 Overlaid contour plot of anthocyanins, tannins, and log reduction

4.4 Conclusions and Recommendations

Lower temperature, shorter holding times, and higher heating rates resulted in a higher anthocyanin concentration and lower tannin content in the HTDT blueberry purée. Accordingly, intensive thermal treatment (high temperature, long holding time, and low heating rate) resulted in lower anthocyanin concentration in the pasteurized product. These results were expected, since anthocyanins are sensitive to thermal treatment. However, these combinations of time, temperature and heating rate more effectively reduced microbial load (desirable for food safety).No significant difference in the effect of holding time and heating rate was detected at a high level of temperature, in contrast to significant effect of these factors at the low level of temperature. Cavitation mode was a non-significant factor, so it was excluded from subsequent models.

The response surface methodology was used to determine the combined effect of time and temperature on anthocyanin concentration, polymeric color (tannins) content, and the microbial load (log reduction). Linear models were effective to describe the response surfaces for anthocyanin concentration and tannin content, while a quadratic model was needed to describe log reduction. An optimal combination of time and temperature was found to achieve the best HTDT blueberry purée quality within specified food safety constraints (Fig. 4.17). Pasteurization temperature in the range of 92 to 95°C in combination with a holding time of 0.5 to 2 minutes provides a safe product (more than 3.8 log reduction) with a high concentration of anthocyanins and a low tannin content. The accuracy of optimization was limited with the sensitivity of microbial (APC) measurements. To increase the accuracy of experiments, provision of the microbial load (by preliminary fermentation our culture inoculation) before thermal processing could be suggested.

5. CONCLUSION

Wild blueberries are a local product of Atlantic Canada and have a high economic importance for the region. Wild blueberries are characterized by the very high content of polyphenolic substances among cultivated crops and have been reported as a product with potential benefits in human health. However, the amount of wild blueberry-based food products presented on the local market is very limited. Consumers are interested in new healthy and natural foods on the market. At the same time, wild blueberry production in Atlantic Canada increases every year. Blueberry producers are also interested in the development of new blueberry-based products, since it will allow them to sell more blueberries locally, nationally and internationally.

Almost all types of food processing used for wild blueberries affect negatively the polyphenolic substances and, by extension, the potential health promoting properties of blueberry-based products. Use of new Hydrothermodynamic technology (HTDT) was indicated to be promising for blueberry food processing in order to minimize polyphenolic degradation.

The research hypothesis was that hydrothermodynamic (HTD) processing would result in a high-quality pasteurized food product. To test this hypothesis, three objectives were formulated: i) comparing HTD technology and conventional blueberry processing technology (using the open contact heater), and study the difference between the HTDT blueberry purée and commercial blueberry-based products; ii) study and model kinetics of quality degradation during the HTDT blueberry purée storage in order to develop shelf-life recommendations; iii) study the effect of the processing factors (temperature, time, heating rate, cavitation mode) on food quality and safety, and optimize time-temperature regimes for HTDT blueberry processing.

The preliminary experiments showed that the quality of the HTDT blueberry purée in terms of anthocyanin concentration and tannin content was higher than that of most commercially available blueberry products. It was demonstrated that HTD technology provided pasteurization of the blueberry purée with significantly lower losses of anthocyanins than that as a result of conventional contact heating, so HTD technology can be successfully used for pasteurized blueberry purée processing with minimal quality degradation.

For HTDT blueberry process optimization, a two stage experiment was used. At the first stage, screening of the processing factors using the unreplicated factorial design showed that the holding time, the temperature of pasteurization and the heating rate were significant factors, while the cavitation mode of HTDT processing was non-significant. At the second stage, the set of

optimization experiments was designed using the response surface methodology. The combined effect of time and temperature on the quality and safety was described as a combined response of anthocyanin concentration, polymeric color (tannin) content, and the microbial load (log reduction). Linear models were effective to describe response surfaces for anthocyanin concentration and tannin content, while the quadratic model was used to describe log reduction. The combination of time and temperature, optimal for the best quality processing within specified food safety constraints, was found. Temperatures in the range 92 to 95°C in combination with holding times of 0.5 to 2 minutes provided acceptable product safety along with high quality in terms of high concentration of anthocyanins and low tannin content.

The investigation of the product shelf life showed that cold storage at 4°C is preferable in terms of lower anthocyanin degradation as compared with storage at room temperature (20 to 25°C). The rate of anthocyanin degradation and tannin formation at 4°C was twice less than that at room temperature. Storage up to 6 months at 4°C and up to 2 months at room temperature resulted in a moderate loss of anthocyanins and can be recommended as a product shelf-life. Fitted exponential models, described in this work, could be used to predict the anthocyanin concentration and tannin content for different storage periods.

Adopting the HTD technology for wild blueberry processing can play a significant role for Atlantic Canada, Nova Scotia in particular. On one hand, a high quality blueberry-based product will be available to consumers, which allows them to have more diverse and convenient ways to consume blueberries. On the other hand, producers will have a chance to launch a new product on the market, therefore, sell more blueberries and meet demands for more health beneficial products. To achieve this, the further investigation might be needed. Developing the continuous system (versus batch processing which was used in this work) and industrial scaling will be necessary for the product manufacture. Further product development (various textures, fruit and berries blends, etc.) will be beneficial for product marketing. Also, testing HTDT blueberry purée health functionality will be helpful in order to investigate and confirm health beneficial properties of the product.

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