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IMPROVING STABILITY OF COLOR, TOTAL PHENOLICS, FLAVONOIDS AND
ASCORBIC ACID IN CRANBERRY JUICE COCKTAIL VIA ALTERNATIVE
PROCESSING AND STORAGE TECHNIQUES

BY

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ABSTRACT OF THE DISSERTATION

IMPROVING STABILITY OF COLOR, TOTAL PHENOLICS, FLAVONOIDS
AND ASCORBIC ACID IN CRANBERRY JUICE COCKTAIL VIA ALTERNATIVE
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Strong evidence suggests potent bioactivity in the phenolic and especially flavonoid fractions of cranberry, particularly anthocyanins (ACYs), flavonols (FLVs) and proanthocyanidins (PACs). Understanding how processing, packaging and storage effects these phytochemicals is crucial to maintaining cranberry nutritional value yet remains a considerable research gap.

This study investigated the impact of oxygen, light, ascorbic acid fortification and heat treatments on the stability of cranberry flavonoids (and color) in cranberry juice cocktail (CJC). Oxygen was expected to play a central directive role, so two innovative conditions were applied to limit oxygen levels during pasteurization and storage: pre-processing degassing – a novel processing step for hot-filled juices – and oxygen-impermeable plastic packaging.

Chromatographic methods were developed to monitor cranberry ACYs and FLVs. CJC was then subjected to combinations of conditions affecting oxidation, including light exposure, ascorbic acid fortification and oxygen exposure over thermal processing and storage to assess their effects and interactions on flavonoids and color stability. Subsequently, such treatments were applied to solutions of purified cranberry flavonoids to identify degradation products. Finally, CJC was bottled in oxygen barrier and standard packaging to see how well oxygen ingress could be limited and the treatments' effects in practical conditions.

Flavonoids, color and added ascorbic acid were indeed destabilized when exposed to oxygen. However fortification with ascorbic acid often destabilized flavonoids and color more so. The effects of nearly all treatments were attenuated by oxygen exposure. In practical packaging, oxygen barrier bottles only marginally reduced dissolved oxygen content. Light and ascorbic acid suppressed dissolved oxygen more thoroughly for bottled CJC, but apparently accomplished this by converting oxygen to more reactive species as these were most often destabilizing factors. Removal of oxygen before pasteurization proved a moderately effective treatment in limiting losses of color, flavonoids and ascorbic acid.

PACs were most stable, followed by FLVs and more unstable ACYs. Deglycosylation appears to be a major route of degradation for ACYs and FLV glycosides. Reactions between ascorbic acid and ACYs seem to be through products of ascorbic acid degradation and direct reaction. PACs or similar compounds seem to be created during the aging of CJC under some conditions.

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Phytochemicals of Cranberry and Cranberry Products: Characterization, Potential Health Effects and Processing Stability

Edward Pappas and Karen M Schaich, © 2009 Taylor and Francis

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1. INTRODUCTION

Vaccinium macrocarpon (American Cranberry) is a woody, perennial plant which produces a bright red berry in its low-growing vines. This berry is prized for its characteristic ‘cranberry’ color and tart, astringent flavor. Human consumption of this berry, indigenous to New England, can be traced back to some of the earliest of European settlers of North America and their accounts of Native Americans’ reverence for this unique fruit. Then Native Americans often sweetened the fruit with maple sugar and valued it for its medicinal qualities.¹ The earliest known recipes for cranberry juices and jellies date back to the late 17th century and still today these are some of the forms in which cranberry is most often consumed. Raw cranberries and single-strength (100%) cranberry juice, on the other hand, are considered unpalatable by many due to high acidity and astringency. Sweetening and/or dilution of cranberry products is necessary to create foods that consumers accept and enjoy. Today, cranberry juice cocktail (generally ~27% juice, sweetened and often fortified with Vitamin C), cranberry juice blends (cranberry juice blended with apple, grape and/or raspberry juice) and cranberry sauce (sweetened and gelled mixtures of cranberries, juice and water) dominate the cranberry marketplace. Sweetened dried cranberries are becoming increasingly popular as well.

Coinciding with a wider trend of promoting ‘health and wellness’ through diet, cranberry foods have recently attracted much attention from the scientific community and general public for their health benefits. Indeed, they have been deemed ‘superfruits’ for their high concentration of phenolic antioxidants.

Moreover, research from the past 25 years has generally supported the long standing folk-lore notion that cranberries are an effective health food, most famously for

preventing urinary tract infections in women. Evidence continually accumulates implying cranberries may provide protection from cardiovascular disease,² inflammation,³ cancer,^{4,5} numerous bacterial infections,⁶⁻⁸ and other degenerative diseases. Emerging research contends that cranberries may offer neuroprotection⁹ and anti-viral properties.¹⁰ Cranberry phenolics (including anthocyanins, flavonols, proanthocyanidins, and numerous phenolic acids) seem to underlie these health benefits along with essential nutrients, most notably Vitamin C.¹¹ Furthermore, many of the same components (e.g. pigmented anthocyanins and astringent proanthocyanidins) are responsible for the primary sensory attributes critical to the quality of cranberry products.^{12,13}

Coupling recognition that cranberries are overwhelmingly consumed in processed forms with evidence that cranberry phytochemicals, flavonoids in particular, are responsible for the majority of cranberry product quality and health benefits^{11,14} raises numerous questions concerning how processing, packaging and storage of cranberry products affect these pharmacologically-active natural components. Instability of flavonoids undermines the quality and health effects of cranberry products when they reach consumers. The loss of red color is particularly troubling to product quality and limits the shelf life of cranberry juice cocktail to 4-9 months. Given these problems, what improvements to traditional thermal pasteurization will be least detrimental to cranberry phytochemicals? What storage and packaging conditions stabilize these components best? Which of these phytochemicals degrade most readily? What improved practices can cranberry processors utilize to ensure that their products provide the best quality and most health promotion possible? The research presented herein attempts to answer these questions as they relate to cranberry juice cocktail.

2. BACKGROUND

2.1 Ascorbic Acid and Its Prevalence in Cranberry Foods

For humans, L-ascorbic acid (AscA, also known as L-ascorbate or vitamin C) is a vital nutrient and an endogenous antioxidant. AscA acts as a potent electron donor and readily undergoes reversible oxidation to form dehydroascorbic acid. Regeneration of AscA occurs when dehydroascorbic acid is reduced, generally by glutathione in biological systems¹⁵ (Figure 1). This cycle affords AscA tremendous utility *in vivo* where it is essential to many critical processes, including collagen synthesis, bone/teeth healing/regeneration and wound healing among others.¹⁶ Furthermore, as an endogenous antioxidant, AscA reduces harmful reactive oxygen species to benign forms, impeding chronic illnesses such as cancer, coronary heart disease (CHD), cataracts, diabetes and neurodegenerative diseases among others.¹⁵ Deficiency in AscA is widely known to cause scurvy in humans.

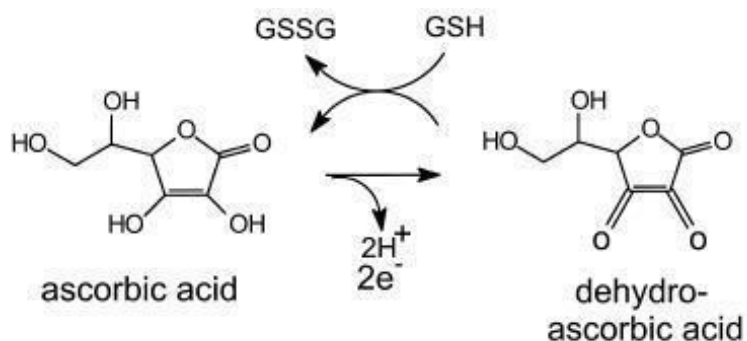


Figure 1: Oxidation and regeneration of ascorbic acid¹⁵

In whole cranberries, AscA is present at low concentrations (~11.5 mg/100g),¹⁷ enough to prevent scurvy among early American settlers.¹ Levels of AscA in cranberry juice cocktail and sauce are lower (2-4 mg and 1 mg /100 g) respectively¹⁷⁻¹⁹ or absent.²⁰

This loss of AscA is likely due to oxidation during processing, but has not been thoroughly explored. Even if AscA were well conserved throughout its processing, cranberry products would not be a remarkable dietary source of this nutrient since the reference daily intake in the United States is 60 mg for vitamin C. Rather, cranberry products, particularly cranberry juice cocktail, are often fortified with AscA to meet consumer expectations. That fortification negatively impacts the stability of cranberry color and anthocyanins¹⁸ has most often been ignored.

2.2 Cranberry Phenolics and Their Functional Properties

2.2.1 Non-Flavonoid Phenolics and Benzoates

Structurally, phenolic compounds are defined as having at least one aromatic ring with at least one hydroxyl group. As such, they are an expansive, diverse class of compounds (Figure 2). Approximately 100 individual phenolic species have been identified in cranberries to date.¹¹ Levels of total phenolics have been estimated at 22,700 $\mu\text{mol/kg}$ (catechin equivalents) in frozen cranberries²¹ and 350 $\mu\text{mol/L}$ (gallic acid equivalents) in commercial cranberry juice cocktail (27% juice)²² using colorimetric assays. Indeed, cranberries may be the most concentrated source of phenolics of all commodity fruits. Two studies found cranberries to be the richest source of total phenols both by fresh weight and per serving.^{21,23} They contain nearly three times the level of total phenolics, on a dry weight basis, than any other of 20 fruits tested.²¹

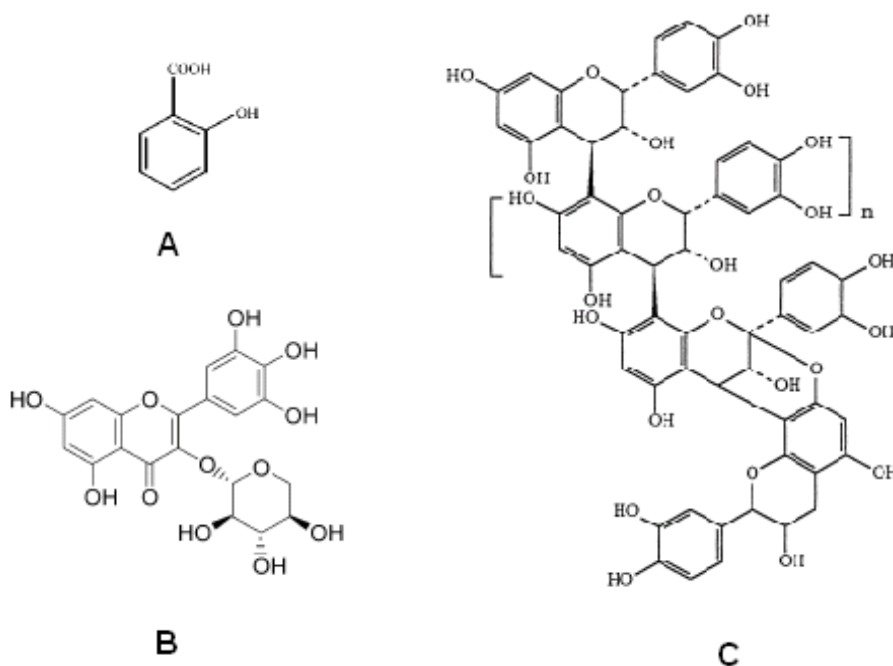


Figure 2: Cranberry phenolics exhibit a diverse array of structures. Presented here is a simple mono-phenolic compound o-hydroxybenzoic acid (also known as salicylic acid) (A), a more complex polyphenolic flavonoid quercetin-3-glucoside (B) and a larger flavonoid polymer A-type proanthocyanidin (C). Adapted from References ⁽²⁴⁻²⁶⁾.

Phenolic compounds, due to their electron-rich aromatic structures, may act as antioxidants through several mechanisms including as reducing agents, hydrogen atom donors, metal chelators and singlet oxygen/free radical quenchers.²⁷ Thus, it is no surprise that cranberries, which are so rich in phenolics, also rank highly when comparing antioxidant capacities of fruits. Measured by the total oxyradical scavenging (TOSC) assay²³ as well as the oxygen radical absorbance capacity (ORAC) assay,²⁸ cranberries have the highest *in vitro* antioxidant capacity by fresh weight among dozens of commodity fruits.

Along with antioxidant activity, phenolics play an important role in cranberry flavor. Aromatic compounds (both phenolics and benzoates) dominate the odor-active fraction of cranberry, an unusual occurrence for fruits.²⁹ Among these compounds, benzoic acid, benzaldehyde, eugenol, vanillic acid, *trans*-cinnamic acids and anisaldehyde are essential to characteristic cranberry aroma^{29,30} though the terpene, α -terpineol, seems to be the single largest contributor to cranberry odor. Benzoic acid which is unusually concentrated in cranberry at 4.7 g/kg,²⁶ contributes to its unique tart taste, as do the many phenolic acids and non-aromatic acids of cranberries.^{19,29}

Aside from antioxidant activity, monophenolic compounds have attracted little attention from researchers studying health effects from cranberries. *o*-Hydroxybenzoic acid is a notable exception. Also known as salicylic acid and chemically similar to acetylsalicylic acid (aspirin), the commonly used anti-inflammatory analgesic medication, this compound is present in cranberry juice cocktail at 7 mg/L after alkaline hydrolysis.³¹ Non-flavonoid polyphenolics present in cranberry, such as *trans*-resveratrol, ellagic acid and secoisolariciresinol have been extensively studied for the health benefits from the consumption of other fruits, but have been largely ignored for their health contributions from consuming cranberry. This is perhaps because of their low concentrations in cranberry; 0.2 mg/L (in cranberry juice), 120 μ g/g (dry weight) and 10.54 μ g/g (dry weight) respectively.³²⁻³⁴

The stability of individual nonflavonoid phenolics and benzoates as well as total phenolics in cranberry juice during processing and storage has not been explored in any targeted studies to date. However, the large discrepancy between levels of total phenolics between cranberries and cranberry juice (mentioned above) strongly suggests that

processing and storage of cranberry products degrades these important compounds. Preventing degradation of cranberry phenolics, critical to cranberries' flavor profile, antioxidant activity and other health promoting properties (see Section 2.3) seems, therefore, a worthwhile endeavor.

2.2.2. Flavonoids

Flavonoids (particularly anthocyanins) dominate research of cranberry chemistry, even more than cranberries' monophenolic compounds, Flavonoids are defined by their C₆-C₃-C₆ skeleton which includes heterocyclic oxygen in its central ring (Figure 3) and represent a class of secondary plant metabolites with a diverse array of properties. The three flavonoid sub-classes most prevalent in cranberry are anthocyanins (red-blue pigments with strong antioxidant potential), flavonols (also colored but more renowned for their health effects), and proanthocyanidins, larger flavonoid polymers known to interact with proteins and to cause astringent flavor and potent health effects. Other flavonoids in cranberries (flavan-3-ol monomers, flavonones and polymeric pigments) have been documented but their low concentrations and relative lack of importance to cranberries' health effects make these components less important.¹¹

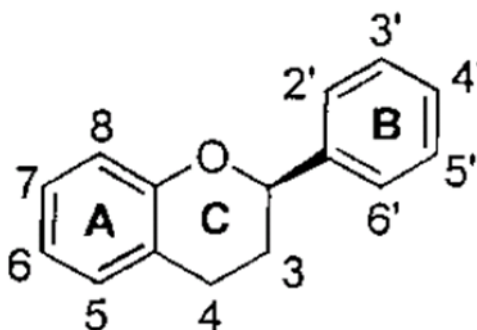


Figure 3: Chemical structure of flavonoid backbone (flavan) with typical numbering system and lettered rings³⁵

2.2.2.1. Anthocyanins (ACYs)

Anthocyanins were identified as the primary pigments responsible for the desirable red color of cranberries decades ago.¹² Several pH-dependent equilibrium structures exist for anthocyanins, but derivatives of the 2-phenylbenzopyrylium cation (the flavylium cation – Figure 4) are most prevalent at the low pH (~2.5) of cranberry products.³⁶ The extensive system of conjugated double bonds of the flavylium cation result in light absorption at a λ_{max} of ~520 nm, giving cranberries red color. Generally, the flavylium cation has hydroxyl and/or methoxyl substitutions at five to six locations (at the 3, 5, 7, 3', 4' and sometimes 5' position) and glycosidic substitution(s) at one to three locations, most often at the 3 position.³⁷ Anthocyanins are often classified by the number of glycosides substituted (e.g. monoglycosides, diglycosides or triglycosides). Though not known to exist in cranberries, acyl substitutions also are common to the sugar moieties of anthocyanins.³⁷ Anthocyanins aglycones are unstable and are rarely found in nature.

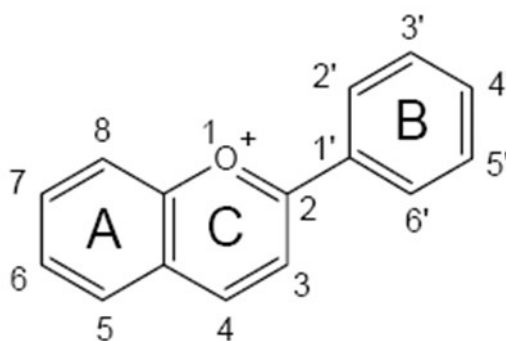


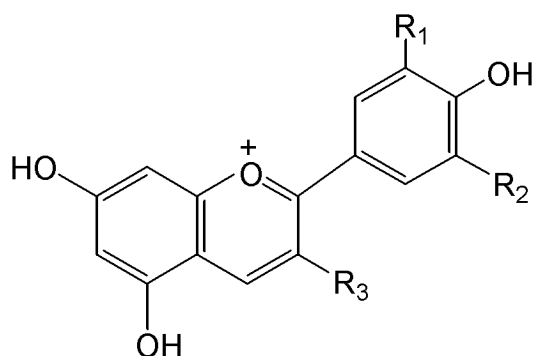
Figure 4: The flavylium cation

Recently, 13 cranberry anthocyanins were identified by LC-MS-MS, (Table 1) and all are glycosylated at the 3 position.^{38,39} However, arabinose, galactose and glucose conjugates of peonidin and cyanidin -- the six anthocyanin cranberry pigments that account for 99% of the total -- are readily visible by less sensitive techniques such as HPLC-UV.⁴⁰ The galactose-bound anthocyanins (peonidin-3-*O*-galactoside and cyanidin-3-*O*-galactoside) are most prevalent in cranberries, followed by the arabinose-bound anthocyanins (cyanidin-3-*O*-arabinoside and peonidin-3-*O*-arabinoside) with one analysis finding concentrations of 2.8, 2.1, 1.4 and 1.1 mg/L respectively in cranberry juice cocktail.¹⁹ Total anthocyanins vary widely in cranberry juice cocktail depending on countless factors, but generally are ~10-50 mg/L.^{19,41} In whole fresh berries, total cranberry anthocyanins range from 136 – 1710 mg/kg, varying widely with cranberry variety, growing location, growing conditions and maturity at time of harvest.⁴²

Color, arguably the most important quality factor for cranberry products,¹⁴ degrades rapidly in cranberry juice products and effectively limits shelf life to 4-9 months. The stabilization of anthocyanins and cranberry juice color is, therefore, of great importance and has been the subject of substantial research. More recently, anthocyanins

from cranberry and other sources have been widely studied for their strong antioxidant capacity and numerous potential health benefits.¹¹

Table 1: Anthocyanins found in cranberries.³⁹



Anthocyanin Name	R ₁	R ₂	R ₃
cyanidin-3- <i>O</i> -galactoside	OH	H	Galactose
cyanidin-3- <i>O</i> -arabinoside	OH	H	Arabinose
cyanidin-3- <i>O</i> -glucoside	OH	H	Glucose
peonidin-3- <i>O</i> -galactoside	OMe	H	Galactose
peonidin-3- <i>O</i> -arabinoside	OMe	H	Arabinose
peonidin-3- <i>O</i> -glucoside	OMe	H	Glucose
malvidin-3- <i>O</i> -arabinoside	OMe	OMe	Arabinose
malvidin-3- <i>O</i> -galactoside	OMe	OMe	Galactose
pelargonidin-3- <i>O</i> -arabinoside	H	H	Arabinose
pelargonidin-3- <i>O</i> -galactoside	H	H	Galactose

2.2.2.2 Flavonols (FLVs)

In cranberries, flavonols are secondary pigments responsible for yellow undertones. Chemical cousins of anthocyanins, flavonols are defined as derivatives of 3-hydroxy-2-phenylchromen-4-one. Notably, they contain a keto group on the 4 position of the central ring (Figure 5). Flavonols may also contain a glycosidic linkage at the 3 position of the central ring. However, unlike anthocyanins, flavonols also exist as stable aglycones, with a hydroxyl group at that position. The basic flavonol structure also contains a system of conjugated double bonds absorbing light at a λ_{max} of ~ 360 nm, giving flavonols a yellow color.

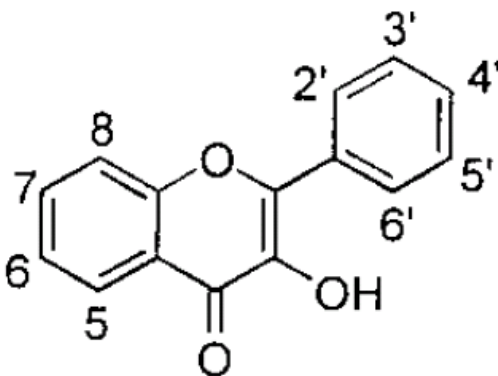


Figure 5: The 3-hydroxy-2-phenylchromen-4-one backbone of flavonols.³⁵

In cranberries, quercetin and its derivatives (particularly quercetin-3-galactoside) are most abundant while myricetin and its derivatives are also prevalent; some reports also include kaempferol and its derivatives as minor components.^{19,24,43-46} In untreated cranberry juice cocktail, quercetin-3-galactoside (hyperin), quercetin, myricetin, quercetin-3-rhamnoside (quercitrin) and quercetin-3-arabinoside (avicularin) were found to be most concentrated at 23.2, 13, 5.3, 5.2 and 1.8 mg/L, respectively, in one report.¹⁹

Targeted investigation of cranberry flavonols revealed an even more diverse profile. Using column chromatography, ^{13}C NMR, ^1H NMR and HPLC-MS, 22 distinct flavonol species were observed from freeze dried cranberry powder and fresh cranberries, and 13 of these were positively identified.^{24,46}

Total flavonols in untreated cranberry juice cocktail (27% juice) at 48 mg/L¹⁹ are comparable to levels in whole fresh fruit, at 200-400 mg/kg^{24,47} if dilution is taken into account. These high levels of flavonols stand out clearly among fruits. Cranberries were the most concentrated source of flavonols of 30 fruits in one study.⁴⁸ Commercial cranberry juice cocktail (25% juice) contained nearly twice the flavonol concentration (~40 mg/L) of any of 12 other juices tested.²² With an estimated 20-22 mg/day combined flavonol and flavonone dietary intake,⁴⁹ cranberries and cranberry juice cocktail are clearly excellent sources of these flavonoids.

It is unclear whether the yellow tones imparted by flavonols to cranberries are desirable or detrimental. More crucially, the health effects from flavonol consumption are considerable and the focus of much research. Flavonol consumption has been inversely correlated to coronary heart disease-related mortality and stroke incidence in epidemiological studies,⁵⁰⁻⁵² and anti-inflammatory properties of flavonols were observed after both consumption and topical application in animal models.^{3,53} *In vitro* anti-cancer activity from cranberry flavonols has also been reported.^{4,5,54,55} Because flavonols exhibit potent beneficial biological effects and are potential contributors to cranberry color, conserving these compounds through the processing and storage of cranberry products is of considerable importance.

2.2.2.3 Proanthocyanidins (PACs)

Proanthocyanidins, also known as condensed tannins, characteristically interact with proteins, often causing denaturation and precipitation. Upon consumption, this activity in the saliva causes the astringent, mouth-drying sensations from foods like cocoa, wine and cranberries.¹³ Beyond these flavor contributions, PACs demonstrate unique bioactivity and are widely studied for antioxidant, anti-cancer, and anti-pathogenic activities among others.⁵⁶ Many believe the unique PACs found in cranberries underlie the fruits' activity in preventing urinary tract infections.¹¹

While also classified as flavonoids, proanthocyanidins display more complex structures than their anthocyanin and flavonol cousins. PACs are oligomers and polymers of flavan-3-ol (or catechin) subunits. Catechins exist as monomers in foods like cocoa, tea, wine and in cranberries,⁵⁷ but appear colorless as they lack a system of conjugated double bonds. Instead, the central ring of catechins contains a hydroxyl group (or sometimes a gallic acid substitution) at the 3 position and no substitution at the 4 position (Figure 6). Catechins may be linked together at the 4 position of one monomer to the 6 or 8 position of another to form PACs. 'B-type' proanthocyanidins contain only these carbon-carbon linkages, while the more uncommon 'A-type' PACs contain an additional ester linkage (Figure 7). Generally from the 2 position of the central ring to the oxygen bound to the 7 position of the C ring, this second linkage adds structural rigidity, bioactivity, and perhaps unique metabolites upon consumption.^{58,59} Cranberries are the only food known to contain substantial amounts of these potentially bioactive A-type PACs, though these are found in peanut and plum in smaller concentrations.⁶⁰⁻⁶²

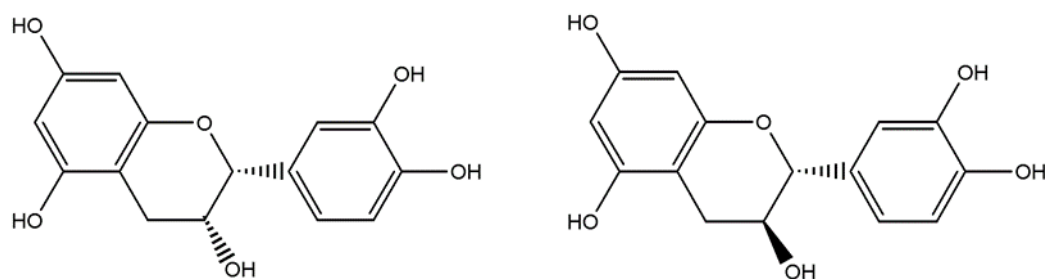


Figure 6: Catechins (-)epicatechin and (+)catechin, monomer units of cranberry PACs.

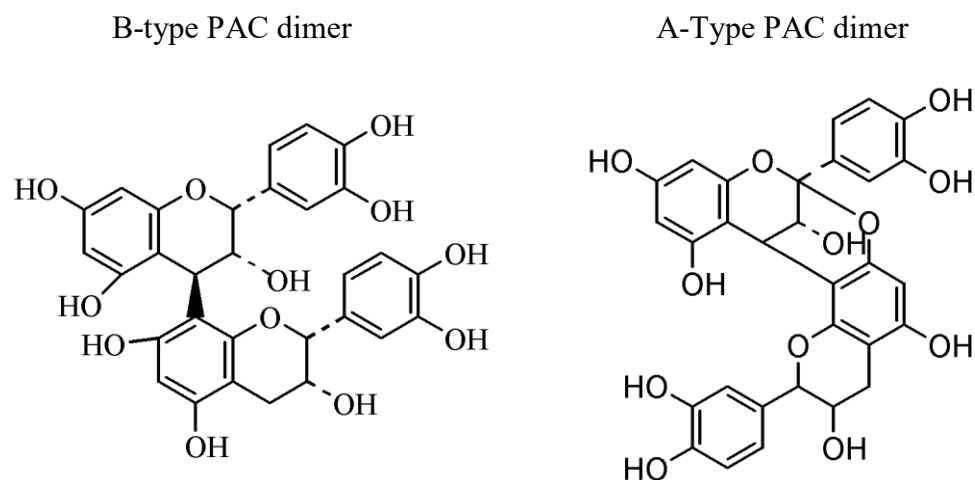


Figure 7: Two proanthocyanidin dimers: a B-type PAC {(EC-(4 β →8)-EC) on left} with single linkage and an A-type PAC {(EC-(4 β →8, 2 β →O→7)-EC) on right} with double linkage.

The possible number of individual PAC species is astoundingly large due to numerous possible linkage types, degrees of polymerization (DP), and monomer substrates (catechin, epicatechin (EC) and epigallocatechin in cranberries).^{61,63} While only several dozen have been observed, this structural diversity makes separation and

analysis of proanthocyanidins painstaking and sometimes impossible with current technology. Isolation of individual species for positive characterization has been successful only up to the 3rd degree of polymerization in cranberry, despite extensive research: LC-MS, NMR and chemical degradation identified 5 cranberry proanthocyanidins: two dimers A2 (EC-(4 β →8, 2 β →O→7)-EC) and B2 (EC-(4 β →8)-EC) as well as three A-type procyanidin trimers: EC-(4 β →6)-EC-(4 β →8, 2 β →O→7)-EC, EC-(4 β →8, 2 β →O→7)EC-(4 β →8)-EC and EC-(4 β →8)-EC-(4 β →8, 2 β →O→7)-EC.⁵⁸

Less conclusive methods, such as matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) have suggested the presence of dozens of other proanthocyanidin species in cranberry. From these analyses, cranberry PACs appear to be mostly DP 4-10, but with DP as high as 23 and with ~60% of PACs containing A-type links.^{57,60,63-67} While epicatechin appears to be the dominant monomer unit, catechin and epicatechin gallate are also present.⁵⁷ Estimates of the average DP of cranberry PACs range from 4.7⁵⁸ to 8.5.⁵⁷

Little quantification data can be found for individual cranberry proanthocyanidin species in today's literature, although quantification by degree of polymerization or by total PAC content is often cited. Quantifications of total PACs reveal that cranberry is a rich source of these micronutrients with 23.1 mg/100 mL in cranberry juice cocktail and 418 mg/100 g in fresh berries.⁶⁸ Only in chokeberry were higher concentrations found among the 21 PAC-rich fruits tested.

With the estimated daily intake of PACs at 57.7 mg/person in the US,⁶⁸ cranberries and commercial cranberry juice cocktail are potentially dominant dietary

sources of these micronutrients. While no data exists on total consumption of A-type PACs, cranberries are likely the major dietary source in the US.¹¹ Because of their high antioxidant capacity, *in vitro* anti-cancer activity, and their ability to prevent adherence of some bacterial pathogens to host cells (see Section 2.3), maximizing the stability of PACs through cranberry products' processing and storage would benefit cranberry consumers.

2.3 Cranberry Health Effects

2.3.1 General Health Benefits From Fruit Consumption

The American Heart Association among many other groups recommends consumption of at least 5 servings of fruit and vegetables daily, and the tremendous health promotion provided by fruits is well established. Indeed, epidemiological studies continually connect fruit consumption with reduced rates of degenerative diseases. In the most significant and recognized connection, coronary heart disease (CHD) incidence and related fatality^{69,70} along with cancer incidence and related fatality⁷¹⁻⁷⁴ inversely correlate to fruit and/or flavonoid intake. Traditional wisdom would suggest that vital nutrients are responsible for these benefits of fruits. However, numerous studies have found that supplementation with synthetic vitamins has no benefit and some have found harmful effects. In contrast, the diverse phytochemicals present in fruits (such as flavonoids) appear to be the active components, perhaps acting synergistically.⁷⁵

The leading cause of death in the US, coronary heart disease, occurs when blood flow is restricted, usually because of atherosclerosis.⁷⁶ Numerous disorders may ensue, including such catastrophic events as heart attack and stroke. For heart attack, relative risk was 0.40 ($P < 0.0006$) between lowest and highest quintiles of fruit intake using data

from the Women's Health Study from 1993-1999.⁷⁰ For stroke, daily consumption of fruit reduced risk by 35% in men and 25% in women ($P < 0.002$) in a prospective cohort study of >40,000 Japanese patients.⁷⁷ How fruits and flavonoids may prevent CHD is debated. Antioxidant mechanisms have been proposed, as oxidation of fatty arterial lesions (mainly from low density lipoprotein [LDL] deposition) is a critical step in the development of atherosclerosis.⁶⁹ Mechanisms involving modulation of platelet activity and/or circulating levels of harmful LDL and beneficial HDL (high density lipoprotein), all well-documented risk factors for CHD, also have been proposed.⁷⁸ Others postulate that mechanisms related to alteration of inflammatory responses, also critical to the progression of atherosclerosis, play a role in fruits' beneficial effects on CHD.⁷⁹ Indeed, all of these factors and others (such as affects related to fiber and folate intake) may contribute to the benefits of fruits towards CHD.⁷⁶

Cancer, the second leading cause of death in the US and the #1 killer for those under 85,⁸⁰ involves DNA mutation and unchecked tumor growth as major mechanisms. Fruit consumption is linked with lowered rates of some cancers. Meta-analysis of case-control studies revealed inverse correlations between fruit intake and stomach, esophageal, bladder, colorectal and lung cancers, but no significant correlation for breast cancer.⁸¹ Data indicates stomach cancer is most affected by fruit consumption, with an odds ratio of 0.69 per serving of fruit per day. While no mechanism is proven, antioxidant related mechanisms seem plausible as reactive oxygen species and free radicals readily damage DNA, a key step in carcinogenesis, but many other mechanisms are continually investigated as well. *In vitro* research reveals that fruit phytochemicals, polyphenols in particular, may modify the expression or activity of critical proteins involved with DNA

repair, cell growth and proliferation, regulation of apoptosis, angiogenesis or maintenance of endogenous antioxidants.^{11,67,82} As with CHD, anti-cancer mechanisms may be numerous, acting additively or synergistically.

Fruits and fruit juice also appear to inhibit age-related loss of neurological function and such degenerative conditions as Alzheimer's disease. In one prospective study of 1836 subjects, those who consumed <1 serving per week of fruit and vegetable juices were four times more likely to develop Alzheimer's disease than those consuming >3 servings per week ($p < 0.01$).⁸³ Numerous animal feeding studies support findings that consumption of fruit or fruit extracts improves neural function and inhibits (or reverses) some age-related neural deficiencies in rodents, as measured physiologically and behaviorally.⁸⁴⁻⁸⁸ Because Alzheimer's disease (and age-related loss of neurological function) are theorized to be caused by oxidative stress and the inability of the aged brain to counter oxidative insult,⁸⁹ it is logical that a diet high in antioxidant-rich fruits would inhibit such conditions.

2.3.2 Health Benefits specifically from cranberries

Little or no epidemiological data exists to confirm that cranberry shares the general benefits that are so apparent for overall fruit consumption, perhaps because it is consumed more rarely than many other fruits and usually in processed forms. However, controlled *in vitro* investigations, animal feeding studies and clinical trials suggest that cranberries do indeed have potent, positive effects on degenerative diseases such as CHD and cancer, neurological health, inflammation, and/or biomarkers of those conditions. Additionally, unlike other fruits, cranberries appear to have unique, clinically significant

effects against bacterial pathogens. A brief overview of cranberry effects is given for some conditions recognized as huge problems for our society.

2.3.2.1. Anti-Degenerative Disease

For CHD, several clinical investigations have revealed promising outcomes due to cranberry consumption. In particular, markers of oxidative stress to the cardiovascular system are consistently lowered after cranberry consumption. These include significantly raised plasma antioxidant capacity using several methods vs. baseline or placebo,⁹⁰⁻⁹³ ~10% reductions in circulating oxidized LDL vs. baseline ($p<0.02$),⁹¹ ~7.5% reduction in plasma nitrate/nitrite levels vs. baseline ($p<0.05$)⁴¹ and ~75% reductions in advanced oxidation protein products (AOPP) in plasma vs. placebo and baseline ($p<0.05$)⁹⁴. Whether cardiovascular antioxidant effects from cranberry consumption linger more than a few hours is debatable. After overnight fasting, no increase in plasma antioxidant status was observed after 2 weeks of daily cranberry juice cocktail consumption.⁹⁵ In contrast, reductions in plasma AOPP lingered for 8 months after 8 weeks of daily supplementation with dried cranberry juice (~50%, $p<0.05$).⁹⁴ After overnight fasting, plasma nitrite/nitrate levels were reduced after 12 weeks of 'lite style' cranberry juice cocktail intake.⁴¹ Overall, cranberry products appear to reduce oxidative stress to the cardiovascular system at least in the short term, probably due to absorption of cranberries' phenolic antioxidants into the bloodstream and their binding to circulating LDL.^{11,93}

Cranberry consumption may also alter blood cholesterol levels and additionally benefit cardiovascular health, although evidence is limited. After 8 and 12 weeks of daily

'lite style' cranberry juice cocktail supplementation, obese men had ~10% increases in HDL levels ($p < 0.05$) and ratios of total cholesterol to HDL ($P < 0.0005$).⁴¹ However, the increase was not observed in healthy women after 2 weeks of cranberry juice consumption.⁹⁵

For cancer, *in vitro* studies of cranberry components effects are promising, but little *in vivo* data exists. One of cranberries' many phytochemicals, quercetin, seems to be particularly active *in vitro*, suppressing proliferation in pancreatic, leukemia, colon, and breast cancer cells by 50% at 15-60 $\mu\text{g/mL}$.^{4,5,54,55} Proanthocyanidins, too, have high *in vitro* bioactivity: cranberry PACs (at 20-70 $\mu\text{g/mL}$) inhibited cell growth by 50% in lung, cervical and leukemia cancer cell lines.⁶⁷ Anthocyanins, while possessing little direct activity against cancer cells, appear to have potent anti-angiogenic effects, which may limit tumor growth and metastasis.^{4,82} One *in vivo* study evaluated the effects of cranberry consumption on cancer and the results were promising. Here, administration of cranberry juice resulted in substantial reductions (77%, $p < 0.05$) in incidence of precancerous cell formations (aberrant crypt foci) in rat intestines after azoxymethane insult as compared to water controls.⁹⁶

Taken together, these *in vivo* and *in vitro* data suggest that cranberry consumption may have potent anti-cancer effects for areas where absorption is not necessary to expose phytochemicals to cells, i.e. the mouth, esophagus, stomach, and colon.¹¹ However, because of the limited bioavailability of cranberry components known to be active *in vitro*, mostly flavonoids, there is little evidence that cranberry consumption would have a significant impact on cancers affecting internal organs and tissue not involved in digestion and excretion.¹¹

For neuroprotective effects, blueberries seem most potent among fruits, but some limited data show cranberries may be active as well. One study evaluated berries' effects on a model of Alzheimer's disease, finding that aged rats fed a diet of 2% freeze-dried cranberries for eight weeks had significantly improved motor skills compared to controls. Their *ex vivo* brain tissue displayed improved nerve signal transmission and response to oxidative stress.⁹ Evaluating blueberries effects on neurological health, the same researchers identified cyanidin-3-galactoside, cyanidin-3-arabinoside and peonidin-3-arabinoside (all present in cranberries) among other intact anthocyanins in the brains of rats.⁸⁶ Indeed, feeding anthocyanin mixtures (38 mg/kg) resulted in lowered levels of stress-induced oxidation in the brain tissue of rats by up to 35% as measured by thiobarbituric acid reactive substance and protein carbonyl assays.⁹⁷ Taken together, research from cranberries and other fruits suggest that anthocyanins may act as potent neuroprotective agents with implications for Alzheimer's disease, Parkinson's disease and general aging.

2.3.2.2 Anti-Pathogen

Most thoroughly documented of cranberries' potential health effects, the anti-pathogenic activity of cranberry has been substantiated in numerous clinical trials, particularly for prevention of urinary tract infections. While the molecular mechanism is still debated, cranberry extracts were shown to inhibit adhesion of urinary pathogens to host cells over 30 years ago.⁹⁸ Today, evidence continually accumulates for similar activity in other areas, particularly the mouth and stomach. No less than five clinical trials involving nearly 500 female patients in total have demonstrated that consuming cranberry

products (including juices, extracts and powders) on a daily basis for at least several weeks prevents UTIs and/or reduces bacteriuria.⁹⁹⁻¹⁰³ One recent study, however, did not find such a link in college-aged females.¹⁰⁴ Several other clinical studies have shown that urine gathered after cranberry intake inhibits binding of uropathogenic *E.coli* to epithelial cells *ex vivo*.^{66,94,98,105} A meta-analysis of clinical data revealed relative risk of 0.65 for symptomatic UTIs and 0.61 for recurrent UTIs when treated with cranberry products as compared to control or placebo groups.⁸

The mechanism underlying cranberry consumption's unprecedented anti-UTI activity continues to be the subject of much research. Prevention of the adhesion of uropathogenic *E. coli* to host tissues by cranberry phytochemicals is well demonstrated,^{58,59,98,106-109} although exactly which components accomplish this *in vivo* and by what specific molecular mechanism(s) remains unproven.¹¹ Early research focused on the cranberry high molecular weight fraction isolated by dialysis (termed non-dialyzable material or NDM), which was shown to be potently active against *E. coli in vitro*.^{106,107} Later research centered upon cranberries' unique lower molecular weight A-type PACs^{58,108} which showed *in vitro* activity against *E. coli* at concentrations as low as 0.3 mg/mL for individual PAC trimers and at 75 µg/mL for mixed cranberry PACs.⁵⁸ Difficulty finding PACs and NDM in urine led some to search for alternate components responsible for cranberries anti-adherence affects.^{39,94,110,111} Some have proposed that flavonols play a role in activity of cranberries against UTI.^{94,111} However, a recent study found that at least one PAC species (PAC A2) can be detected in urine after cranberry consumption, albeit in low concentrations (<25 ng/mL).¹¹²

Absorption of PAC and other cranberry components is not necessary for effects in the mouth and stomach. Cranberry components demonstrate potent activity against numerous dental and oral pathogens *in vitro*, including *P. gingivalis*, *T. forsythia*, *T. denticola*, *S. Sobrinus* and *S. mutans* and their virulence factors.¹¹³⁻¹¹⁸ Limited *in vivo* data also support that cranberry is active against these pathogens in counteracting tooth decay and periodontal disease. Mouthwashes containing cranberry NDM lowered bacterial counts of *Streptococcus mutans* by two orders of magnitude versus a control mouthwash.⁶ Applying PACs topically to rat teeth lowered incidence of severe cavities by ~40%.¹¹⁹ While most such research focuses on cranberry PACs and NDM as the active components, flavonols seem to have some limited impact as well.¹¹⁶

Similarly, cranberry components appear to have activity against the stomach pathogen *H. pylori* and its ability to adhere to host cells. Much *in vitro* evidence demonstrates that cranberry NDM and also PAC rich extracts interfere with *H. pylori* infectivity, and one even found cranberry components affect more strains than antibiotic treatments.¹²⁰⁻¹²⁴ *In vivo* data also supports cranberries activity against this stomach pathogen. In two clinical trials, daily consumption of cranberry juice for >35 days resulted in modestly reduced rates of infection compared to controls, 14-17%.^{124,125} These effects in the mouth and stomach are particularly intriguing in suggesting that cranberry may be an effective natural therapy for treating (as well as preventing) certain bacterial infections, a rare effect for a plant food.

2.4 Factors affecting stability of cranberry phenolics and ascorbic acid

Because cranberry phytochemical antioxidants, particularly its flavonoids, monophenolics and ascorbic acid, are critical to the observed health effects from cranberry consumption, understanding the factors affecting the stability of these components is essential. While much research has focused upon degradation pathways, stabilizing factors and destabilizing factors for anthocyanins and ascorbic acid, relatively little of this information is known for flavonols and proanthocyanidins.

2.4.1 pH

The crucial role pH plays in anthocyanin stability is well documented, but little or no data exists for effects of pH on the stability of flavonols and proanthocyanidins.

For anthocyanins in aqueous solutions, a complex set of pH-dependent equilibrium reactions exist, with important implications for stability and perceived color (Figure 8). At very low pH (<2.5), the red-colored and relatively stable flavylium cation predominates. Addition of water and loss of proton yields the colorless carbinol form while loss of only a proton yields the blue-hued quinoid base form. Both reactions are promoted by higher pH, although the quinoid base is never the predominant form. At pH >3 , the carbinol form is favored and more modest concentrations of the blue quinoid base are present. The fourth major anthocyanin equilibrium form, the colorless chalcone, results from ring opening of the carbinol form. This highly unstable species also is promoted by higher pH, becoming more prevalent than the flavylium form at pH >3.5 . At pH >5 , only negligible amounts of the stable flavylium form remain while the chalcone predominates.³⁶

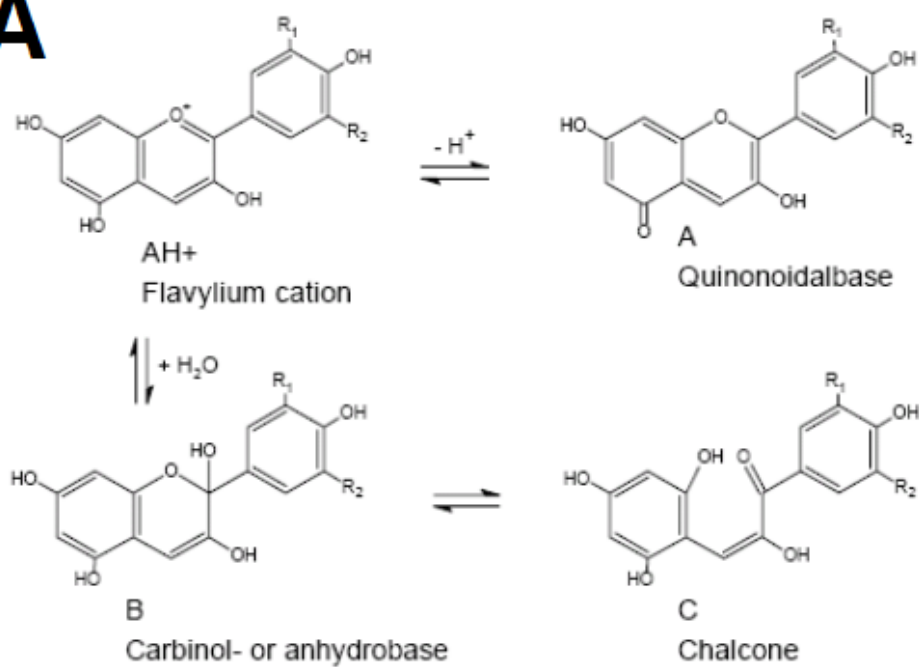
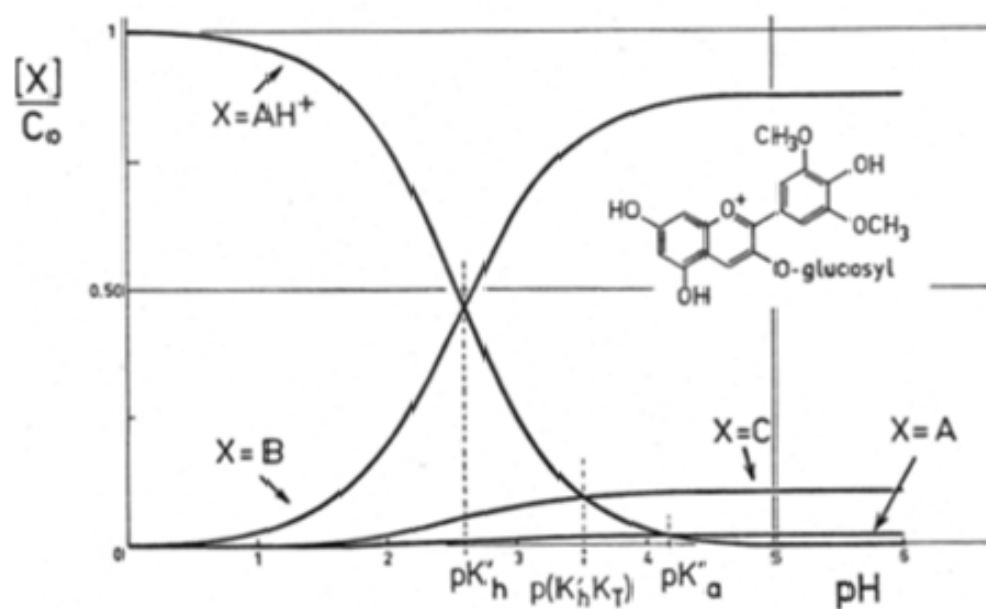
A**B**

Figure 8: The major equilibrium forms of anthocyanins (A).¹²⁶ Relative concentrations of anthocyanin equilibrium forms at differing pH values (B).³⁶

Minor adjustment in cranberry juice pH can increase stabilization of anthocyanins: cranberry juice adjusted to pH 2.2 retained ~5% more anthocyanins over 12 weeks at ambient storage than did unaltered cranberry juice (pH 2.7).¹²⁷ However, the practicality of such a pH adjustment is questionable. Adding acid to cranberry juice would increase the perceived ‘sourness’ of the juice, which is already powerfully sour in its native state, and any acid that could reach this pH would necessarily also affect the flavor of cranberry juice.

As a further complication, anthocyanins still degrade at low pH where the most stable flavylum form is most predominant and color is most red. Often, the mechanism is acid hydrolysis of the glycosyl moiety yielding the unstable anthocyanidin (aglycone) form, and ultimately ring opening with associated loss of color.¹²⁸

For ascorbic acid, pH has a substantial impact on stability. Ascorbic acid in canned tomato juice degraded most readily ($k = 2.48 \times 10^{-3}/\text{day}$) at pH 4.06, very close to its pKa, while first order reaction rate constants were reduced at higher pH (pH 4.35, $k = 2.28$) and at lower pH (pH 3.78, $k = 2.27 \times 10^{-3}/\text{day}$).¹²⁹

Little is known about pH effects on stability of flavonols or PACs. The low pH of cranberry juice cocktail that favors relatively good stability of anthocyanins and ascorbic acid may promote hydrolysis of glycosyl moieties of flavonoids, especially over long periods of storage and/or elevated temperatures. Any increase in pH would promote instability of anthocyanins and vitamin C, while addition of acids to lower pH of cranberry juice would magnify its already sour flavor. Therefore, adjustment of pH to promote phytochemical and nutrient stability is impractical for cranberry juice.

2.4.2 Light

Light radiation accelerates degradation of anthocyanins, flavonols, ascorbic acid and possibly other cranberry flavonoids. While light may excite these molecules leading to photo-induced auto-degradation, the photo-stability of hydrocarbons is generally evaluated in air-saturated solutions¹³⁰ because photo-photosensitized oxidation is generally the primary means of photo-degradation for organic chemicals.

Storage studies have extensively documented the destabilizing effects of light on anthocyanin integrity and color as well as ascorbic acid content in a variety of processed foods (including soft drinks, wine, syrups, infant formula, jellies and jams).¹³¹⁻¹³³ For example, in a carbonated grape drink, ambient storage over 135 days resulted in ~30% loss of anthocyanins when stored in the dark, ~50% loss when exposed to day light and ~70% loss when exposed to continuous fluorescent light (80 foot candles) as occurs in grocery stores.¹³³ Vitamin C oxidation is drastically accelerated by light. Exposure of infant formula to 1756 lux (160 foot candles) of fluorescent light increased the second-order oxidation rate constant for ascorbic acid oxidation by ~1800%.¹³¹ In model systems, the rate of degradation of isolated cranberry anthocyanins (at 40 °C, pH 2.5, under air) increased by ~450% during exposure to fluorescent light (400 foot candles) compared to storage in the dark. Crucially, when oxygen was removed, light had no significant effect on anthocyanin retention. The researchers concluded that molecular oxygen was involved in the photo-degradation of anthocyanins, perhaps through a singlet oxygen intermediate.¹³⁰

In a study of anthocyanidin solutions (anthocyanin aglycones) subjected to UV radiation (313 nm), photo-degradation was attributed to direct excitation of the flavylum

cation and subsequent autodegradation. As in thermal degradation (see Section 2.4.3), photo-degradation yielded calchone as an intermediate and the final cleavage products were identified as various hydroxybenzaldehydes from the A ring and benzoic acid derivatives from the B ring. However, this study did not consider the effect of oxygen.¹³⁴ It is unclear whether these differing conclusions concerning the mechanism of anthocyanin/anthocyanidin photo-degradation can be attributed to the control of oxygen levels or to the different chemical species that were evaluated.

Unlike anthocyanins, flavonols show remarkable photo-stability. Indeed, some authorities propose that the central biological role of flavonols is to protect plants from harmful UV radiation.¹³⁵ After 15 hours of exposure to intense light radiation ($90 \mu\text{W}/\text{cm}^2$) in air-saturated solutions, quercetin (the aglycone form) lost 17% while quercetin-3-galactoside lost only 2% of its absorbance, and this trend was observed in a number of other flavonols and flavones. Hence, blocking the -OH group at the 3 position was found to be critical to photo-stability. This 3 position was theorized to be the major site of photo-induced electron transfer to molecular oxygen in flavonol aglycones. The presence of metal ions greatly accelerated flavonol photo-bleaching. For example, with 2:1 ratio of aluminum to flavonol, quercetin lost all absorbance after 8.5 hours under the same conditions.¹³⁶

It is clear that light plays a major role in the degradation of cranberry anthocyanins, vitamin C, and color, at least in the presence of oxygen. Flavonols appear to have superior photo-stability, especially flavonol glycosides. What role light may play in proanthocyanidin stability remains unknown.

2.4.3 Heat

Because heat accelerates chemical reactions, it is detrimental to the stability of flavonoids and ascorbic acid. In particular, even short periods of thermal abuse may allow oxidation reactions to become established, initiating a cascade of events that lead to red color loss, browning and degradation of nutrients and nutraceuticals. This is tremendously important for commercial cranberry juice because thermal processing (often to temperatures exceeding 90 °C) is the traditional mode of microbial inactivation. The effect of thermal processing has been evaluated directly on cranberry anthocyanins and vitamin C (see Section 2.5.1). In addition to processing effects, heat plays an important role in storage. Bottled CJC is stored refrigerated after it is opened, but it spends more time stored at ambient temperatures (which includes trucks and warehouses exceeding 40°C during the summer). Thus, the effect of heat is critical to the shelf life of cranberry juice and the stability of its components.

High temperatures devastate anthocyanins under first-order kinetics.^{137,138} In model solutions, the half-life of cyanidin-3-glucoside was less than 2.1 hours at 100 °C at pH<4.¹²⁸ Storage at 20 and 3.5 °C, on the other hand, yielded >60% and >90% of anthocyanin retention after 135 days, respectively, in a model beverage at pH 3.7.¹³⁸

One mechanistic study explored heat induced anthocyanin degradation.¹²⁸ Observations that at 100 °C and pH 2-4, free sugars were released at the same rates as anthocyanin degradation and red color loss led to the conclusion that glycosidic hydrolysis is the major pathway and the rate-determining step of anthocyanin thermal degradation, rather than opening of the pyrylium ring. Although not critical to hydrolysis, the presence of oxygen had a profound effect by greatly accelerating the reaction and

altering the number and nature of degradation products. Of more than seven degradation products observed, only a chalcone aglycone and flavylium aglycone were identified. These were present only when anthocyanins were heated under anaerobic conditions and these products degraded rapidly when exposed to oxygen. Recent work evaluating thermal stability of strawberry pigments also concludes that glycoside hydrolysis is the first step in thermal degradation of anthocyanins.¹³⁹ During 7.5 hours heating, first aglycones were observed and then cleavage products including phenolic acids and aldehydes appeared (Figure 9).

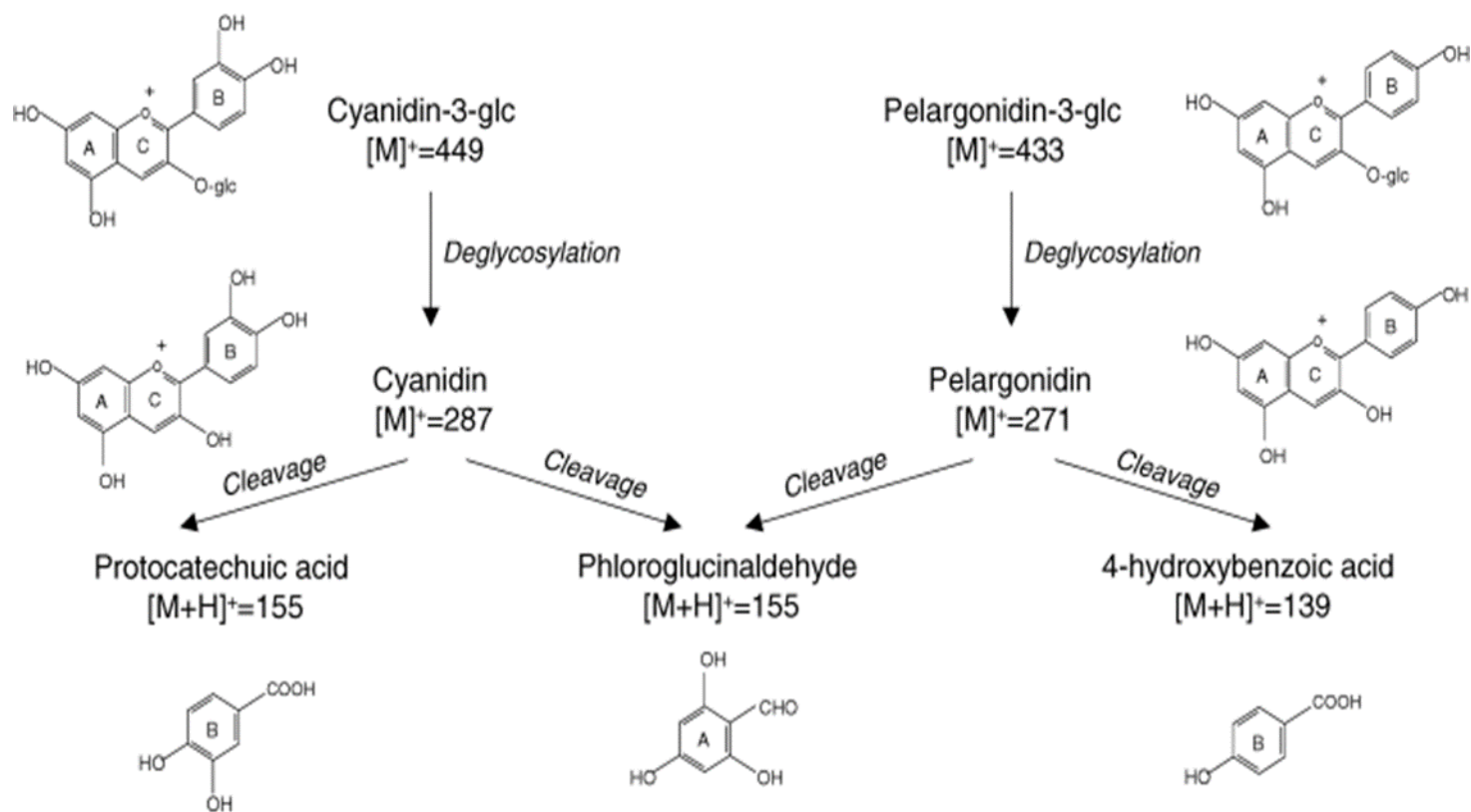


Figure 9: Thermal degradation scheme for strawberry anthocyanins¹³⁹

Coumarin glycosides also appear to be major thermal degradation products of anthocyanins,¹⁴⁰ formed via an alternate in which the bond between the B and C ring is cleaved, yielding a monophenol and a coumarin derivative (Figure 10).³⁷

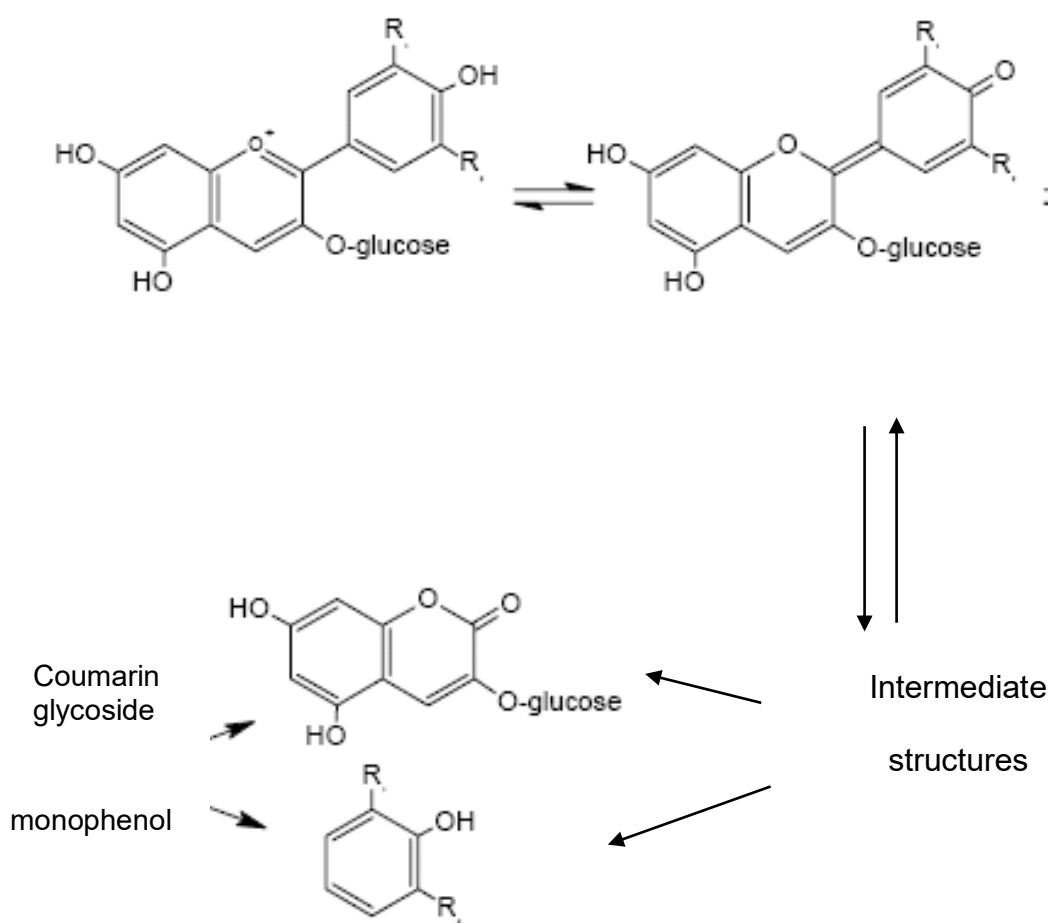


Figure 10: Scheme of heat accelerated degradation of anthocyanin monoglucoside³⁷

In model system studies, heat affects stability of flavonols less dramatically than that of anthocyanins, although this has not been shown in cranberry products directly. In blueberry extracts, 60 days of storage at -20°C, 23°C and 35°C resulted in 90%, 74% and 61% retention of quercetin, respectively,¹⁴¹ yet high heat treatments seem to have little effect on flavonols (see Section 2.5.1). Mechanisms underlying thermal degradation of flavonols remain unknown.

The heat stability of proanthocyanidins in foods has not been thoroughly documented but some limited data suggests that PACs have intermediate heat stability. High heat treatments caused small proanthocyanidin losses in cranberry juice (<10%)¹⁴² and larger losses in blueberry juice (40-60%).¹⁴³ Storage at high temperature (35°C vs. 22°C) accelerated proanthocyanidin losses by a factor of ~4 in cranberry juice stored for one month.¹⁴²

Degradation of total phenolics, like individual phenolics, is accelerated by heat during extended storage. For example, in clarified banana juice, 24 weeks of storage at 4°C, 25°C and 37 °C caused 95%, 77% and 74% retention of total phenolics, respectively, as measured by the Folin-Ciocalteu method.¹⁴⁴ Perhaps less dramatic losses are observed for assays measuring total phenolics (relative to individual species) because such methods are blind to conversions of native phenolics to nonnative phenolics.

2.4.4 Oxygen, Oxidizers and Browning Reactions

2.4.4.1 Molecular Oxygen and Oxidation

Molecular oxygen (O₂) displays some unusual chemistry that is best explained by molecular orbital theory. In its ground state, O₂ exists as a stable diradical; it contains two

unpaired electrons (one on each oxygen) in degenerate molecular orbitals (Figure 11A).¹⁴⁵ This ground form is in an open-shelled triplet state ($^3\text{O}_2$) with the unpaired electrons having parallel spin, giving electronic characteristics with broad implications for the reactivity of molecular oxygen.¹⁴⁵ Triplet species cannot react directly with molecular species such as double bonds having closed electron shells (singlets) (Figure 11B)¹⁴⁵ Rather, at ambient temperature, ground state triplet oxygen will react most readily with species in doublet or triplet states (i.e. radicals).¹⁴⁵

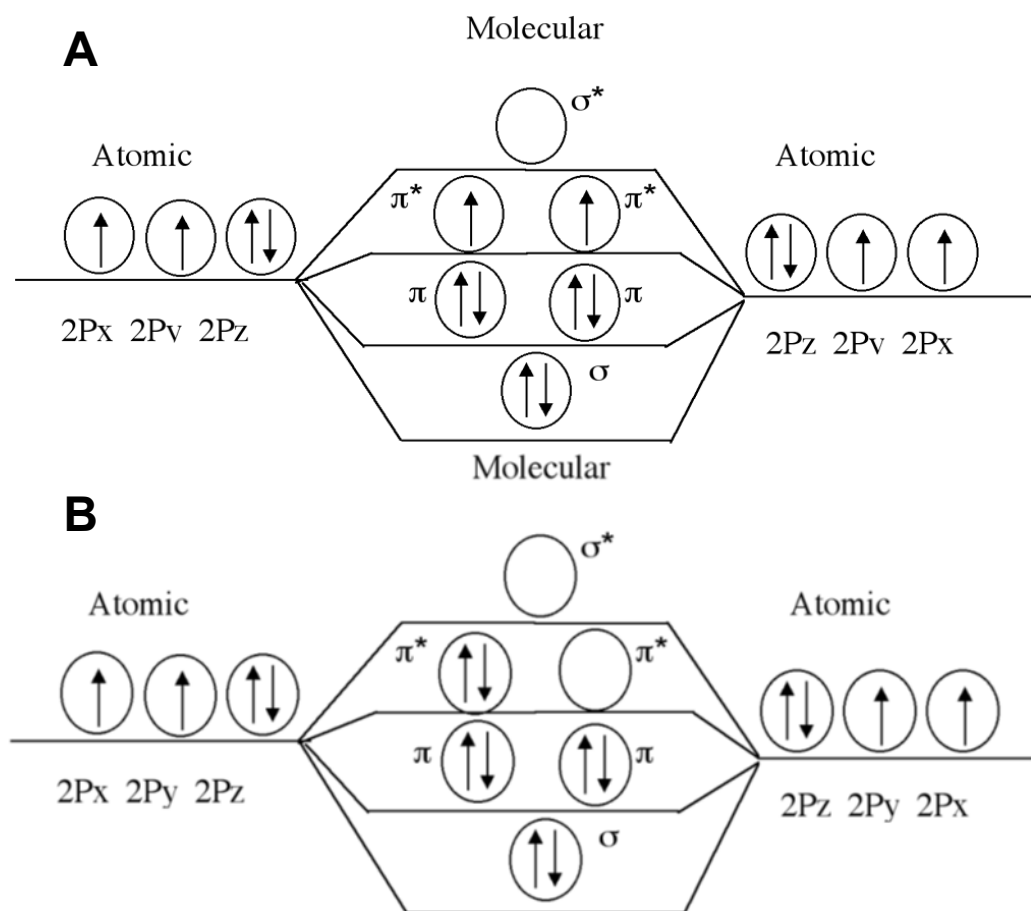


Figure 11: The valence molecular orbitals of triplet oxygen (A) and singlet oxygen (B).

Adapted from ¹⁴⁵

In excited state of O₂, those valence electrons that had been parallel in the ground triplet state become antiparallel, residing either within the same orbital or across the two degenerate orbitals (Figure 11B).¹⁴⁵ This state is referred to as singlet oxygen, ¹O₂. The antiparallel spins of the valence are now free to react with other closed shell singlet species (e.g. double bonds) without the activation energy required to form the intermediate triplet state within the substrate. Hence, singlet oxygen (¹O₂) is extremely reactive at ambient temperatures and is considered to be a reactive oxygen species. With an activation energy from 0 – 6 kcal/mol, high temperatures are not required for singlet oxygen reactions.¹⁴⁶ In aqueous foods, the lifetime of singlet oxygen is short (<700 microseconds) but nevertheless long enough to react and degrade other compounds.¹⁴⁵

Singlet oxygen reportedly may be generated from the triplet form via catalysts, photosensitization and/or chemical means (Figure 12). However, reactions generating ¹O₂ in dark chemistry remain highly controversial because the analytical methods used to detect ¹O₂ are too non-specific. Photosensitization is the most important source of ¹O₂ in foods. In this reaction, a number of natural pigments such as chlorophyll, riboflavin and pheophytins, among others, harvest low energy from visible light, transform it, and transfer it to molecules as to high level chemical energy. Anthocyanins, too, may act as photosensitizers, and indeed have been shown to be effective photosensitizers in solar cells,¹⁴⁷ but this activity that has been little explored in food systems. Two major pathways are known to occur in this photo-oxidation. In both, the photosensitizers absorb light energy and become an excited triplet. In Type 1 reactions, the excited photosensitizer reacts with another compound (such as a phenol or fatty acid) directly, creating a radical compound which may then react with triplet oxygen. While this

pathway does not involve formation of singlet oxygen, it is a very important reaction catalyzing oxidation of foods and may have importance in oxidative degradation of cranberry juice. Alternatively, in the Type 2 pathway, the excited photosensitizer reacts directly with triplet oxygen via triplet-triplet annihilation, forming singlet oxygen and ground-state photosensitizer. In the case of cranberry juice, riboflavin is present (albeit in small quantities – 0.18mg/kg)¹⁴⁸ and so photo-oxidation surely occurs when oxygen and light are present. Likewise, the presence of reactive oxygen species, such as the degradation products of ascorbic acid may also spur oxidation reactions.

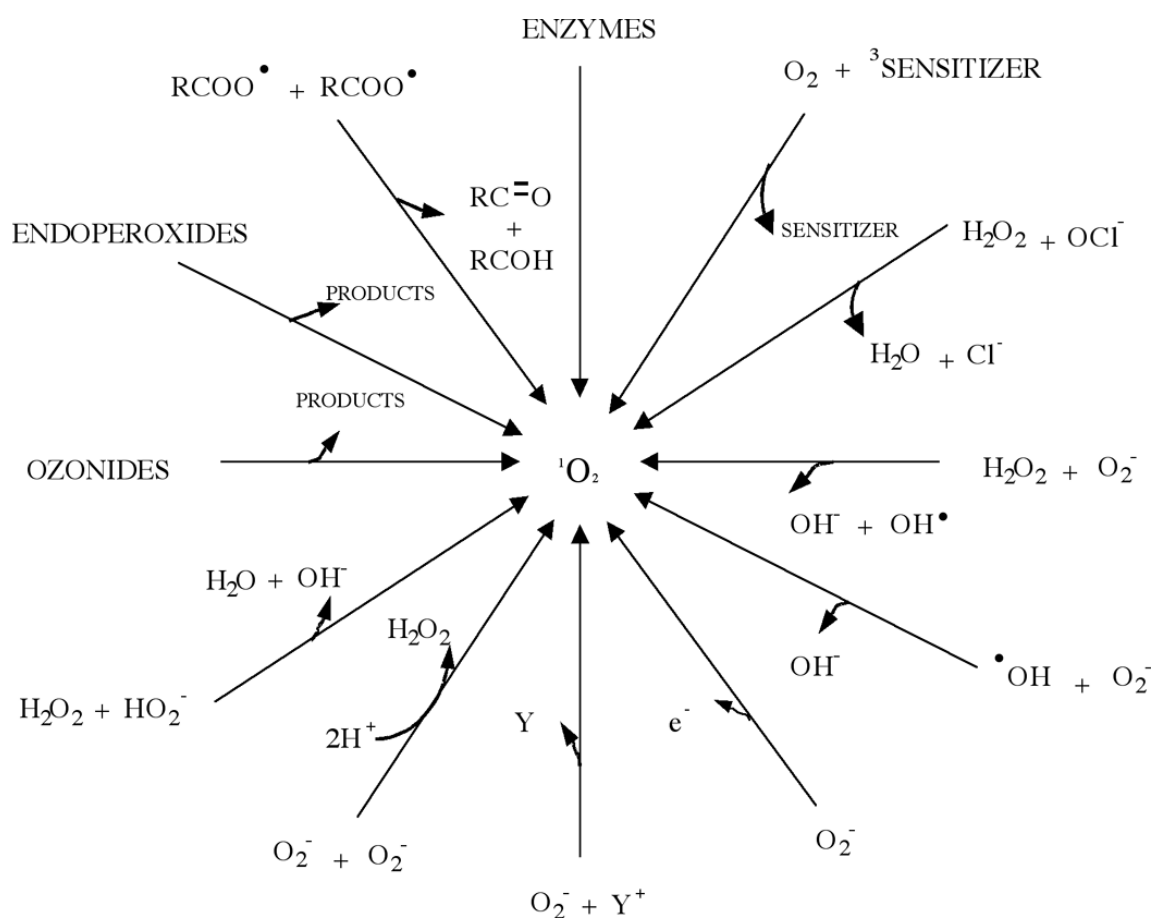


Figure 12: Various pathways to singlet oxygen formation (adapted from ¹⁴⁹)

2.4.4.2 Ascorbic Acid Interactions

The instability of vitamin C presents challenges to the food industry on many levels. One notorious example is the phenomenon of ascorbic acid browning and its sometimes devastating, if unrecognized, impact on color quality, nutritional quality and shelf life of foods. For cranberry juice, it is well known that the presence of ascorbic acid accelerates degradation of anthocyanins under aerobic conditions.¹⁸ It is not known whether similar interactions occur between AscA and other cranberry phenolics and flavonoids.

AscA is a powerful reducing agent and this property underlies its instability and reactivity. AscA reduction of oxygen in the presence of transition metals creates superoxide anions, reactive oxygen radicals that dismutate to hydrogen peroxide (and ultimately to hydroxyl radicals), while degrading AscA to ascorbyl radicals, dehydroascorbic acid, furfural and 2-furoic acid.³⁷ Hence, although AscA is known to be a strong antioxidant, it also has pro-oxidant activity, particularly at high concentrations.

The effect of varying levels of vitamin C and bottle headspace oxygen on anthocyanins has been studied in cranberry juice directly.¹⁸ After 32 weeks storage, those samples exposed to the highest headspace (12.5% by volume) and ascorbic acid (177 µg/mL) resulted in 83% loss of total anthocyanins over 36 weeks storage, while low headspace (<1% by volume) and minimal ascorbic acid (23 µg/mL) lost only 52% of the anthocyanins. The observed ~3 fold difference in anthocyanin retention underscores the remarkable inverse influence of both ascorbic acid and molecular oxygen on anthocyanin retention in cranberry juice. This research however, predates the use of [oxygen

permeable] plastic packaging as well as sensitive dissolved oxygen measuring technology and did not investigate the role of dissolved oxygen.

Multiple mechanisms appear to be active in ascorbic acid degradation of anthocyanins. Highly oxidizing chemical species stemming from degradation of AscA, such as hydrogen peroxide and hydroxyl radicals, degrade anthocyanins.¹⁸ A direct reaction between AscA and anthocyanins (alone or in addition to the aforementioned mechanism) has also been postulated, because AscA caused increased anthocyanin losses even with protection from oxygen exposure.¹⁵⁰ One well-established pathway is that degradation products of ascorbic acid lead to condensation reactions involving anthocyanins, with resultant formation of brown pigments (see Section 2.4.5).^{150,151}

Flavonol-AscA interactions appear to be less active than anthocyanin-AscA interactions. A recent study of effects of added vitamin C (at 0.5 g/kg) on stability of flavonols in apple purees during processing showed puzzling disparate results. Here, vitamin C-fortified Idared apple puree had 20-50% lower flavonol retention after processing than unfortified apple puree. The opposite was observed with another variety (Shampion apples) where flavonol content over 6 months storage did not appear to be affected by vitamin C fortification, with losses of 30-80% depending on the individual flavonol species.¹⁵² Clearly, more information is needed to fully elucidate potential reactions of AscA with flavonols.

Similarly, little is known about proanthocyanidin-AscA interactions. As with flavonols, vitamin C fortification exerted inconsistent effects on PAC retention during processing of apple puree.¹⁵² However, there was a clear trend toward accelerated degradation of PACs by vitamin C during 6 months storage at 30°C. For example,

fortified apple puree retained only ~10% of procyanidin B2 after 6 months storage while the unfortified control retained nearly 50%.¹⁵² In model solutions, the presence of ascorbate (0.3 mg/mL) greatly decreased retention of catechin (a PAC monomer) during storage for 130 days under oxygen (from ~90% to ~40%).¹⁵⁰ These data suggest that combinations of vitamin C and oxygen accelerate the degradation of PACs, most likely via a mechanism involving the reactive degradation products of vitamin C.

2.4.4.3 Phenolic Browning Reactions

Some of the most complex and most intensively-researched reaction pathways in food chemistry are those involving browning. These pathways include enzymatic browning, Maillard (carbonyl-amino) browning, ascorbic acid browning, and phenolic browning among others. Phenols of all kinds are well-known to be substrates for enzymatic browning catalyzed by polyphenoloxidase enzymes widely distributed in fruits. Because enzymes are deactivated by the traditional heat treatments used for commercial cranberry juice cocktail, enzymatic browning will not be discussed here in detail. However, it is important to note that once enzymes catalyze the oxidation of phenolics compounds, the reaction pathway of enzymatic browning is similar to some other browning pathways as they relate to phenolics.

When high concentrations of phenols are present or when polyphenoloxidase has been inactivated, phenolic browning proceeds via direct oxidation of phenol groups to quinones (dicarbonyls) catalyzed by several species, including polyphenoloxidase, metals, ascorbyl radicals, and others^{35,153} (Figure 13). Quinone products of initial oxidations then undergo various further reaction with both phenolics and non-phenolic substrates,³⁵ e.g. condensing with hydroxyl groups on adjacent phenols. Serial

condensations eventually lead to polymer formation and brown melanoidin pigment development (Figure 14). For cranberry juice, phenolic browning is doubly detrimental to color quality - in addition to undesirable brown pigment formation from condensed phenols, the destruction of anthocyanin pigments results in loss of desirable red color.

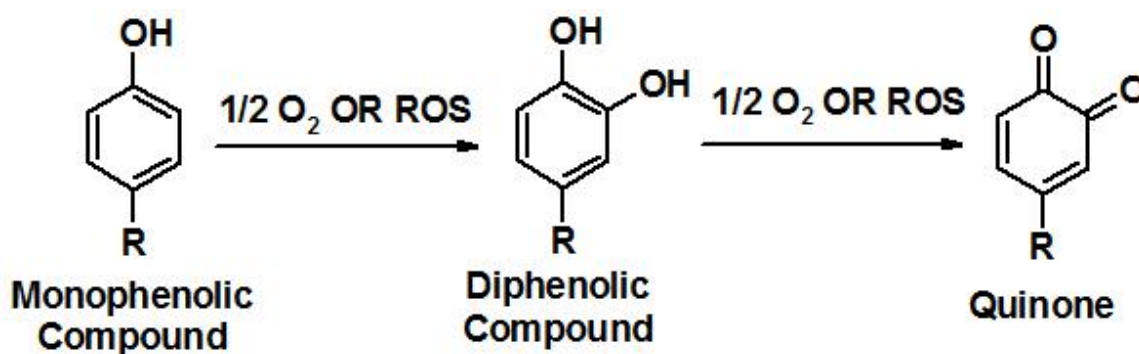


Figure 13: Oxidation of phenols to *o*-quinones (adapted from ³⁵). ROS denotes reactive oxygen species

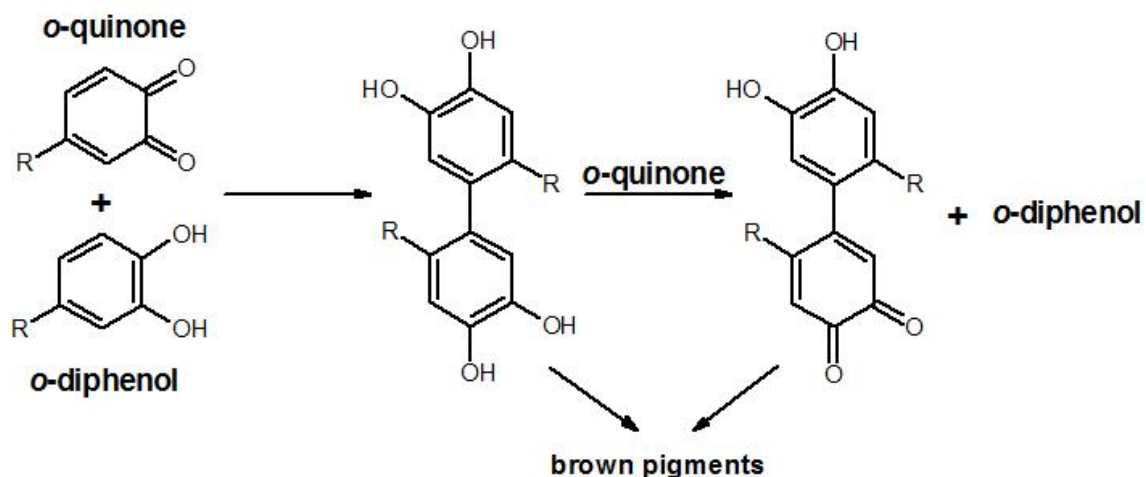


Figure 14: Condensation of *o*-quinones and *o*-diphenols to form brown pigments (adapted from ¹⁵⁴).

Trace metals are surely present in the water used to make cranberry juice cocktail and may have a role in catalyzing browning reactions. Catechins seem especially susceptible to metal ion-catalyzed auto-oxidation. In a model system, the presence of ferrous iron ions (Fe^{2+}) at 20 mg/mL accelerated catechin degradation, resulting in 70% catechin loss after 30 days, compared to only 10% loss when no iron was present.¹⁵⁵ Because PACs are polymers of catechins, PACs should be similarly prone to metal catalyzed auto-oxidation and subsequent browning. In contrast, effects of metals on cranberry anthocyanins are less dramatic and perhaps are even favorable to their retention. Copper, iron and tin added to cranberry juice at 50 ppm were mildly protective to anthocyanins under most conditions.¹²⁷ The researchers speculated that binding metals to the diphenol functions might block their oxidation, thereby stabilizing ACY pigments.

Interestingly, some condensation products of anthocyanins retain red color character and are estimated to account for 10% of the color in freshly prepared whole cranberry juice.¹⁵⁶ Indeed, specific assays have been long been utilized to determine ‘polymeric color’ from anthocyanin condensations and to differentiate these from ‘monomeric anthocyanins’.¹⁵⁷ It is widely believed that these polymeric pigments produce reddish-brown tones, and as such, values from the ‘polymeric color’ assay are often used as part of calculations to determine various browning indices.¹⁵⁸ Whether such polymeric pigments occur naturally in cranberries is uncertain. However, because amounts of polymeric pigment increase with the age of cranberry juice, it is likely that these condensations result from oxidation and browning that occur during processing/storage. Modern mass spectrometric techniques (matrix assisted laser desorption ionization mass spectrometry, MALDI-MS) have tentatively identified dozens

of anthocyanins-PAC conjugates with degrees of polymerization as high as six,^{63,159} but none of these compounds have been isolated for positive identification. More recently, studies using model solutions consisting of quinones (mostly of hydroxycinnamic acids) and anthocyanins have shown that these substrates condense to form both colored and colorless products. The exact molecular structures of the condensation polymers have not yet been elucidated.¹⁶⁰

As with other aspects of flavonol chemistry, little research is available regarding browning of flavonols. In pureed pear, levels of flavonols did not correlate to browning as measured by colorimetry. During controlled browning, pears lost ~80-100% of catechins and hydroxycinnamic acids but only ~0-30% of flavonols.¹⁶¹

Taken together, these data indicate the phenolic condensation and browning reactions are perhaps one of the primary modes of flavonoid degradation in juices. Experimental data is lacking for cranberry juice cocktail and its phenolics components, but catechins appear to be most susceptible to condensations, and perhaps their PAC polymers are similarly susceptible.

2.4.5 Dissolved Oxygen – The Common Thread

Data presented in all the previous sections demonstrates that molecular oxygen is a required reactant in nearly all degradation pathways of the phenolics found in cranberry juice cocktail. Cranberry juice is exposed to several sources of molecular oxygen throughout its processing and storage (cf Figure 15 for sources of dissolved oxygen exposure and Figure 16 in Section 2.5 for the traditional process for producing cranberry juice cocktail). Raw cranberry juice is exposed to atmospheric oxygen during pressing,

clarification and storage. Furthermore, the water used to dilute cranberry juice to make cocktail also represents a significant source of dissolved oxygen (as well as metals).

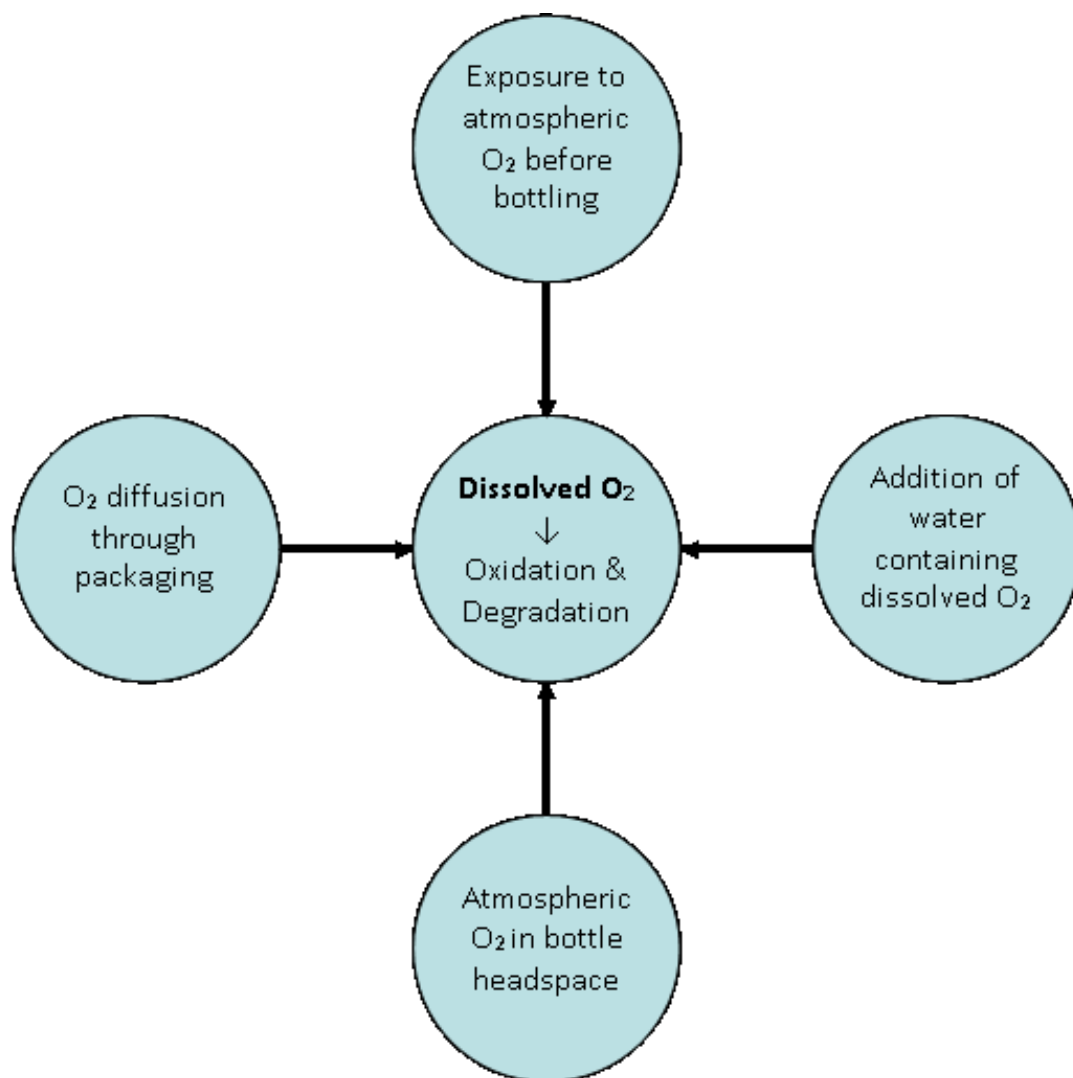


Figure 15: Sources of exposure to dissolved oxygen for bottles juices

Measurements taken at a New Jersey cranberry processing facility indicate that immediately before processing, cranberry juice cocktail is ~75% saturated with dissolved oxygen (~6 ppm) in large scale production in industry.

Heating during pasteurization drives molecular oxygen out of the juice due to the lower solubility of gases in liquids at higher temperatures. Even so, dissolved oxygen undoubtedly catalyzes many of the above mentioned reactions at these increased temperatures during pasteurization and cooling. Headspace in the bottle offers an additional source of molecular oxygen, so some industrial capping equipment displaces headspace air with nitrogen or other gasses before the bottle is sealed. Oxygen enters into juice after bottling as well, partly because of the small size of molecular oxygen. Plastic bottles and caps used as standard packaging for CJC are oxygen permeable. Cooling juice in sealed bottles creates a vacuum inside the bottle, which may in turn accelerate oxygen permeation through most plastic packaging in common use for commercial CJC. Furthermore, it is widely known that oxygen passes through the miniscule space between the threads of the cap and bottle. Industry literature estimates approximately 50% of oxygen permeation through the closure and 50% through the bottle in modern plastic bottles. After heating and bottling, low but detectable levels of dissolved oxygen typically remain in freshly bottled cranberry juice cocktail produced by industry (0.07 ppm) (unpublished data).

2.5 Processing, Packaging and Storage

Traditional processing of cranberries to make cranberry juice cocktail (CJC) is outlined in Figure 16. Briefly, cranberries are harvested, cleaned and stored frozen. Frozen storage and ice crystal formation dramatically increases pressing yields and anthocyanin recovery.⁴² Juice extraction is mainly by mechanical means but increasingly, countercurrent extraction of cranberry juice is utilized in order to preserve

the cranberry shell. After pressing, juice is clarified using filtration and cellulase and/or pectinase enzymes. Then, juice is stored under refrigerated temperatures for use within ~7 days. Alternatively, juice may be concentrated and stored frozen for up to ~2 years.

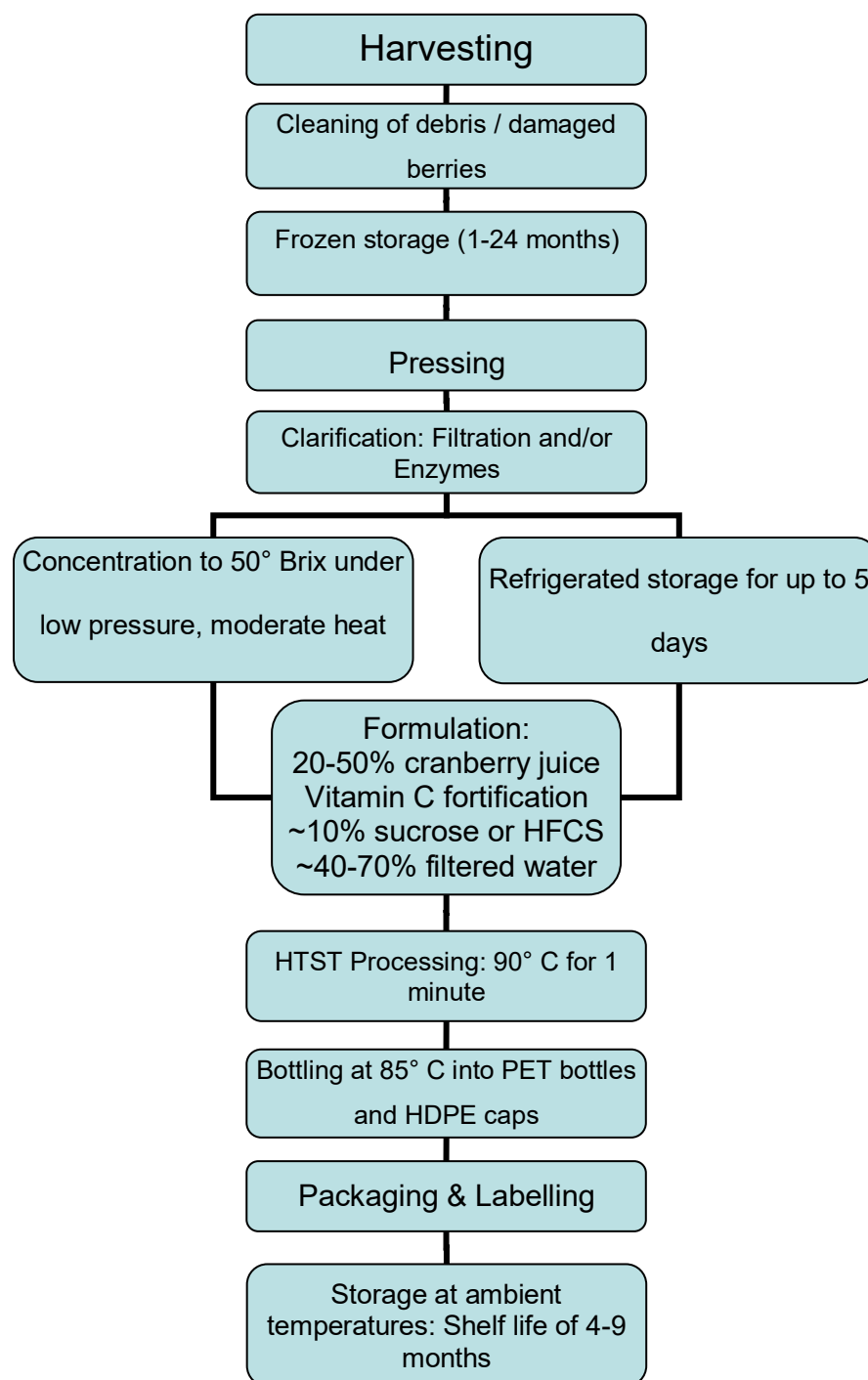


Figure 16: Diagram outlining steps in cranberry juice cocktail processing

Cranberry juice cocktail is then formulated from the fresh juice and/or juice concentrate. Blending of cranberry juices is commonly practiced to produce consistent color and acidity. Traditionally, 27% cranberry juice is present in the final product, with ~12% sugar added to balance the natural tartness of cranberry. Variations on the traditional form of cranberry juice cocktail exist, with percent cranberry juice ranging from 5-50%, use of non-nutritive sweeteners for 'light' and 'diet' products, or the use of other fruit juices in place of added sugar; however, 27% juice with sugar or high fructose corn syrup remains the industry standard.

Once formulated, the CJC is most often processed by high temperature short time (HTST) pasteurization. The United States Food and Drug Administration (FDA) mandates a strict protocol of food safety in the production of fruit juices and juice cocktails. Among the requirements, juices must be processed to achieve at least 5-log reduction in the most resistant pathogen that might be present in the juice. In practice, for shelf-stable juice manufacturers use processing parameters that provide a much higher level of pathogen reduction (100+ log reduction of *E. coli*) to provide so-called 'commercial sterility' of the more resilient spoilage microorganisms such as yeasts and molds. Generally, HTST processing in commercial production of CJC uses continuous heat exchangers to rapidly heat the juice to ~90°C and the juice is generally held at high temperature for at least 15 seconds before filling into bottles at ~85°C. This 'hot-fill' process ensures adequate inactivation of any microbes present in the packaging. Bottles are then cooled via sprayed water in a tunnel cooler.

Alternatively, some cranberry juice is aseptically packed using HTST or ultra high temperature (UHT). For reasons not well understood, the color stability of aseptically packed CJC is generally quite poor compared to hot filled CJC. However, UHT-processed cranberry represents a small fraction of the total cranberry juice cocktail market and will not be discussed here in detail.

Standard packaging of CJC (as well as most shelf stable juices in the United States) consists of polyethylene terephthalate (PET) bottles and high density polyethylene caps (HDPE). Over the past 20 years, such plastic packaging has replaced glass due its lighter weight, lower cost, durability and increased safety (broken glass is an occupational and consumer hazard). PET is the preferred plastic polymer for hot-filled beverages applications because of its excellent clarity, good heat stability, relatively low oxygen permeability and relatively low cost. As mentioned above, plastics are oxygen permeable. According to one recent study, clear PET bottles of 300 μm thickness had oxygen transmission rates of 15.4 mL (~ 0.7 millimoles)/ m^2 per day at 1 atmosphere and 25°C.¹⁶² PET bottles used for hot fill processes have similar, but slightly higher thickness of 400-500 μm ¹⁶³ so presumably have slightly lower oxygen permeability. As hot fill plastic packaging and bottling technology has evolved, bottles have become lighter and thinner to reduce costs. If this trend continues, increased rates of oxygen permeability will present ever greater challenges to bottlers.

Storage of CJC is almost always at ambient temperature; even if sold refrigerated, the majority of storage time is in ambient warehouses. CJC has a shelf life of 4 to 9 months at ambient temperatures, depending on several factors and limited by color deterioration (Figure 17). Many other juices have a shelf life of 12-24 months, so

extending cranberry juice shelf life is a goal of the industry. To achieve 9 months shelf life, CJC processors often add colorants including artificial color (FD&C Red #40) or natural coloring from other fruits and/or vegetables. Premium CJC formulations with high percent juice and high initial anthocyanins content may have 9 months shelf stability without added color if bottled in large containers. These larger containers provide for longer shelf life presumably because they limit oxygen ingress due to a lower bottle surface area to volume ratio. For CJC in smaller, conventional PET containers (8-32 oz.) and with no added colorants, CJC generally has a shelf life of 6 months or less.

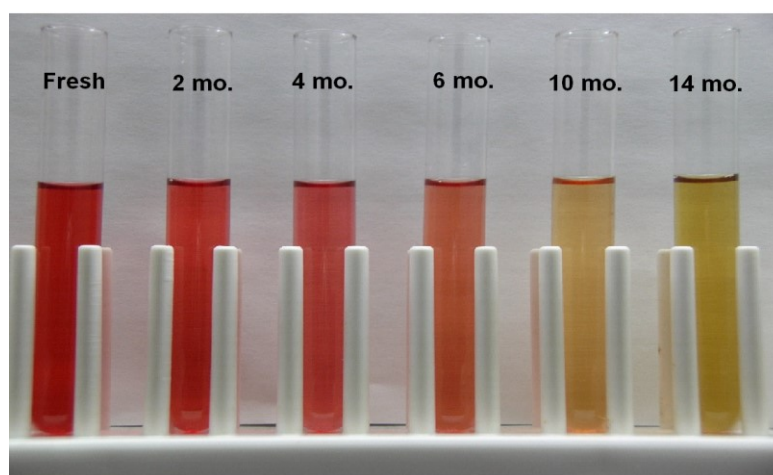


Figure 17: Color changes in cranberry juice cocktail during storage in 64 oz. PET bottles

2.5.1 Traditional Storage and Packaging Effects on Retention of CJC Phenolics and Ascorbic Acid

Several studies have directly investigated the effects of traditional heat treatments and storage on phytochemicals in CJC directly.^{18,127,142,164,165} As noted above, color degradation limits the shelf life of CJC. Consequently, most of these studies have focused

on anthocyanin retention in attempts to prolong CJC shelf life. Few published studies have considered the effects of high heat treatments or periods of storage on other cranberry phenolics that are increasingly of interest due to their potential health promoting qualities.

Losses of vitamin C during HTST processing are about 25% in CJC.¹⁸ Ascorbic acid losses during storage vary depending on the headspace, container, exposure to light and storage conditions. 20-40% Vitamin C losses were observed after 9 weeks storage of CJC in glass bottles, depending on the amount of bottle headspace.¹⁸ Higher losses are observed in industry where plastic, oxygen-permeable bottles are used. Up to 100% overages of vitamin C are added to CJC prior to processing to meet labeling claims at the end of shelf life.

High heat treatments degrade anthocyanins in CJC. One study found ~15% losses of total anthocyanins compared to untreated, refrigerated controls after heating to 90°C for 90 seconds followed by rapid cooling.¹⁶⁴ Anthocyanins degraded 35-55% over 12 weeks of storage in glass bottles, depending on the presence of ascorbic acid and metal ions.¹²⁷ About 5 mg/L anthocyanins were found in the 6 month old sample in Figure 17 which was at the end of its shelf life, as measured by the pH differential method (unpublished data). Some studies have found increases in anthocyanin content after processing of cranberry juice.¹⁴² Here, a plausible explanation is that anthocyanins may have been bound to cell wall structures or trapped in membranes in the raw juice making them unavailable for detection; the heating process then released such anthocyanins allowing for their detection.

Heat treatments seem to affect flavonols less than anthocyanins. Baking and sautéing onions significantly increased amounts of total measurable flavonols.¹⁶⁶ Pasteurization led to ~15% loss of quercetin in blueberry extract and only 1-7% losses of total flavonols in pear juice, though neither result was shown to be statistically significant.^{141,167} In another study, pasteurization did not significantly change total flavonol levels in cranberry juice.¹⁶⁵

As discussed in Section 2.4.3, little research has documented stability of cranberry PACs during processing and storage. One study showed PACs were more stable than ACYs in cranberry juice during pasteurization and storage.¹⁴² Another study found that total PAC oligomers were not significantly affected by pasteurization but PAC polymers (DP>10) were decreased by 0-30%, depending on pretreatments including blanching and grinding of berries prior to juice extraction.¹⁶⁵

Taken together, these data indicate that anthocyanins and ascorbic acid are least stable, proanthocyanidins have intermediate stability and flavonols are most resilient. However, we have found no targeted studies of the stability of flavonoids and ascorbic acid in CJC in modern, practical packaging yet published.

2.5.2 Emerging Processing and Packaging

Over the past few decades, several new pasteurization technologies have been developed, studied, and compared to traditional thermal treatments. Among these, pulsed electric field (PEF) pasteurization, high hydrostatic pressure (HHP) pasteurization and ohmic heating have shown promise for beverage application. Other emerging processing technologies include gamma irradiation, ultraviolet light irradiation, ozone treatments, radio frequency heating and microwave heating. Few of these have been studied in

cranberry juice and none have yet proven that any of these new processes provide the combination of efficacy and practicality needed for use in industry.

PEF processing of cranberry juice has been evaluated.¹⁶⁴ Here, only the most intense PEF treatment (40kV/cm for 150 μ s) and traditional thermal treatment provided 5-log microbial reduction but improvement in color quality and anthocyanin retention were observed in PEF treated samples over thermally treated samples. Negligible anthocyanin losses were observed in PEF processed cranberry juice, while thermally processed juice lost approximately 15% of total anthocyanins, though results were not statistically significant. While promising in showing greater retention of phytochemicals, PEF treatment of CJC would require more study to assess its commercial viability, especially how to achieve the higher levels of microbial reduction necessary to commercially sterilize juice with high loads of spoilage microbes.

HPP was recently evaluated as a means to process whole cranberry juice.¹⁴² Here, HPP treatments increased levels of detected anthocyanins, as did thermally treated samples, which may be attributed to incomplete clarification and release of anthocyanins from cells and particles during processing. Over 4 weeks storage, anthocyanins degraded very similarly in HPP treated and thermally treated samples. PACs were marginally better preserved after HPP treatment compared to thermal treatment, with 11% greater concentrations of PACs after 30 days storage at 37°C.¹⁴² Still, the research concluded that HPP treatment did not offer a significant overall benefit compared to conventional thermal processing.

Emerging packaging has shown perhaps more promise than nonconventional processing techniques for improving CJC quality, phytochemical retention and shelf life.

PET packaging dominates the shelf stable juice market, having replaced glass gradually over the past 25 years. In hot fill juice applications, plastic packaging has drawbacks such as oxygen and UV light permeability. However, recent advances in PET bottle technology have begun to address these problems. Of particular interest to CJC application are inclusions of oxygen scavenging materials and functional polymer barrier layers ‘sandwiched’ between layers of conventional PET (Figure 18). Advances in closures are also intriguing for CJC applications. The impact that these advances in packaging technology may offer in CJC applications has not been evaluated, but some of the more promising developments will be discussed briefly. Such functional bottles and caps are now widely available. Indeed, other oxygen sensitive beverages have utilized these new plastic packaging technologies with limited commercial success, most notably for beer applications.

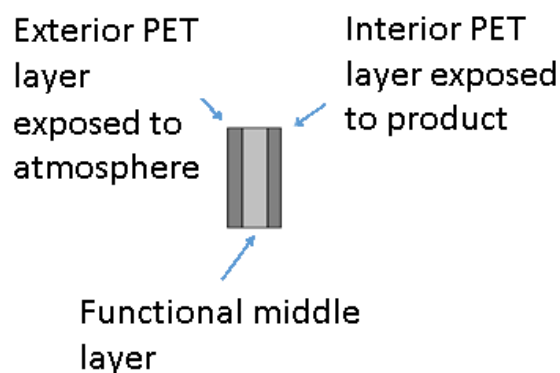


Figure 18: A representation of a cross section of a tri-layer bottle with functional layer sandwiched between two layers of conventional PET.

Many packaging innovations involve the use of oxygen scavenging and numerous patents have been granted for oxygen barrier/scavenging material incorporated into the

PET resin of a monolayer bottle,¹⁶⁸ the PET resin of the middle layer of a tri-layer bottle sandwiched between conventional PET layers,¹⁶⁹ and the linings of bottle caps.¹⁷⁰ The oxygen scavenging materials used most commonly include a transition metal catalyst copolymerized to the primary polymer. Examples of commercially available oxygen scavenging systems include OXBAR (United Closures) and Amosorb™ (BP Chemicals), both of which utilize a cobalt salt mechanism to scavenge oxygen. Indeed, one study convincingly demonstrated that monolayer PET bottles with differing levels of oxygen scavenging material (Amosorb™) reduced oxygen ingress, AscA degradation and nonenzymatic browning products in a model beverage. Here, AscA degraded by 42% over 16 weeks of storage at 35°C in the model beverage in conventional PET bottles and only 20% in bottles with 3% oxygen scavenging material ($p < 0.05$). Brown color development (Abs at 420 nm) increased 911% in controls but only 533% in the active packaging. Oxygen permeation was reduced greatly, but not entirely; oxygen transmission rates were measured at 0.021 and 0.003 cm³/(package · day) for the PET and PET with 3% oxygen scavengers bottles respectively. The authors concluded that the permeation through the closure was negligible, though provided no supporting evidence.¹⁷¹ Nonetheless, these results demonstrate the promise of active packaging in improving quality and the retention of beneficial chemical components in beverages.

There are limitations and challenges of the use of oxygen scavenging materials in beverage packaging, however. Most crucially, available oxygen scavenging materials become ‘spent’ as they absorb oxygen, leading to gradual loss of their effectiveness over time. Therefore, these materials need to be used very shortly after their production, presenting a logistical hurdle. Further, they may not be suitable for applications with long

shelf lives. This is a problem particularly in monolayer bottles and in cap liners as the oxygen scavenging layer is directly exposed to atmospheric oxygen. Also, such packaging is expensive relative to conventional packaging.

Other innovations include the use of oxygen barrier technology. Certain plastic resins, particularly ethylene vinyl alcohol (EvOH), have much lower oxygen permeability than PET. While these resins are not practical for use in monolayer bottles for various reasons (particularly heat stability and cost), they can be used as the center layer of a tri-layer bottle, sandwiched between conventional PET layers. In bottle caps, oxygen barrier technology generally consists of modified cap linings that provide a secondary seal. Some such caps include ‘oxygen barrier’ plastic resins such as EvOH in these linings.

2.5.3. Research Gaps and Opportunities

Decades of research has focused upon anthocyanins and their relationship to cranberry color. As public and scientific focus has shifted to the health benefits that phenolics compounds impart, similar research is now necessary to determine how storage and processing parameter affects other flavonoid and phenolic components. In cranberries, the components most studied for their health effects are proanthocyanidins and flavonols, along with anthocyanins. Is oxygen exposure as detrimental to these components as it is to anthocyanins?

Limiting oxygen exposure to beverages is not a novel concept. Beverage manufacturers have used various means reduce oxygen, most often purging bottles with nitrogen before filling. In aseptic juice bottling, it is fairly common practice to deaerate processed juices before or after pasteurization and cooling in order to prevent oxidation. However, limiting oxygen exposure prior to hot fill processing of CJC and other

cranberry beverages has not yet been explored (at least not reported in the published literature).

Bottles designed to limit oxygen provide the possibility of containing beverages in plastic packaging in an anoxic (or nearly anoxic) environment for some or all of the shelf life of the beverage. Previous research has evaluated the effect of limiting dissolved oxygen in CJC or in model systems through controlling headspace. However, no studies have evaluated whether oxygen can be limited or eliminated from CJC in practical modern packaging. Furthermore, until now, no published research has targeted the relationship between dissolved oxygen content and the retention of phytochemical components of CJC over processing and periods of storage in modern, practical packaging.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

Dissolved oxygen is involved in most reaction pathways resulting in the degradation of pigments and nutraceuticals in cranberry juice cocktail. Therefore, limiting dissolved oxygen via practical processing and packaging changes should extend the period over which the color and nutraceutical content of cranberry juice can be stabilized.

3.2. Objectives

1. Develop robust analytical procedures to isolate, separate, identify, and quantify anthocyanins, flavonols, and proanthocyanidins in cranberry juice.
2. Test the concept that oxygen is a main controlling factor in degradation of the test compounds using cranberry juice cocktail.
3. Determine magnitude of effects of oxygen, light, heat, and ascorbic acid on degradation rates of anthocyanins, flavonols, and PACs in CJC under laboratory conditions.
4. Use model systems of cranberry juice components to identify flavonoid degradation products and potential degradation pathways formed under conditions tested.
5. Determine magnitude of interactions of dissolved oxygen with formulation, processing and storage variables affecting color, individual anthocyanins, individual flavonols, total vitamin C, total proanthocyanidins and total phenolics in cranberry juice cocktail on a laboratory scale.
6. Use this information to develop and evaluate practical processing and storage approaches for stabilizing color, nutrients and nutraceuticals in cranberry juice cocktail.

4. MATERIALS AND METHODOLOGY

4.1 Overview of Study Design and Flow

This study is divided into five phases, starting from methods development and progressing to CJC in model systems then actual cranberry juice cocktail with test processing and packaging (Figure 19).

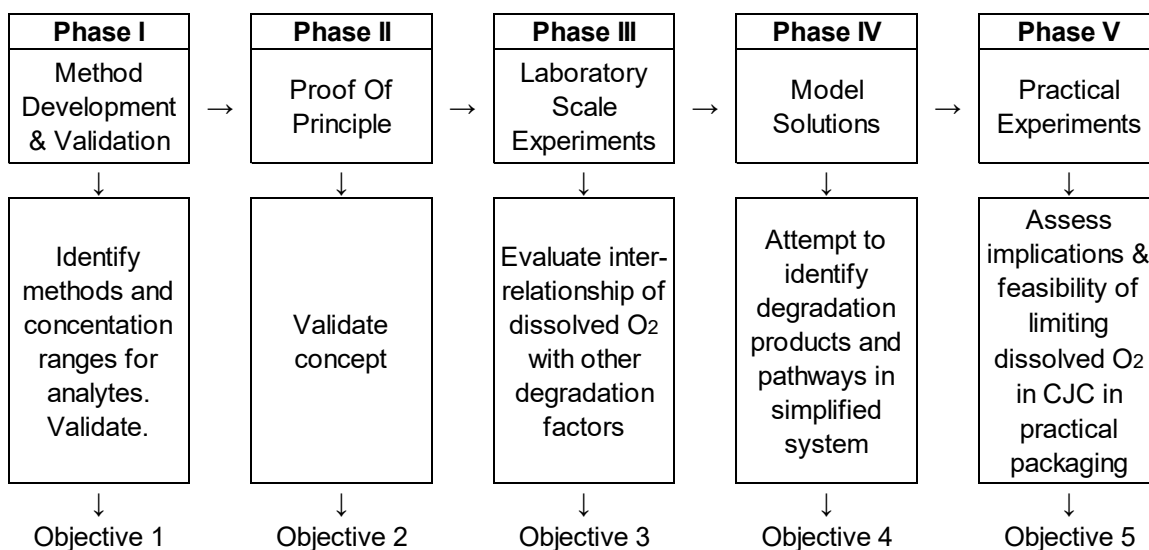


Figure 19: Overview of research project to progressively test and evaluate factors contributing to degradation of anthocyanins, flavonols, and PACs in cranberry juice.

Phase I: Method Development and Validation

Methods were developed and validated for routine and robust isolation and quantitation of compounds of interest in a cranberry juice cocktail matrix, including:

Solid phase extraction (SPE) of CJC to concentrate and isolate anthocyanins and flavonols for chromatographic analysis

Quantification of major cranberry anthocyanins by HPLC-PDA (photodiode array) with identification of analytes by HPLC-MS (mass spectrometry)

Quantification of major cranberry flavonols by HPLC-PDA with identification of analytes by HPLC-MS

Phase II: Proof of Principle Experiments

In simple experiments, we evaluated how removing dissolved oxygen from cranberry juice cocktail affects the retention of anthocyanins and color in cranberry juice cocktail following:

High heat treatment

Storage with exposure to light

Storage protected from light

Phase III: Laboratory Multivariable Analysis of CJC

Under laboratory conditions (in sealed glass bottles contained within a desiccator to closely control atmosphere), we evaluated the interrelationship of dissolved oxygen with multiple variables that affect retention of phenolics, flavonoids and total vitamin C in cranberry juice cocktail over 6 week periods at ambient temperature. A flow diagram of this study is shown in Figure 20. All combinations of 4 variables were analyzed resulting in 16 variable combinations (Table 2).

Table 2: Group legend for Phase III experiments showing the various treatment combinations applied.

Group	Ascorbic Acid Fortification	Preprocessing Treatment	Storage Atmosphere	Light Exposure
1	500 mg/L	Aerated	Protected	Protected
2	500 mg/L	Aerated	Protected	Exposed
3	500 mg/L	Aerated	Exposed	Protected
4	500 mg/L	Aerated	Exposed	Exposed
5	500 mg/L	Degassed	Protected	Protected
6	500 mg/L	Degassed	Protected	Exposed
7	500 mg/L	Degassed	Exposed	Protected
8	500 mg/L	Degassed	Exposed	Exposed
9	0	Aerated	Protected	Protected
10	0	Aerated	Protected	Exposed
11	0	Aerated	Exposed	Protected
12	0	Aerated	Exposed	Exposed
13	0	Degassed	Protected	Protected
14	0	Degassed	Protected	Exposed
15	0	Degassed	Exposed	Protected
16	0	Degassed	Exposed	Exposed

Parameters measured before and after processing and weekly thereafter were:

Individual anthocyanins, flavonols, and total proanthocyanidins

Total phenolics by Folin-Ciocalteu method

Total Vitamin C

Dissolved Oxygen

Color

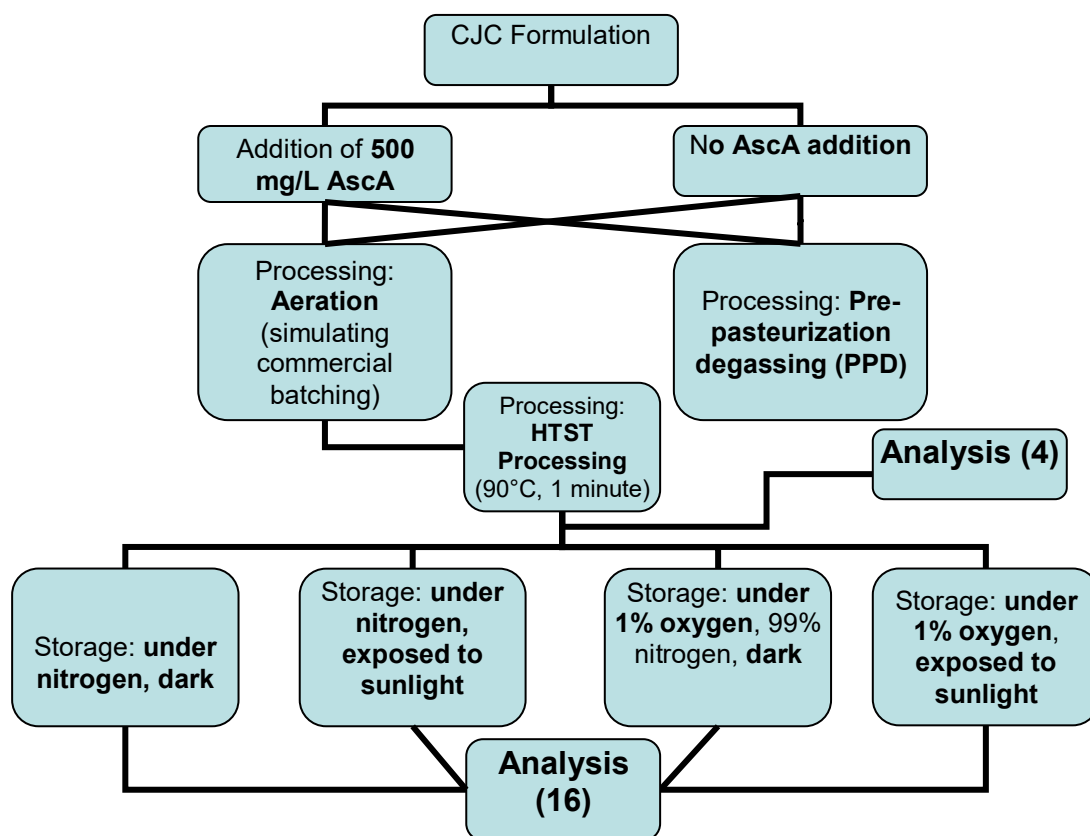


Figure 20: Flowchart outline for Phase III experiments. Numbers represent the number of treatment combinations analyzed at each step.

Phase IV: Identification of Primary Degradation Products & Pathways

Model solutions (mock cranberry juice cocktail) composed of purified chemicals were incubated for 4 weeks under the same laboratory conditions used in Phase III, then degradation products of flavonoids were identified using LC-MS. A flow chart for this process is presented in Figure 21. The model solutions consisted of:

25 mM Citrate buffer at pH 2.7

Anthocyanin: Cyanidin-3-glucoside (15 mg/L)

Flavonol: Quercetin-3-galactoside (50 mg/L)

Proanthocyanidin: PAC A2 (10 mg/L)

Ascorbic acid (500 mg/L) in some groups

Phase V: Determine effects of oxygen limitation by degassing and oxygen barrier packaging on CJC degradation under simulated real world conditions

In the final phase of the research, cranberry juice was evaluated under conditions simulating the real world. Juice was bottled into 10 ounce PET bottles with and without an oxygen barrier layer. Other subtle differences between here and Phase III include longer storage duration (3 months), turbulent mixing to approximate industrial batching (rather than aeration) and the use of ground water rather than high purity water in making CJC. A flow diagram of this study is shown in Figure 21. Variables were similar to Phase III and are shown in Table 3.

Parameters measured before and after processing and monthly thereafter were:

Individual anthocyanins and flavonols by HPLC-PDA

Total proanthocyanidins by DMAC (dimethylaminocinnamaldehyde) method

Total phenolics by Folin-Ciocalteu method

Total Vitamin C

Dissolved Oxygen

Color

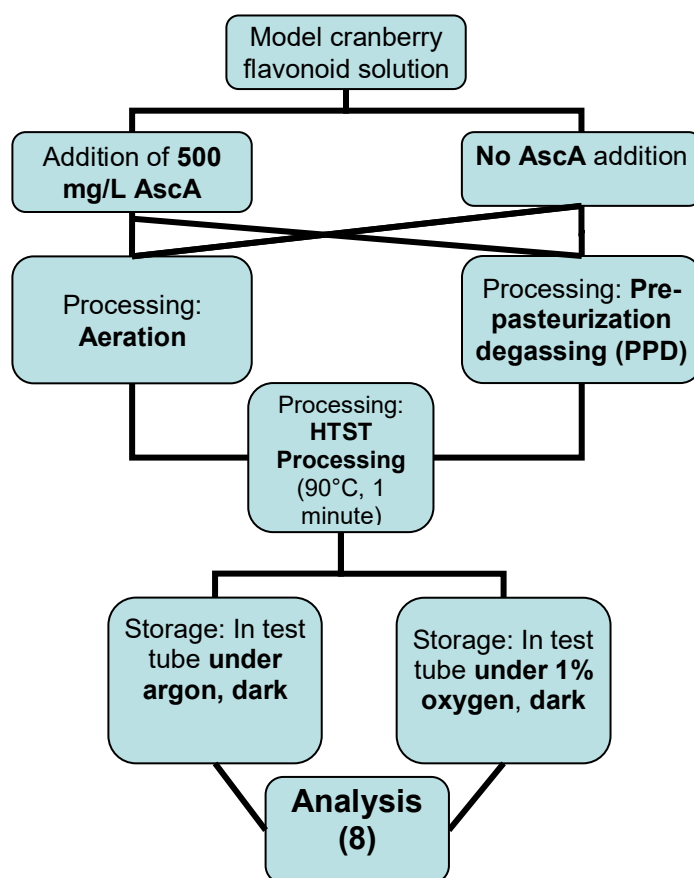


Figure 21: Flowchart outline for Phase IV experiments. Numbers represent the number of treatment combinations analyzed by HPLC-MS.

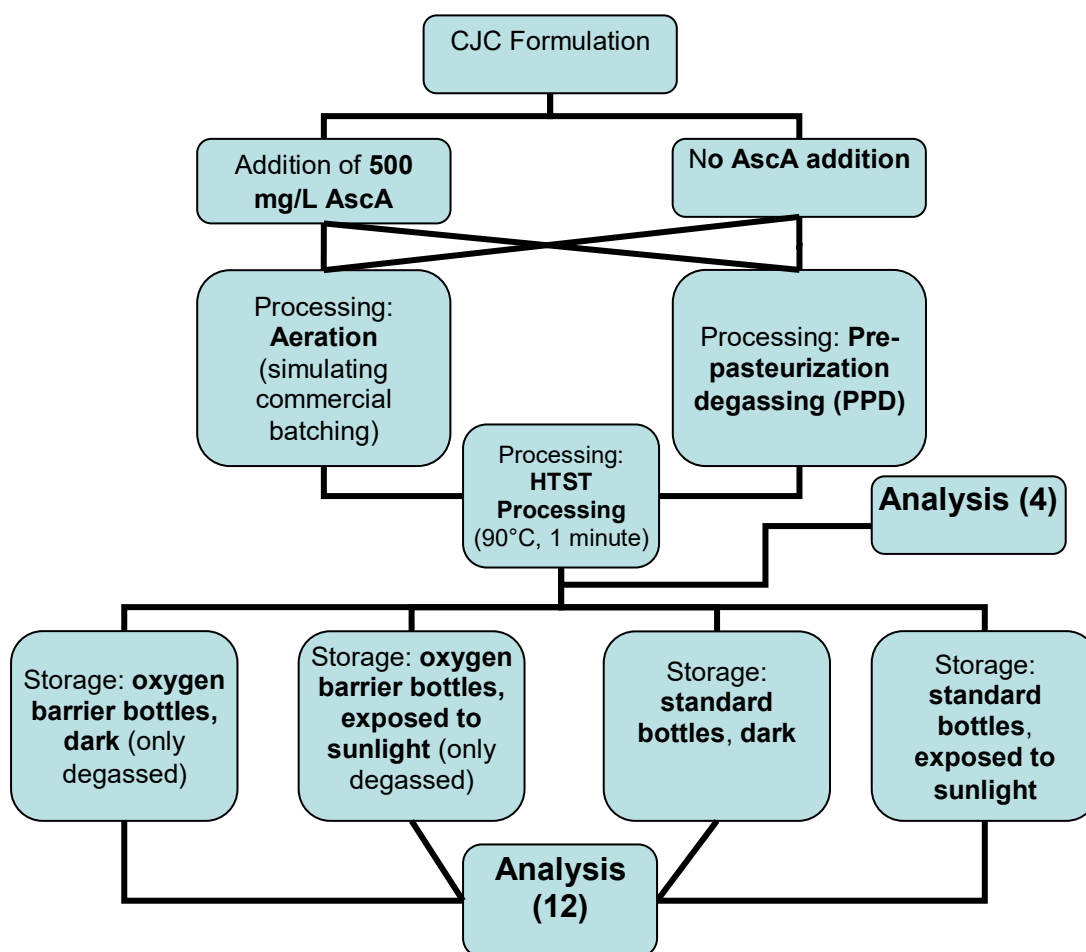


Figure 22: Flowchart outline for Phase V experiments. Numbers represent the number of treatment combinations analyzed for flavonoids, AscA, total phenolics and color.

Table 3: Group legend for Phase III experiments showing the various treatment combinations applied.

Group	Ascorbic Acid Fortification	Preprocessing Treatment	Bottle Type	Light Exposure
1D	0	Degassed	Barrier	Protected
1L	0	Degassed	Barrier	Exposed
2D	500 mg/L	Degassed	Barrier	Protected
2L	500 mg/L	Degassed	Barrier	Exposed
3SD	0	None	Standard	Protected
3SL	0	None	Standard	Exposed
3BD	0	None	Barrier	Protected
3BL	0	None	Barrier	Exposed
4SD	500 mg/L	None	Standard	Protected
4SL	500 mg/L	None	Standard	Exposed
4BD	500 mg/L	None	Barrier	Protected
4BL	500 mg/L	None	Barrier	Exposed

4.2 Materials

4.2.1 Cranberry Juice Cocktail Components

Clarified Cranberry Juice (CCJ): Freshly cold-pressed and clarified cranberry juice was donated by Clement Pappas & Co. (Carneys Point, NJ). The juice was obtained from mixed varieties of cranberries (*Vaccinium Macrocarpon Ait.*) including Ben Lear, Stevens and Early Black cultivars from various growing regions (Massachusetts, Wisconsin, and Quebec). This juice ranged from 7.4-8.2° Brix with 1.8-2.2% titratable acidity. The juice was frozen ($-5\pm 3^{\circ}\text{C}$) immediately after clarification until used for making CJC.

Granulated sucrose: Food grade granulated cane sugar was donated by Clement Pappas & Co.

Ascorbic acid: Food grade synthetic ascorbic acid (USP grade) was donated by Clement Pappas & Co.

4.2.2 Reagents, Gases and Analytical Standards

Solvents/Reagents: Acetonitrile (ACN), methanol (MeOH) of HPLC grade, glacial acetic acid, formic acid (>90%) and ethanol (99%) as well as reagent grade potassium chloride, Folin reagent, sodium carbonate, gallic acid, 4-dimethylaminocinnamaldehyde (DMAC), concentrated hydrochloric acid, dimethylsulfoxide (DMSO) and ascorbic acid (>99%) were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA).

Analytical standards: Cyanidin-3-glucoside, quercetin-3-galactoside and proanthocyanidin A2 of analytical grade (>99%) were obtained from Indofine Chemicals, NJ. Quercetin of analytical grade (>99%) and ascorbic acid of reagent grade was obtained from Sigma-Aldrich (Saint Louis, MO).

Gases: N₂ gas (Food Grade), 99% Nitrogen and 1% O₂ gas mixture, Helium gas (99.999%) and Argon gas (99.999%) were obtained from Airgas (Piscataway, NJ)

10 ounce plastic bottles were supplied anonymously by a bottle manufacturer. Standard bottles were made from polyethylene terephthalate (PET) while barrier bottles contained a ~2% ethylene vinyl (EvOH) layer sandwiched between PET layers. Total wall thickness and dimensions of the two bottle types were identical.

All reagents were prepared with high purity **water (18 MΩ resistivity)** from a Milli-Q four cartridge water purification system in which two of the cartridges were ion exchange (Millipore, Bellerica, MA).

4.3 Procedures

4.3.1 Phase II – Proof of Principle

CJC was prepared by combining 270 mL clarified cranberry juice with 120 grams of sucrose and 350 mg of AscA in a 1000 mL volumetric flask then bringing the mixture to volume with 18 MΩ water.

Effect of degassing on anthocyanins during heat treatment: For the degassed sample, a 100 mL aliquot of CJC was placed in 250 mL flask and purged with argon for ~5 minutes. The flask was stirred under vacuum for ~15 minutes and the vacuum was broken with argon gas. Another 50 mL aliquot of the untreated CJC was purged with air to simulate the turbulent mixing processes of commercial juice batching. Informal trials had shown that a heat block set to 105 °C would heat a test tube filled with 5 mL of water to approximately 90°C in 10 minutes. Thus, 5 mL aliquots (in triplicate) of both the degassed and aerated CJC were treated in this fashion followed by cooling for 1 minute in a warm water bath and 1 minute in a cold water bath. All samples were then analyzed for total monomeric anthocyanins (TMA) by the method described in Section 4.4.3.

Effect of storage atmosphere under low stress conditions (refrigerated and protected from light): From another 100 mL aliquot of the CJC, 20 mL were removed and immediately analyzed for TMA. A 40 mL aliquot was placed in a round bottom flask and aerated by purging with air for 5 minutes. The remaining 40 mL was placed in a

round bottom flask and degassed in the same fashion as the above experiment, with vacuum broken by argon gas. Each sample was then stoppered and refrigerated for 7 days. At the end of the seven days, each sample was analyzed for TMA.

Effect of storage atmosphere at higher stress conditions (exposed to light at ambient temperature): 100 mL of CJC were degassed in the same fashion as in the first proof of principle experiment. Here, the entire 100 mL aliquot in a round bottom flask was heated rapidly to 90 °C in a heating mantle heater while under a blanket of argon gas. The flask was cooled for 5 minutes in a warm water bath followed by 5 minutes in a cold water bath. 20 mL of the treated CJC was removed for TMA analysis. 40 mL of the treated CJC was placed in a separate round bottom flask, blanketed with air for 20 minutes, and then stoppered. The remaining 40 mL in the original flask was purged with argon for 5 minutes and stoppered. Both samples were placed on a lab bench next to a window for maximal sunlight light exposure. On the seventh day after heat treatment, the samples were analyzed for TMA.

4.3.2 Phase III – Laboratory Scale Experiments

Raw cranberry juice (5 L) was collected immediately after commercial pressing and clarification. CJC was prepared by mixing 2.70 L of the raw juice with 1.20 kg of sucrose and bringing to 10 L volume with 18 MΩ water. This batch was split into two 5 L portions. 5 g ascorbic acid was added to one portion. 200 mL aliquots were removed from each portion for analysis and then immediately frozen for later analysis.

Each 4.8 L of CJC was split into 6 parts of 0.8 L each to provide triplicates for degassed and aerated groups. For the degassing treatment, CJC was placed in a flask and

purging with argon and vacuum (~200 mbar) was applied for ~5 minutes each. This degassing procedure was repeated three times before heat treatment. For aerated groups, CJC was purged with purified air for ~2 minutes.

Each aliquot was processed separately on a stove top. Degassed groups were blanketed with argon. After heating to 90°C, a 50 mL aliquot was taken for immediate analysis and the remainder of juice was split equally into 4 acid-washed 500 mL glass bottles (about 175 mL of CJC in each) for incubation. Care was taken to not introduce air into the headspace during cooling by purging bottles with argon gas prior to filling and capping them promptly. The bottles were placed in a warm water bath for ~5 minutes followed by a cold water bath for ~5 minutes.

These CJC samples were then subjected to combinations of protection/exposure to light and storage atmosphere. For protection from light, bottles were wrapped with aluminum foil. For storage atmosphere, bottles were purged with 1% oxygen in either nitrogen or argon for ~2 minutes, sealed and then placed in a desiccator. The atmosphere within the desiccator was controlled by applying a vacuum and breaking it with the intended atmosphere, either 1% oxygen or argon. Each bottle was analyzed at weekly intervals for 6 weeks as described below. First, dissolved oxygen readings were taken and then 25 mL aliquots were removed from each bottle and analyzed for anthocyanins, flavonols, PACs, phenolics, vitamin C and color as described in **Section 4.4**. Each bottle was then purged for ~5 minutes with either 1% oxygen or argon, as appropriate and placed back in the appropriate desiccator until the next analysis time point.

4.3.3 Phase IV – Model Solutions

Model solutions were prepared by mixing 60 mg/L quercetin-3-galactoside, 40 mg/L PAC A2 and 30 mg/L cyandin-3-glucoside in 25mM sodium citrate buffer, pH 3.0. Solutions were either degassed or aerated prior to processing, with and without 500 mg/L ascorbic acid added. Degassing was accomplished by sparging with argon for 2 minutes followed by applying a 10 mbar vacuum for 2 minutes and repeating the cycle five times. Heat treatments were applied on a hot plate, bringing juice to 85 °C followed by cooling in warm water for 5 minutes and cool water for 5 minutes. Samples to be stored in anoxic environment were again degassed after cooling. Samples were then placed in chambers of an OxipresTM oxygen bomb (Mikrolab, Aarhus, Denmark) under either argon or 1% oxygen in either nitrogen under 1.2 bar pressure at 25°C. Headspace gas was purged weekly with the appropriate storage atmosphere. After 1 month, samples were extracted and analyzed by HPLC-MS as per **Section 4.4.9**.

4.3.4 Phase V – Practical Experiments

CJC was formulated according to the same proportions as in Phase III (Section 4.3.2) except using ground water and a separate batch of cranberry juice. Again, 500 mg/L ascorbic acid was added to appropriate groups. In non-degassed groups, juice was turbulently mixed to approximate industrial batching but no aeration treatment was performed. Degassing was accomplished by applying a 100 mbar vacuum for 5 minutes, followed by sparging with helium for 3 minutes. This process was repeated five times and a final, brief vacuum was applied, broken by argon gas. Batches were heated to 195°F on a hot plate, with degassed groups blanketed by argon. Each set of conditions

was repeated three times. Bottles were purged with argon prior to bottling and were cooled in a cool running water bath after filling. All bottles were stored within the same room with those protected from light sealed within cardboard boxes and those exposed to light were placed near a window. At monthly intervals for 3 months, 3 bottles from each set of conditions were analyzed for dissolved oxygen, anthocyanins, flavonols, PACs, phenolics, vitamin C and color as described in Section 4.4.

4.4 Methodology

4.4.1 Colorimetry

For Phase III, a D-25-9000 Hunter colorimeter (Reston, VA) was used to measure color parameters L^* (lightness), a^* (green – redness) and b^* (blue – yellowness). In Phase V, a Kinoca Minolta CP-410 handheld colorimeter was used to obtain the same color parameters. For the latter, 50 mL of sample was added to a #3140 80x40 mm Corning pyrex dish over white paper. The reading was taking after placing the handheld colorimeter so it sat flush on the bottom of the dish, partially submerged in the sample. A reading taken through the dish without any sample yielded $L^* = 90.78$, $a^* = 0.68$, $b^* = 0.13$. Calibration of the Hunter instrument was performed prior to each set of analyses using Hunter supplied color calibration tiles. Calibration of the Kinoca Minolta instrument was performed prior to each day of analysis with the supplied blank white tile.

4.4.2 Dissolved Oxygen

For Phase III, a VWR (Bridgeport, NJ) Symphony SP80PD unit equipped with a dissolved oxygen probe was used to measure dissolved oxygen. For Phase V, a YSI 5300A oxygen meter was used. For the latter, $O_2\%$ (of air saturated solution) was

converted to parts per million (mg/L) O₂ using a conversion factor of 0.08224 mg/L/O₂%. Calibration was performed prior to each analysis using an anoxic solution (0.00 mg/L) and an air saturated water solution (8.224 mg/L O₂ at 25 deg C, 1 atmosphere). The anoxic solution is prepared prior to each analysis by adding 2.5 g of sodium sulfite to 250 mL of distilled water and stirring for 3 minutes.

4.4.3 Determination of Total Monomeric Anthocyanins (TMA)

In Phases I and II of this research, total monomeric anthocyanins were analyzed according to AOAC official method 2005.02, known as the pH differential method. This has been shown in collaborative studies to yield higher anthocyanin quantifications than HPLC methods.¹⁷² The mechanism of the assay relies on the pH-dependent equilibrium reactions known to exist for anthocyanins (described in **Section 2.2.2.1**) and the extinction coefficients known for the flavylium cation form of anthocyanins prevalent at low pH. Samples are diluted in both pH 1.0 and pH 4.5 buffers. Absorbance measurements are taken for each pH buffered samples at 520 nm and 700 nm.

Buffers at pH 1 (0.025 M potassium chloride) and pH 4.5 (0.40 M sodium acetate) were prepared. Cranberry juice cocktail was diluted 1:5 in each buffer and these solutions were allowed to equilibrate for at least 15 minutes but no longer than 1 hour. Absorbances of solutions at both 520 nm and 700 nm were then measured in 1 cm path length cuvettes using a Cary 50 Bio Spectrophotometer (Varian Inc, Palo Alto, CA). Total monomeric anthocyanins, expressed as cyanidin-3-glucoside equivalents, were calculated using the following equation:

$$\text{TMA (mg/mL)} = \frac{A \times \text{MW} \times \text{DF} \times 10^3}{\epsilon}$$

where $A = (Abs_{520} - Abs_{700})_{pH1} - (Abs_{520} - Abs_{700})_{pH4.5}$,

MW = Molecular weight (449.2 g/mol for cyanidin-3-glucoside),

DF = Dilution Factor (5),

ϵ = molar extinction coefficient (26900 L/(cm • mol) for cyanidin-3-glucoside).

4.4.4 Determination of Total Phenolics

Total phenolics were determined by the Folin-Ciocalteu (F-C) method as adapted from the method of Waterhouse.¹⁷³ While the molecular mechanisms of the reaction underlying this method are unclear, it is known that reaction relies on electron transfer from the phenols to the molybdotungstophosphoric heteropolyanion reagents in the Folin reagent, resulting in a color change from the reduction of molybdenum:¹⁷⁴

molybdotungstophosphoric heteropolyanion reagent + phenol \rightarrow Mo-phenol complex

Mo(VI) (yellow) + e^- \rightarrow Mo(V) (blue)

Sodium carbonate solution (1 M) was prepared in 18 M Ω water. Standard solutions of gallic acid (0, 50, 100, 250 and 500 mg/L) were prepared in 10% sucrose solution. 20 μ L of standards and samples (diluted as required to fit within the standard curve) were pipetted into a 96 well plate and the plate was inserted into a Mithras LB 940 plate reader (Berthold Technologies, Zug, Switzerland). Then, 80 μ L of 1:10 Folin reagent (diluted in water) was added to each well by the plate reader dispenser. Samples were shaken for 3 minutes by the plate reader before 100 μ L of the 1 M sodium carbonate solution was

dispensed automatically to each well. The solutions were shaken on the plate reader for 60 minutes before absorbance was read at 620 nm. Analyses were performed in triplicate.

A linear calibration curve was then prepared using the “LINEST” function of Excel. Calibration curves with $r^2 > 0.98$ were considered acceptable for quantification, expressed as gallic acid equivalents (GAE). Corrections for ascorbic acid interference were made by subtracting 0.65 mg/mL GAE for each 1 mg/mL of ascorbic acid that was present in the sample as was previously described¹⁷⁵ and informally verified.

4.4.5 Determination of Total Proanthocyanidins

This method was adapted from a recently validated method¹⁷⁶ known as the DMAC assay. 1 mg/mL solution of 4-dimethylaminocinnamaldehyde (DMAC) was prepared in a 25% concentrated HCl / 75% methanol solution. Standards of proanthocyanidin A2 were prepared at 25, 50, 75 and 100 mg/L in ethanol.

In a 96 well plate, 40 μ L of standards (in triplicate) and 20 μ L of samples were plated (yielding a final 2:1 dilution of the samples). For matrix matching, 40 μ L of 18 M Ω water was added to standard wells while 60 μ L of 2:1 ethanol:water was added to sample wells. The plate was inserted into a Mithras LB 940 plate reader and 200 μ L of the 1 mg/mL DMAC solution was dispensed automatically to each well. The mixture was shaken for 20 minutes and the absorbance was read at 620 nm.

A linear calibration curve was then prepared using the “LINEST” function of Excel. Those calibration curves with $r^2 > 0.98$ were considered acceptable for quantification, expressed as PAC A2 equivalents.

4.4.6 Determination of Ascorbic Acid

In Phase III, a plate reader method was used to determine ascorbic acid concentration, adapted from the method described by Vislisel¹⁷⁷. In this method, ascorbic acid is oxidized using tetramethyl-piperidinyloxy stable nitroxyl radical (Tempol) and subsequently reacted with o-phenylenediamine (OPDA) to form a fluorescent condensation product.

Here, 2M sodium acetate buffer was prepared and adjusted to pH 5.5 with acetic acid, then run through a 20 mm x 50 cm glass column containing ~3 g of chelating resin to remove trace metals that could interfere with the assay. Fresh ascorbic acid standards were prepared directly before each analysis at 0.1, 0.3, 0.6 and 1.0 mM in 20% MeOH with 250 μ M diethylenetriaminepentaacetic acid (DTPA). 2.32 mM solutions of Tempol and 5.5 mM OPDA were prepared in the 2 M sodium acetate buffer. 60 μ L of standards and 15 μ L samples were plated in triplicate on 96-well plates. 45 μ L of 20% MeOH with 250 μ M DTPA diluent was added to each sample well. 60 μ L of the 2.32 mM tempol solution was dispensed automatically to each cell by the plate reader, then the plate was shaken for 5 minutes. 60 μ L of the OPDA solution was dispensed automatically to each cell and the plate was shaken in the plate reader for 20 minutes at ambient temperature before fluorescence emission was recorded (355 nm excitation, 400 nm emission). The average of 10 readings was recorded in a Microsoft Excel spreadsheet. A standard curve was generated using the polynomial best fit function of Excel.

While this method provided highly correlated standard curves ($>0.985 r^2$), it proved laborious and would often not produce a fluorescent response, so an HPLC method was used in later stages of the research.

For Phase V, ascorbic acid was quantified by a simple HPLC method, adapted from the method described by Ullah et al.¹⁷⁸ Here, all glassware and vials were left in 0.1 N HCl overnight to remove metal contaminants before being rinsed with 18 M Ω water and dried before use. Fresh ascorbic acid standards of 50, 100, 250 and 500 mg/L were prepared and were analyzed within 1 hour. Cranberry juice samples were injected without treatment within 2.5 hours of opening the bottles. The HPLC system was a Shimadzu (Kyoto, Japan) AVP10 system with SPD-10AVP dual wavelength UV detector and Perkin Elmer Analyst (Version 1.4.2) software. The column used was a Reliasil C-18, 5 μ m 250 x 4.6mm and the mobile phase was 20 mM potassium phosphate buffer, pH 3.0. A flow rate of 1.0 mL/minute was used with an injection volume of 10 μ L. The ascorbic acid peak eluted at about 4.5 minutes and was detected at 280 nm (Figure 23). Total run time was 8 minutes. Peak areas were transferred to Microsoft Excel and linear calibration curves were calculated using the “LINEST” function. Acid washing of the glassware and prompt analysis of samples after opening were critical for producing acceptable ($r^2 > 0.99$) standard curves.

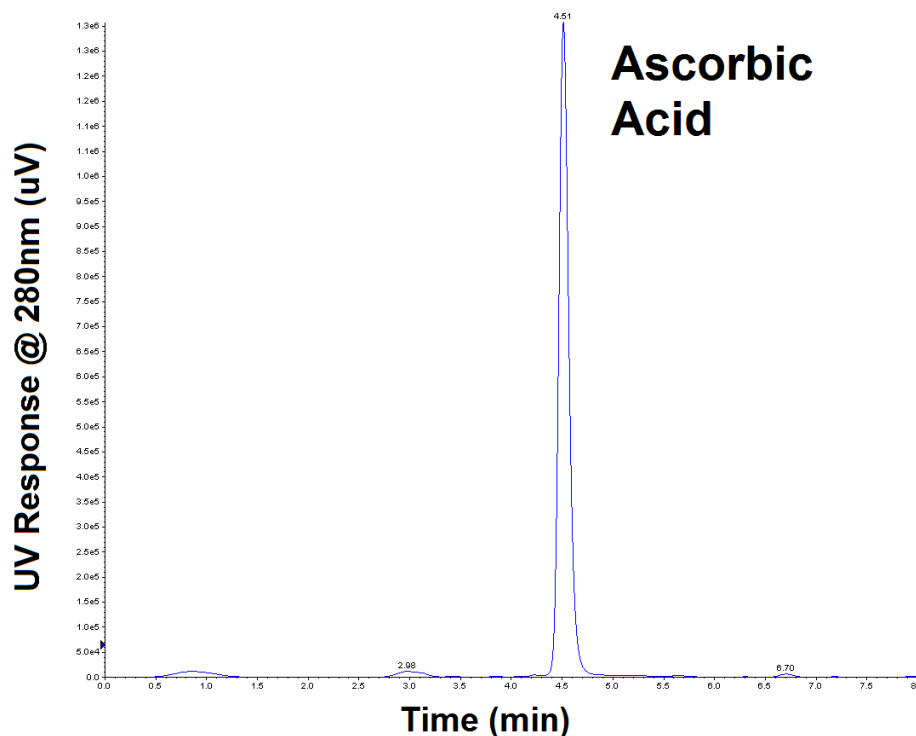


Figure 23: Sample chromatogram of the ascorbic acid HPLC method.

4.4.7 Solid Phase Extraction for HPLC Analysis

Solid phase extraction was performed to clean up CJC samples and isolate anthocyanins and flavonols for HPLC analyses. 5 mL columns packed with 2 g C18 resin (Varian Bond Elut or equivalent) were used with the following elution scheme:

2.5 mL MeOH (column wetting)

2.5 mL 0.5% formic acid (equilibration)

5 mL sample/standard (column loading) (collected as fraction #1)

2.5 mL 0.5% formic acid (collected as fraction #2)

2.5 mL 0.5% formic acid in MeOH (collected as fraction #3)

2.5 mL 0.5% formic acid in MeOH (collected as fraction #4)

10 mL MeOH (column wash for re-use)

For validation, all fractions were collected separately in each analysis. >95% of anthocyanins and flavonols were recovered in fraction #3 and #4 so these fractions were pooled for routine analysis.

4.4.8 Simultaneous Determination of Flavonols and Anthocyanins by HPLC-PDA and HPLC-MS

Flavonols and anthocyanins in solid phase extracts were analyzed and quantitated by HPLC-UV using various HPLC systems, primarily a Shimadzu AVP10 system with SPD-10AVP dual wavelength UV detector and Perkin Elmer Analyst (Version 1.4.2) software. Mobile Phase A (MPA) was 80% 18 M Ω water, 15% ACN and 5% formic acid. Mobile Phase B (MPB) was 5% 18 M Ω water, 94% methanol and 1% formic acid. 20% methanol, 80% 18 M Ω water was used as autosampler needle rinse. HPLC conditions are presented in Table 4.

Table 4: HPLC Conditions for analysis of anthocyanins and flavonols.

Injection Volume	50 μL
Flow Rate	1 mL/min
Equilibration time	10 minutes
Column	Reliasil C18, 5 μ , 250x4.6mm
Column Temperature	25° C
UV Detector wavelengths	360 nm, 520 nm
Pump Program:	
Time	%MPB
0	0
30	0
50	50
60	60
61	100
70	100

Anthocyanins eluted during the initial isocratic stage of the pump program, over ~5-25 minutes. Flavonol glycosides and aglycones eluted from ~7-60 minutes. Anthocyanins were quantified at 520 nm using 10 mg/L cyanidin-3-glucoside as a single point calibration standard. Flavonols were quantified at 366 nm using 40 mg/L quercetin-3-galactoside as a single point calibration standard.

HPLC-MS analysis was performed to confirm peak identities where possible. The same HPLC system was used, with 1/10th effluent flowing to a Sciex API 400 mass spectrometer (Perkin Elmer, Waltham, MA). Electrospray (ESI) ionization was used with standard operating parameters. Mass to charge ratios (m/z) of 250-750 at 1 Hz were monitored with default MS parameters.

4.4.9 HPLC-MS Analysis of Model Solutions

Model solutions were solvent exchanged and concentrated prior to analysis. Solid phase extract cartridges (Varian BondElut C18, 2g) were wetted with 2.5 mL MeOH and equilibrated with 2.5 mL 0.5% formic acid. 15 mL of extracts of the model solutions were passed through the cartridges, then phenolics bound to the solid support were eluted with and collected in 2.5 mL of 0.5 % formic acid in MeOH. This was repeated until 50 mL of each model solution was solvent exchanged. Extracts were dried in a Buchi R-210 rotary evaporator at 150 mbar, 35°C, and reconstituted with 2.5 mL 0.5% formic acid in MeOH, creating a 20 fold concentrated extract.

Extracts (10 μ L) were analyzed by HPLC-MS on a Thermo Surveyor HPLC system with an Ascentis C18 5 μ m, 50 x 2.1 mm column (Supelco) and detection by photodiode array detector (PDA) and Thermo LCQ mass spectrometer. The mobile phase

consisted of 50% acetonitrile in water with 0.1% formic acid delivered at a flow rate of 200 $\mu\text{L}/\text{min}$ over a run time of 20 minutes. The PDA monitored 220-600 nm. The mass spectrometer was operated in positive ion mode, using electrospray ionization, scanning masses 100-2000 at 1 Hz. A Thermo Xcaliber data system was used to acquire and process data.

4.4.10 Statistical Analysis

All analyses in Phases II, III and V were conducted in experimental triplicates, usually with a single measurement of each experimental replicate. For phases I and II, student's t-test were calculated with Microsoft Excel using the program's "Data Analysis" feature. For Phase III, analysis of variance (ANOVA) was performed via SAS version 9.3 (Cary, NC) using the "PROC ANOVA" function. For Phase V, general linear model (GLM) analysis was performed via SAS software (because this dataset is not balanced, ANOVA is not appropriate) using the "PROC GLM" function and type III sum of squares. For both Phase III and V, Duncan multiple range tests (MRT) were used to assess differences between groups where appropriate as calculated by SAS. The percent retention of compounds over storage was calculated in relation to the concentrations found directly after processing (rather than initial concentrations before processing) so as to examine only effects after heat treatments. Results with $p < 0.05$ were considered significant.

Phase IV was qualitative and no statistical analysis was performed.

5. RESULTS

5.1 Phase I: Method Development and Validation

During development of chromatographic methods, peaks were identified using a variety of methods: use of authentic analytical standards, comparison of retention order/pattern to chromatography from published literature, and mass spectrometry (Table 5). The chromatographic profile of major cranberry anthocyanins is well established in the literature^{38,39,179,180} and the HPLC method developed exhibited a typical peak pattern. HPLC separation of cranberry flavonols has been studied,²⁴ but the peak pattern is less established and was deemed unsuitable for establishing flavonol peak identities in this study. LC-MS was used to confirm peak identifies of four anthocyanins and three flavonols; m/z values for ion peaks corresponded to those available in the literature for both anthocyanins³⁹ and flavonols.²⁴ HPLC chromatograms typical of CJC anthocyanins are presented in Figure 24; Figure 25 shows flavonol chromatograms.

Table 5: Methods used for chromatographic peak identification.

Analyte	Retention Order	Authentic Standard	[M + H] ⁺ in HPLC-ESI-MS
Anthocyanins			
cyanidin-3-galactoside	X		449
cyanidin-3-glucoside	X	X	
cyanidin-3-arabinoside	X		419
peonidin-3-galactoside	X		463
peonidin -3-glucoside	X		
peonidin –arabinoside	X		433
Flavonols			
myrecetin-3-galactoside	X		481
quercetin-3-galactoside	X	X	465
quercetin-3-rhamnoside	X	X	449
Myricetin		X	
Quercetin		X	

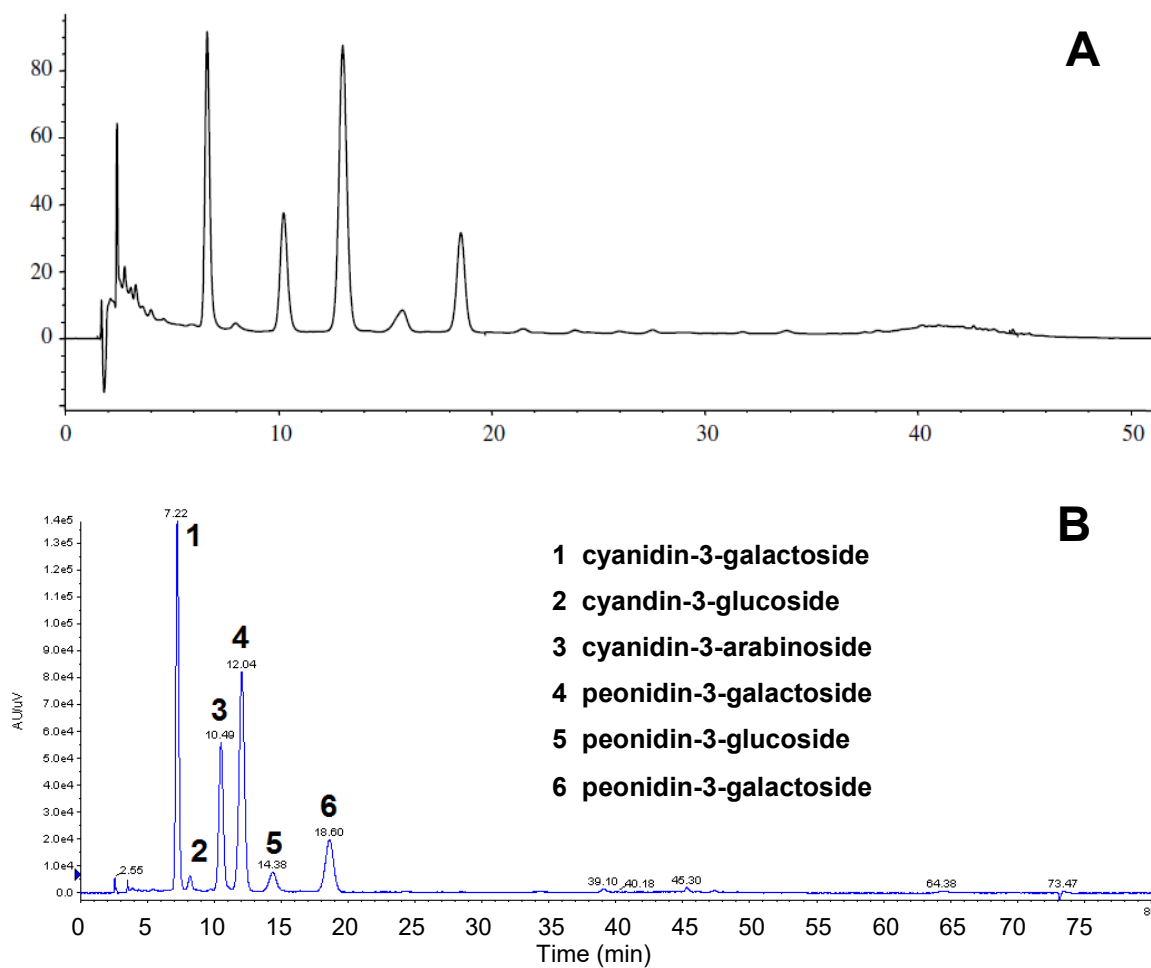


Figure 24: Typical cranberry anthocyanin chromatogram from the literature (A),¹⁷⁹ typical chromatograms of anthocyanins using the method described here detected by UV at 520 nm (B).

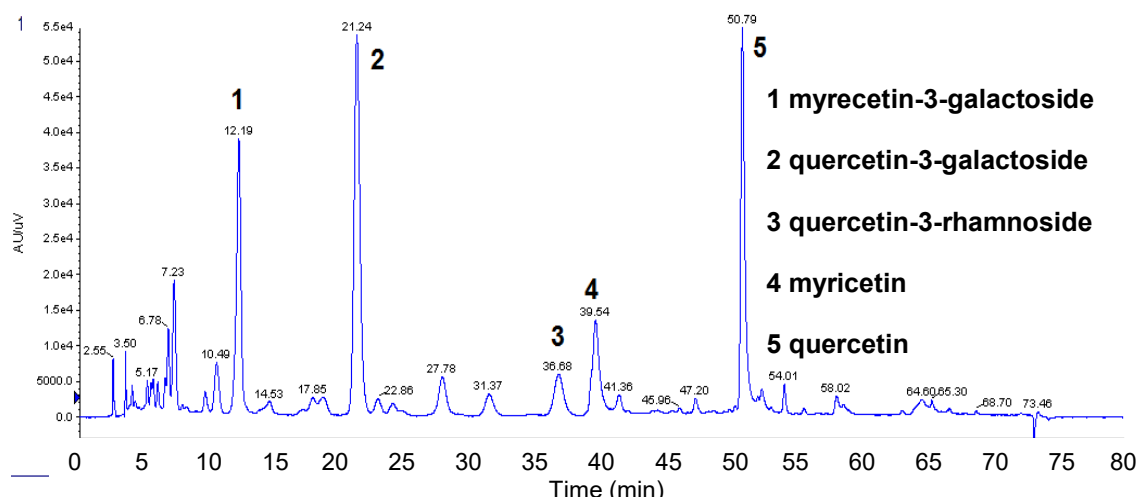


Figure 25: Typical chromatogram of CJC flavonols, detected by at 360 nm.

Linearity of responses was excellent for both anthocyanin ($r^2=0.998$) and flavonol ($r^2=0.9999$) standards (Figure 26). Precision of low and high standards was 1.97% and 4.05% for cyanidin-3-glucoside and 3.81% and 0.43% for quercetin-3-galactoside. Accuracy of the highest concentration standards was excellent, averaging -0.2% error (0.2% lower than expected response) for anthocyanins (25 mg/L) and 0.0% error for flavonols (100 mg/L). Accuracy of lowest concentration standards was adequate: -16.0% error for anthocyanins (0.5 mg/L) and 16.0% error for flavonols (2 mg/L) (Tables 6 and 7, respectively). Accuracy in mock samples was also adequate. High concentration mock samples averaged -3.0% error for anthocyanins and 5.1% for flavonols while low concentration mock samples averages 15.1% error for anthocyanins and 9.3% for flavonols.

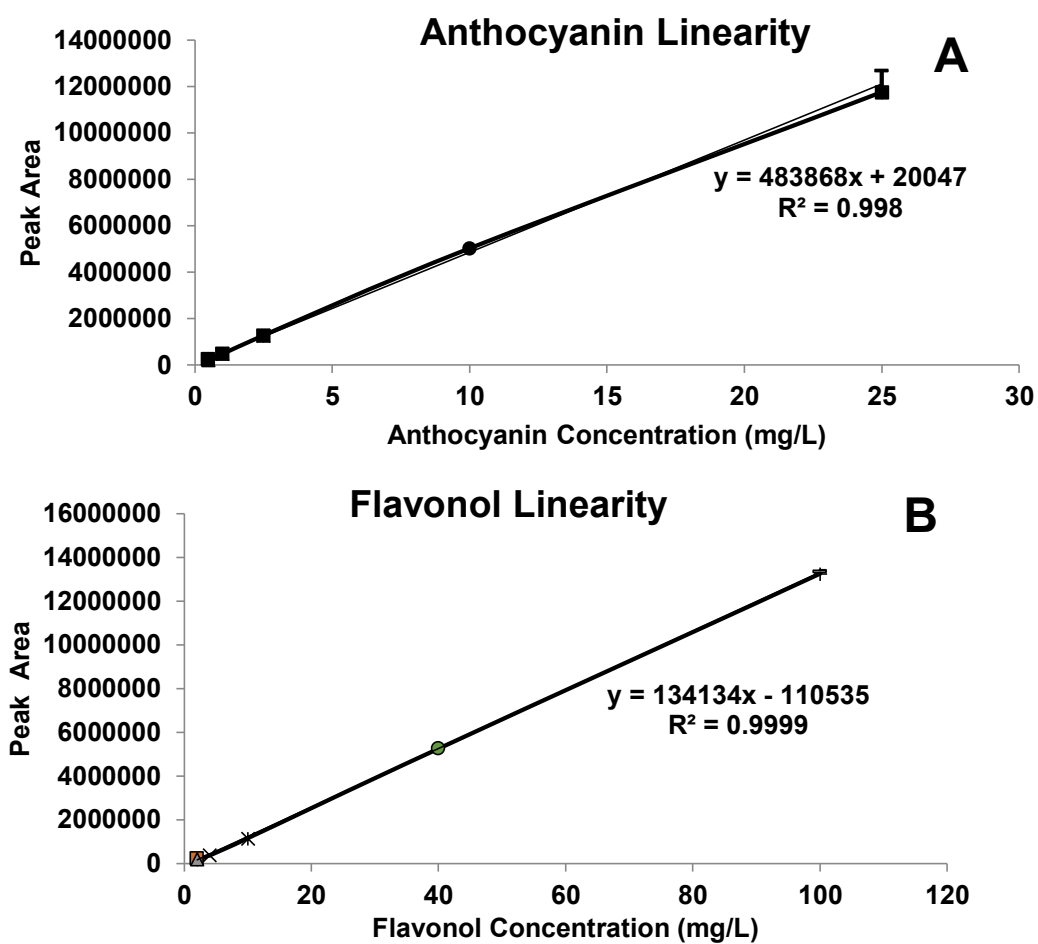


Figure 26: Calibration curves demonstrating linear responses for cyanidin-3-glucoside (A) and quercetin-3-galactoside (B).

Table 6: Accuracy and reproducibility of peaks areas for HPLC analysis of cyandin-3-glucoside as an anthocyanin standard: HPLC-PDA validation.

Standard	Concentration (mg/L)	Peak Area	Calculated Conc. (mg/L)	Error
1a	0.5	227775	0.43	-14.1%
1b	0.5	222890	0.42	-16.2%
1c	0.5	219011	0.41	-17.8%
2	1	483248	0.96	-4.3%
3	2.5	1271525	2.59	3.5%
4	10	5018989	10.33	3.3%
5a	25	11751842	24.25	-3.0%
5b	25	11877253	24.51	-2.0%
5c	25	12656038	26.11	4.5%
Blank	0	0	0	0.0%

Table 7: Accuracy and reproducibility of peaks areas for HPLC analysis of quercetin-3-galactoside as a flavonol standard: HPLC-PDA validation.

Standard	Concentration (mg/L)	Peak Area	Calculated Conc. (mg/L)	Error
1a	2	195196	2.28	14.0%
1b	2	209491	2.39	19.3%
1c	2	197580	2.30	14.9%
2	4	378852	3.65	-8.8%
3	10	1139469	9.32	-6.8%
4	40	5256458	40.01	0.0%
5a	100	13240536	99.54	-0.5%
5b	100	13347277	100.33	0.3%
5c	100	13328570	100.19	0.2%
Blank	0	0	0	0.0%

5.2 Phase II – Proof of Principle

In the first proof of principle experiment, total anthocyanins were measured before and after heating CJC to 90°C and quickly cooling. Here, degassed CJC lost 9.2% of total anthocyanins while aerated CJC lost 15.4% of total anthocyanins (Figure 27), a difference that was statistically significant ($p < 0.005$ by paired, two-tailed t-test). These results verify that removal of dissolved oxygen in CJC prior to processing improves retention of anthocyanins during short term heating at high temperatures, i.e. under conditions similar to pasteurization.

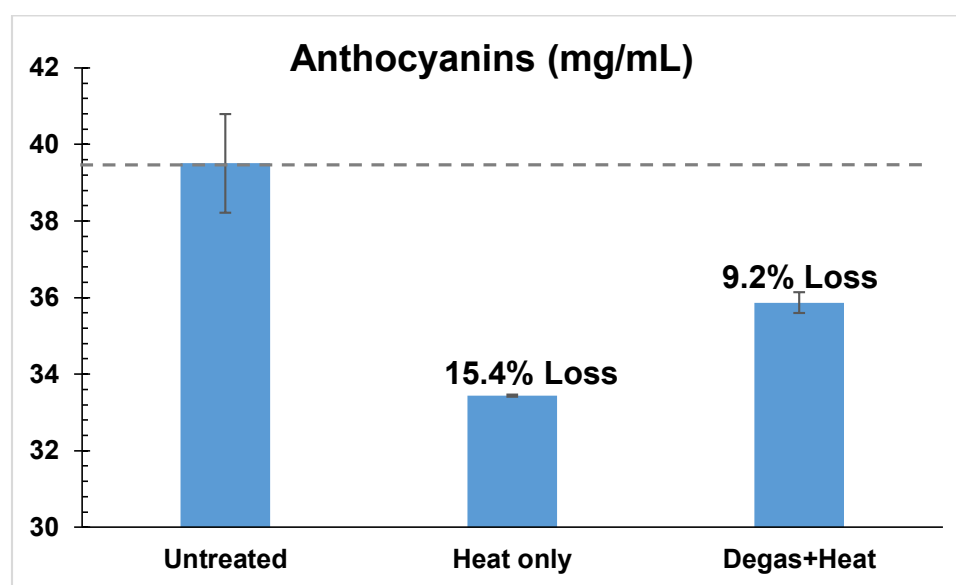


Figure 27: Effects of degassing on retention of anthocyanins during short-term heating. The dotted line indicates the initial, untreated ACY content. Results are total monomeric anthocyanins, expressed as cyanidin-3-glucoside equivalents.

In a second proof of principle experiment, total anthocyanins were measured before and after 1 week cold, dark storage of CJC. Here, the degassed sample lost 2.2%

of total anthocyanins while the aerated sample lost 4.9% (Figure 28), a difference that was once again statistically significant ($p < 0.05$ by paired, two-tailed t-test). These results verify that removal of dissolved oxygen in CJC promotes retention of anthocyanins during periods of storage at low temperatures when protected from light, e.g. similar to storage in warehouse in winter or in a refrigerated case.

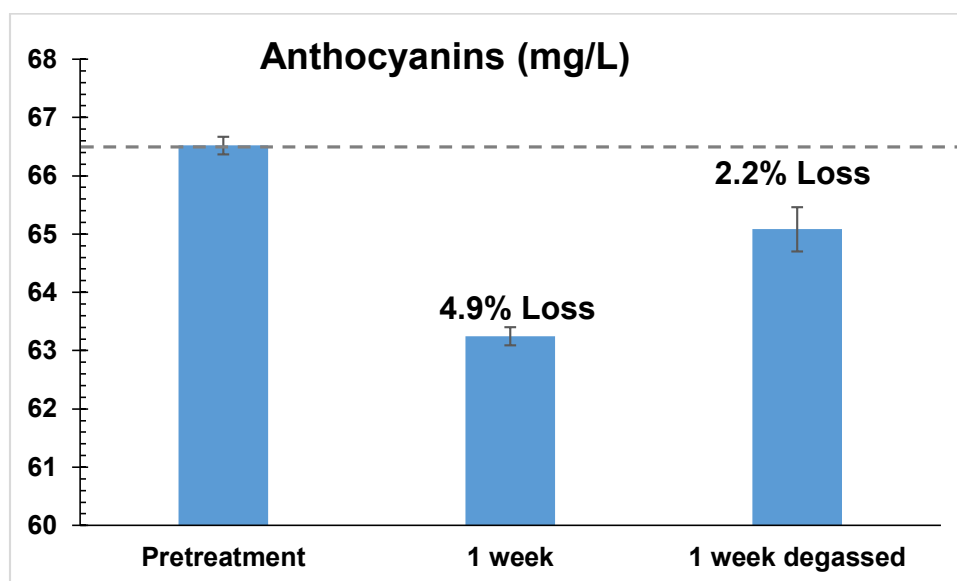


Figure 28: Effects of oxygen on anthocyanin losses in CJC after storage for one week in the dark at 5 °C. The dotted line indicates the initial, pretreatment ACY content. Results are total monomeric anthocyanins, expressed as cyanidin-3-glucoside equivalents.

A third proof of principle experiment evaluated total anthocyanins in CJC samples stored at ambient temperature and exposed to light after processing. Here, air blanketed samples lost 15.8% while nitrogen blanketed samples lost only 8.2% total anthocyanins (Figure 29). Again, differences were statistically significant ($p < 0.05$ by

paired, two-tailed t-test). These results indicate that protection from oxygen stabilizes anthocyanins in CJC under conditions similar to a grocery store shelf.

Taken together, these experiments show that removing sources of oxygen exposure has a dramatic effect on anthocyanin retention in a variety of environments. The protection provided by limiting oxygen exposure was fairly consistent over these 3 experiments: anthocyanin losses were approximately 40%, 56% and 48% less when oxygen exposure was limited.

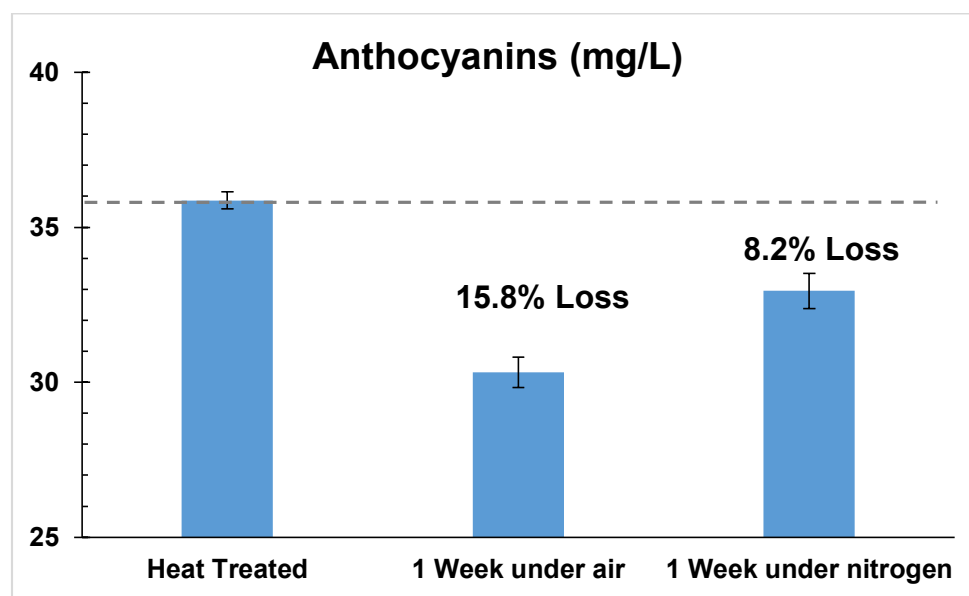


Figure 29: Effects of oxygen on anthocyanin losses in CJC after one week storage exposed to light at ambient temperature. The dotted line indicates the initial, heat treated ACY content. Results are total monomeric anthocyanins, expressed as cyanidin-3-glucoside equivalents.

5.3 Phase III: Controlled Laboratory Experiments

5.3.1 Overview

In Phase III, we investigated how multiple variables that influence oxidation impact CJC during thermal treatment and subsequent storage in atmosphere-controlled desiccators. The Phase III data set is large and complex and so a brief overview is presented here for clarity. Figure 30 shows the simple effects of the treatments relative to the average effect of all treatments. For processing (Figure 30A), degassing treatments enhanced anthocyanin, ascorbic acid and color retention, while ascorbic acid fortification resulted in higher amounts of flavonols and corrected total phenolics (CTP) after processing. At the same time, AscA increased loss of anthocyanins and red color.

During storage, protection from oxygen was universally stabilizing. Protection from light was also stabilizing, but its effect was smaller. Here, ascorbic acid fortification was mostly damaging, particularly for anthocyanins but was moderately stabilizing for flavonol aglycones. With 16 groups monitored during storage, one for each variable combination, graphs depicting all groups are cumbersome to follow. Rather, in the sections below (Sections 5.3.2 – 5.3.7), graphs depict the average of the eight groups subjected to a common treatment in order illustrate the simple effect of that treatment. Generally, only those treatment effects found to be significant in the ANOVA data analysis are included. Statistically significant interactions are shown in data tables and are discussed in the text.

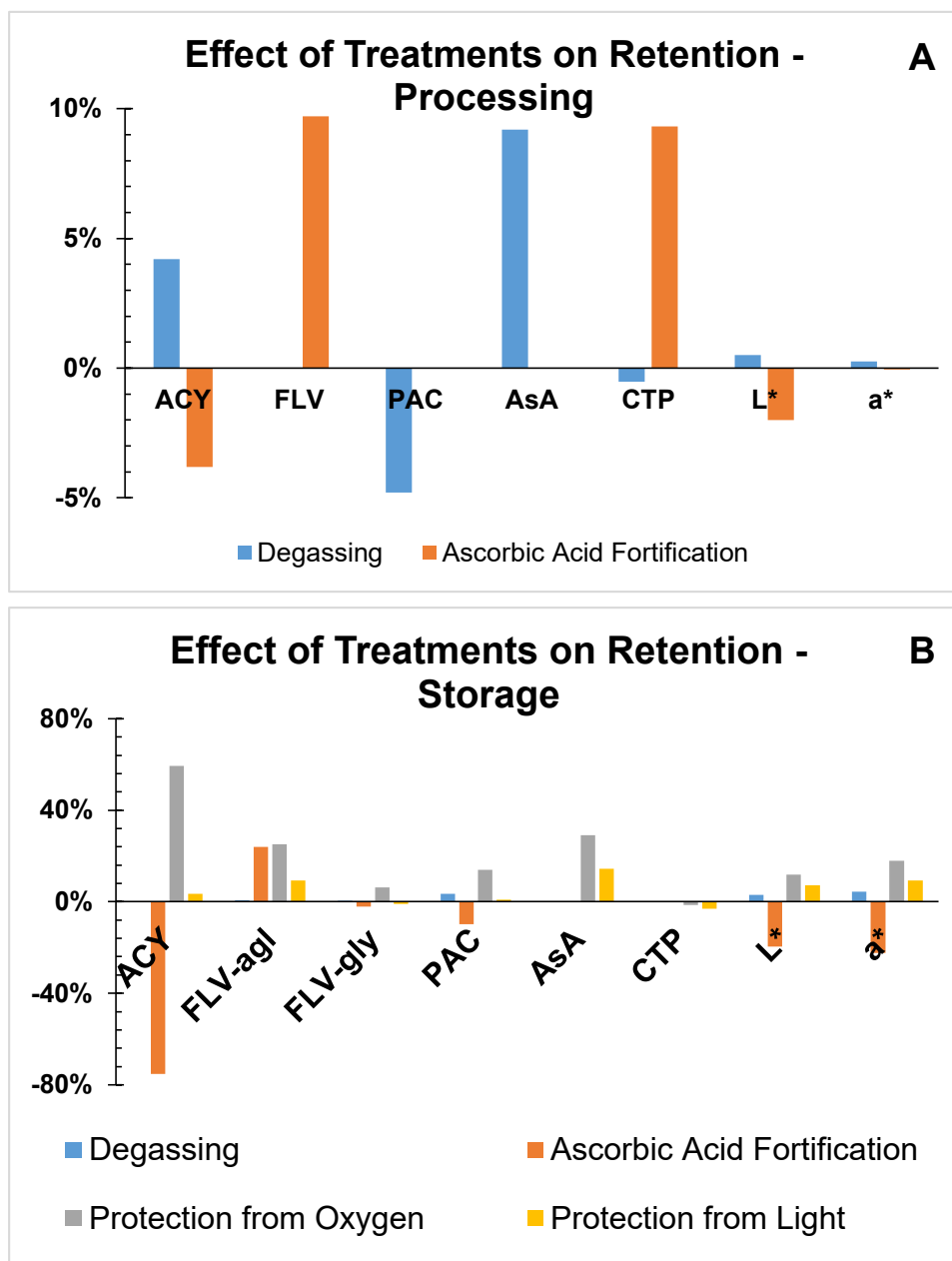


Figure 30: Overview of the main effects of treatments for Phase III experiments after processing (A) and 6 weeks of storage (B) of CJC. Results are expressed as values relative to the average of all conditions tested (i.e. zero line is overall average from all treatments). Only statistically significant effects are shown ($p < 0.05$).

5.3.2 Dissolved Oxygen (DO)

Prior to processing, aerated juice measured 6.92 mg/L dissolved oxygen (DO) while DO levels were reduced to 0.06-0.08 mg/L in the degassed groups. Figure 31 shows the significant effects of various treatments on DO during the storage period. DO was limited to very low levels (average of ~0.025 mg/L) when stored under argon and higher levels when under 1% O₂ (average of ~0.3 mg/L). Ascorbic acid fortification suppressed DO levels throughout the storage period by approximately 40% due to its autoxidation. Degassing the juice before processing did not yield any reduction in the DO levels during storage; in fact, by the second week of storage, aerated samples showed slightly lower DO levels. The average DO for all groups during storage was 0.16 mg/L.

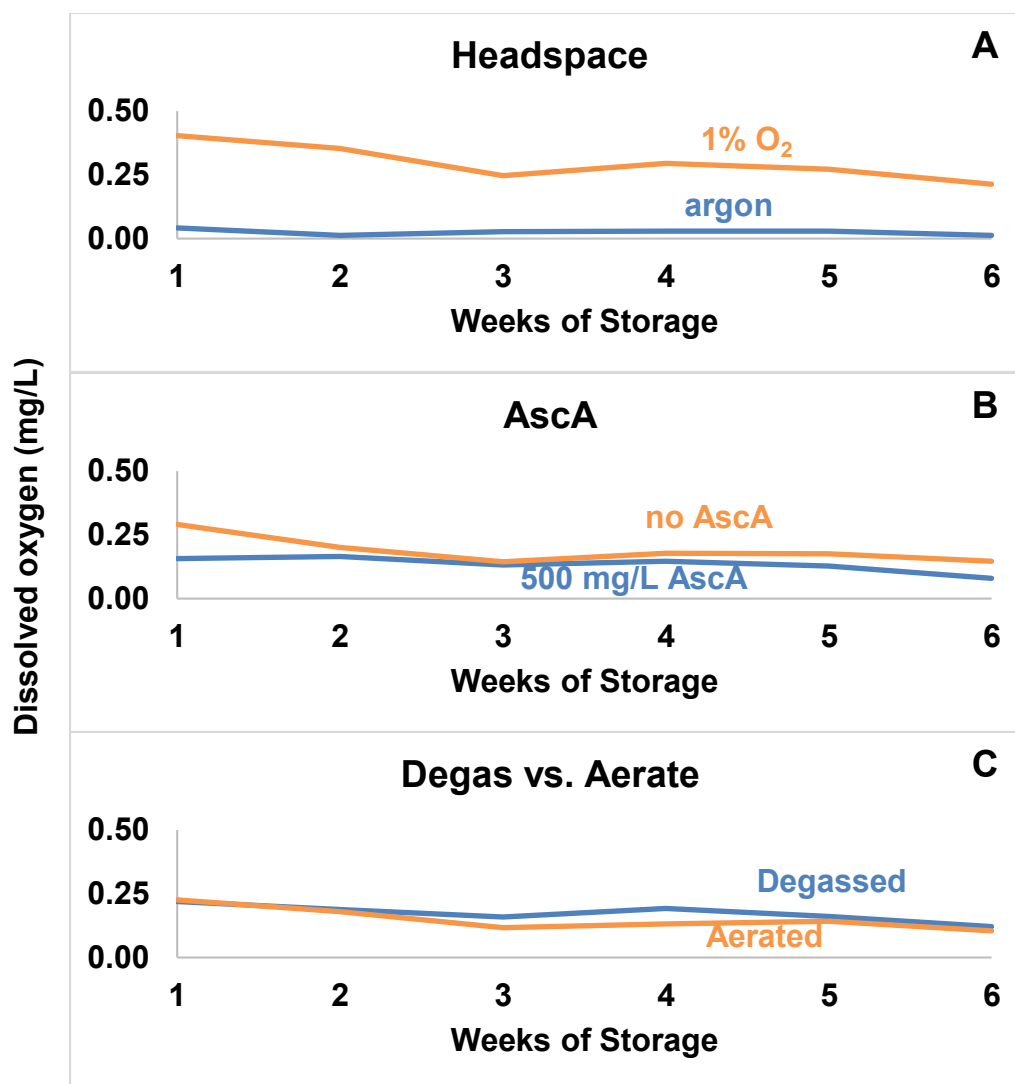


Figure 31: Effects of treatments on dissolved oxygen in CJC stored for six weeks under ambient conditions and include groups stored exposed to light and protected from light (A) groups stored under argon vs 1% O₂; (B) groups stored with or without ascorbic acid fortification; (C) groups degassed or aerated before processing (C). Values reported are averages of all conditions with and without each test factor. n=24 for each data point.

5.3.2 Anthocyanins

Processing. During processing, only juice that was both aerated and fortified with ascorbic acid before HTST yielded a significant loss of total anthocyanins (Figure 32.) All other groups showed small non-significant gains of anthocyanins during processing. ANOVA indicated both ascorbate fortification and preprocessing degassing as significant main effects, along with their interaction (Table 8). It is notable that the individual anthocyanins reacted very similarly to the heat treatment, with no one species particularly more stable or unstable.

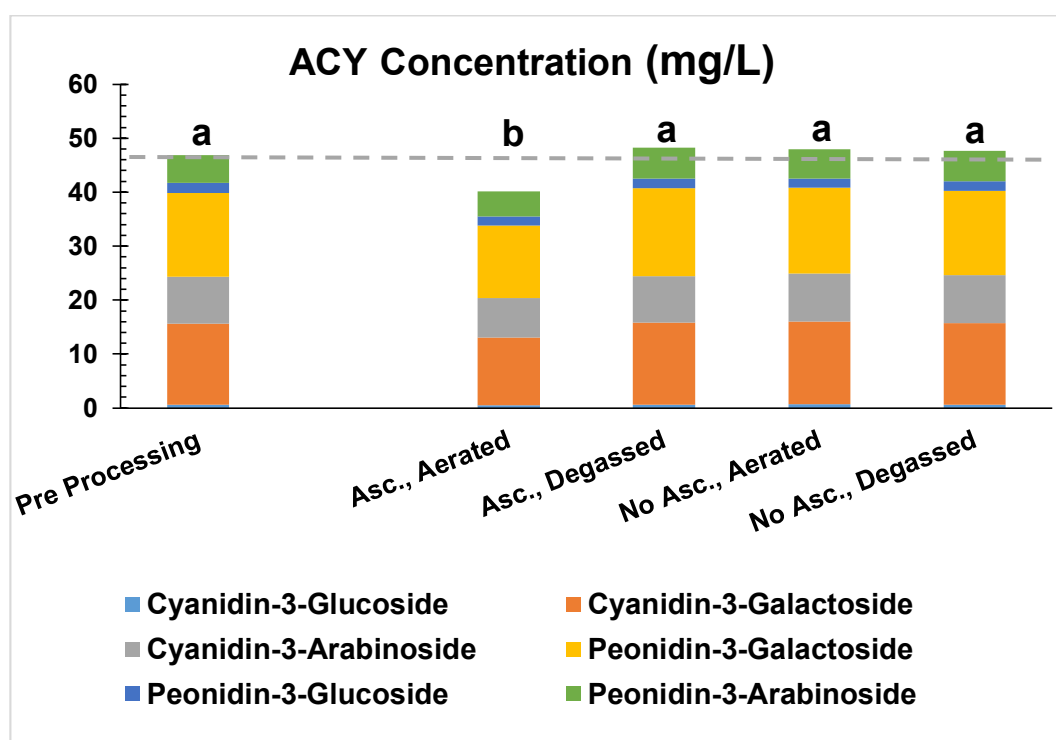


Figure 32: Effects of treatments on levels of individual anthocyanins in CJC before and after processing. Groups labelled with a common letter have total anthocyanin levels that are statistically the same ($p < 0.05$). The dotted line indicates the initial, pre-processing ACY content.

Table 8: Sources of variance from ANOVA analysis of anthocyanin retention after HTST processing.

Source	p	Notes
Ascorbic Acid Fortification	0.0168	
Preprocessing Degassing	0.0109	
Ascorbic Acid x Degassing Interaction	0.0071	Only combination of fortification and no degassing was different than other groups

Storage. During storage, anthocyanins were the most unstable of the cranberry flavonoids in the CJC, with average losses of ~75%. Several factors had significant impacts on anthocyanin retention. The effect of ascorbate fortification was most dramatic with nearly seven times more anthocyanins remaining in the non-fortified group at the end of the six week storage period (Figure 33A). Oxygen exposure also had a large effect, with four times more anthocyanins remaining at the end of storage when CJC was stored under argon vs 1% O₂ (Figure 33B). Surprisingly, the effect of light was small: ~10% more remained after six weeks when stored in the dark vs exposed to light (Figure 33C). Degassing showed no significant main effect for storage ($p=0.062$).

ANOVA indicates interactions were present along with these main effects (Table 9). Effects of degassing before processing diminish slightly over the storage period, light was detrimental to anthocyanin retention only when combined with O₂ exposure (storage atmosphere x light interaction) or at the final time point when not exposed to O₂ (time x storage atmosphere x light interaction), the combined effects of ascorbate and O₂ exposure were much more than additive (ascorbate x storage atmosphere interaction), and degassing had a greater protective effect in CJC fortified with ascorbate (ascorbate x

degassing interaction).

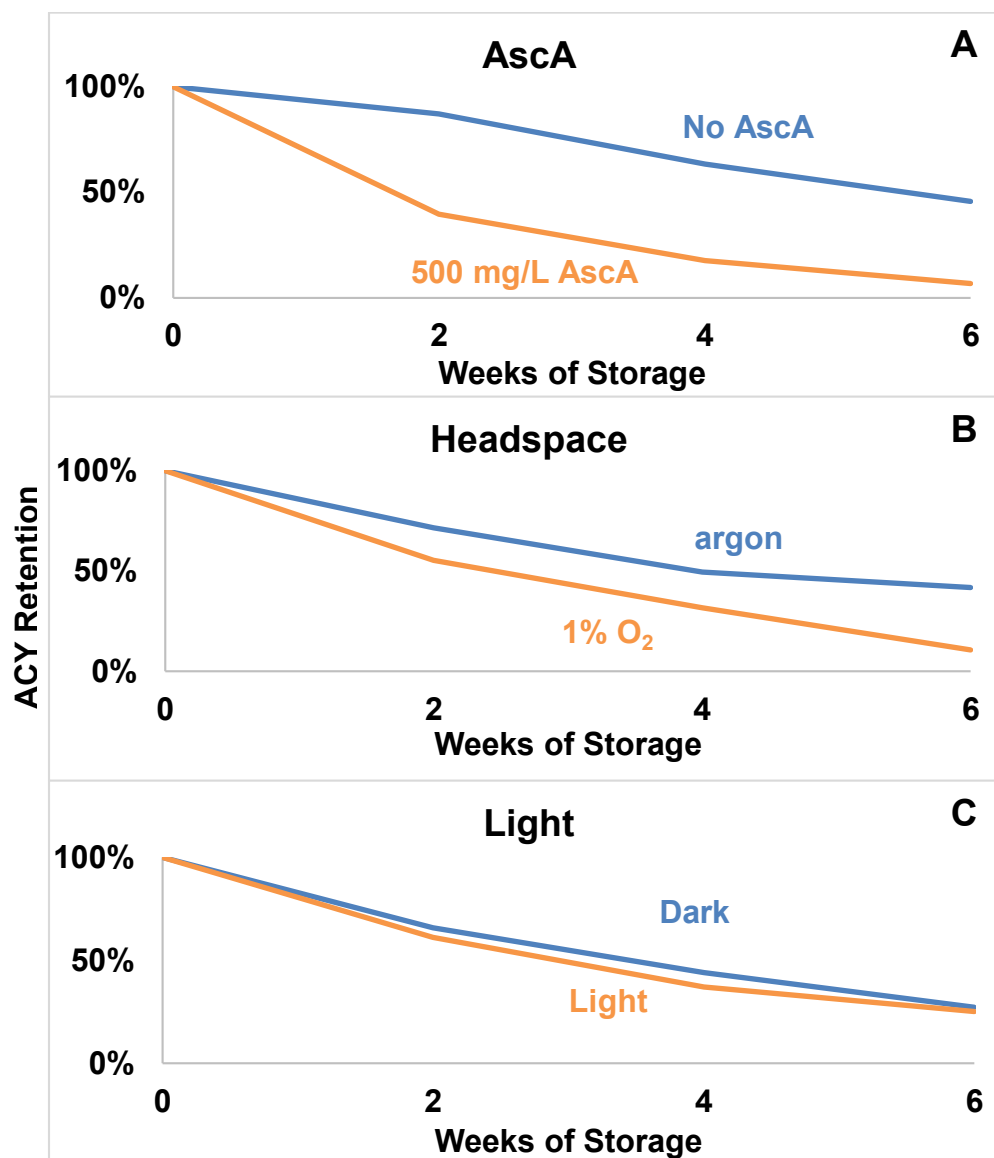


Figure 33: Effects of formulation and conditions on anthocyanin retention in CJC stored for six weeks under ambient conditions: A) fortified with 500 mg/L ascorbic acid versus not fortified, B) stored under argon vs 1% O₂, C) stored in the dark vs exposed to light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

Table 9: Significant sources of variance from ANOVA analysis of total anthocyanin retention in CJC stored for six weeks under ambient conditions. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbate Fortification	<0.0001	Ascorbate promotes degradation
Storage Atmosphere	<0.0001	1% O ₂ promotes degradation vs. argon
Light Exposure	<0.0001	
Time x Ascorbate	<0.0001	Effect of Asc. increases over time
Time x Degassing	0.0003	After 6 weeks, degassing no longer had effect
Time x Storage Atmosphere	<0.0001	Impact of storage atmosphere greater over time
Time x Light	0.0101	Effect of light most pronounced at week 4
Ascorbate x Degassing	<0.0001	Degassing had protective effect only when fortified with ascorbate
Ascorbate x Storage Atmosphere	<0.0001	Combined effects more than additive
Ascorbate x Light	0.0008	Light exposure slightly more damaging when fortified with ascorbate
Storage Atmosphere x Light	<0.0001	Light exposure is damaging only with oxygen exposure
Time x Ascorbate x Storage Atmosphere	<0.0001	Combined effects more than additive
Ascorbate x Degassing x Storage Atmosphere	<0.0001	Combined effects more than additive
Time x Degassing x Light	0.0231	Effects more pronounced at earlier time points
Ascorbate x Degassing x Light	<0.0001	Light exposure not damaging in degassed, fortified groups
Time X Oxygen x Light	<0.0001	Effect of combination of light and oxygen diminishes over time; light only damaging with no oxygen exposure at last time point
Degassing x Oxygen x Light	<0.0001	No effect from light when degassed and protected from O ₂

5.3.3 Flavonols

Processing. During processing, total flavonol content increased by ~20% with degassing + ascorbate fortification and decreased by ~10% when degassed without

ascorbate fortification (Figure 34), but did not change significantly for other treatments. ANOVA results (Table 10) indicate ascorbate fortification and its interaction with degassing were significant factors in flavonol degradation for processing. Degassing was only protective when combined with ascorbic acid fortification.

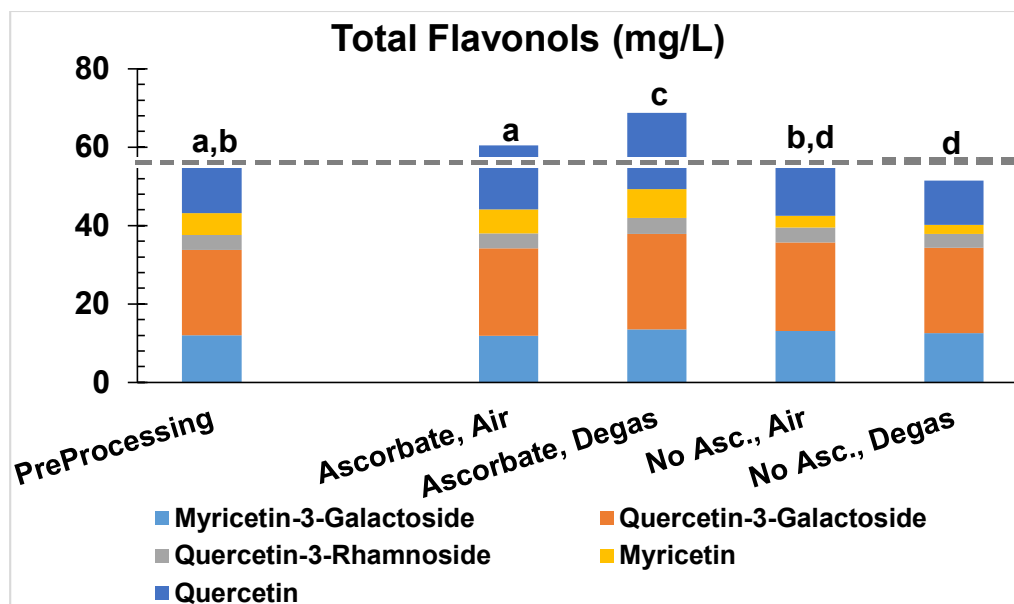


Figure 34: Effect of formulation and processing conditions on flavonol contents of CJC before and after processing. Groups labeled with a common letter have total flavonol levels that are statistically the same ($p < 0.05$). The dotted line indicates the initial, preprocessing FLV content.

Table 10: Sources of variance from ANOVA analysis of flavonol retention for HTST processing.

Source	p	Notes
Ascorbate Fortification	<0.0001	Ascorbate is protective
Ascorbate x Degassing Interaction	0.004	Degassing is protective only when ascorbate is present

For aglycones, substantially increased concentrations were evident in groups fortified with ascorbic acid but losses were observed when no ascorbate was added. Changes in flavonol glycosides were less dramatic. Indeed, after 6 weeks of storage, it is evident that the aglycones and glycosides are impacted differently by the various treatments, as is demonstrated in Figure 35. Because of this, storage effects were evaluated for aglycones and glycosides separately rather than as total flavonols (Figures 36 and 37).

Storage. Flavonols were more degraded in storage than during processing. Nevertheless, they were still more stable than anthocyanins, showing ~25% losses for glycosides and ~30% for aglycones when averaged over all conditions. This difference will be discussed in further detail later. Strikingly, ascorbic acid fortification yielded disparate effects on flavonol aglycones and glycosides. Ascorbate fortification protected aglycones over most of the storage period, with an average of 7% more aglycones remaining with ascorbate. Conversely, ascorbate caused 8% lower retention of glycosides (Figure 36A). On an absolute basis, the difference is even more striking for aglycones: 16.7 mg/L flavonol aglycones remained with ascorbic acid fortification versus 10.2 mg/L without fortification at the end of the storage period. However, the protective effect of ascorbate for flavonol aglycones was present only in samples stored under 1% oxygen. Under argon, ascorbate was damaging and caused about 15% greater aglycone losses than argon alone. Thus, the interaction of atmosphere and ascorbic acid was highly significant for aglycones (Table 11). Oxygen itself was damaging, even at low levels (1% O₂), and again effects were much larger for aglycones than for glycosides: ~80% more aglycones were retained at the end of six weeks under argon versus ~15% more for

glycosides than the same systems under 1% O₂ (Figure 36B.) Light exposure was harmful to aglycones (~25% more retention after six weeks in the dark), but there was no significant affect for glycosides (Figure 36C).

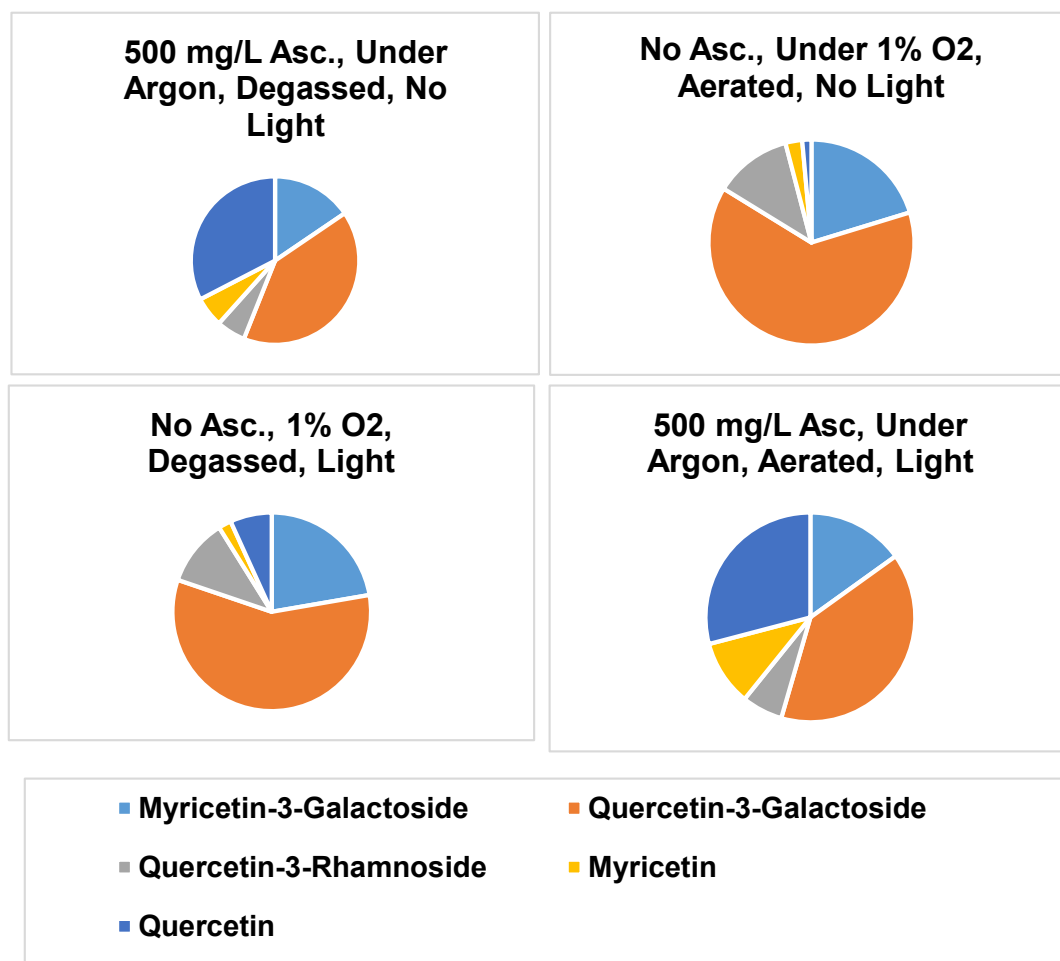


Figure 35: Effects of processing and handling treatments on relative flavonol contents of CJC stored for six weeks under ambient conditions. Shift in graphs segment proportions reveal differential degradation among individual flavonols.

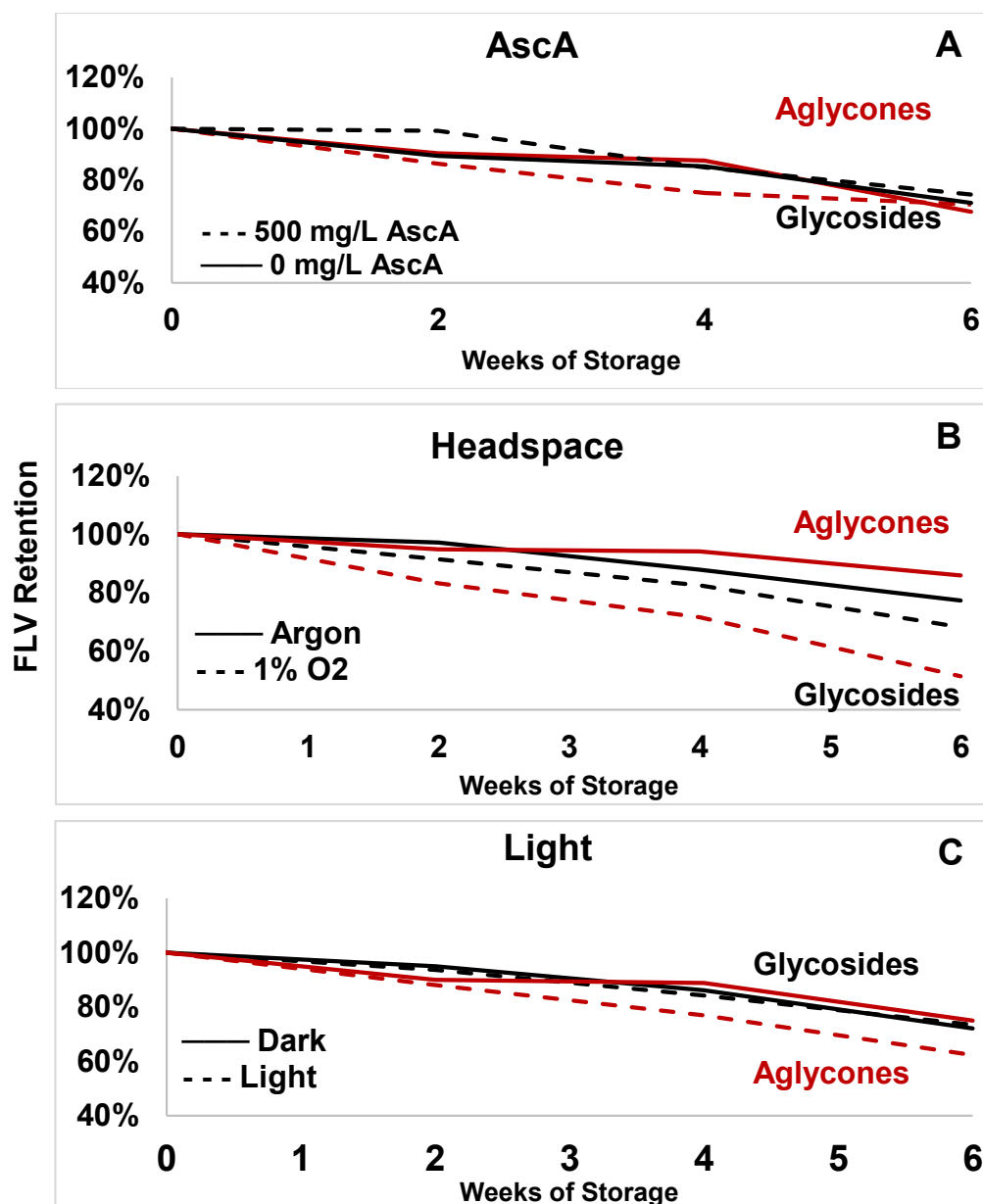


Figure 36: Effects of treatments on retention of flavonol aglycones and glycosides during six weeks storage of CJC under ambient conditions. A) with/without fortification with 500 mg/L ascorbic acid, B) stored under argon vs 1% O₂, C) stored in the dark vs exposed to light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

ANOVA indicates that degassing prior to processing led to a significant detrimental effect on flavonol aglycones and glycosides during storage; however, this result is misleading. Our ANOVA analysis used data that was expressed as % retention from the post processing concentration (Figure 37A). When charted in absolute terms (Figure 37B), it is clear that preprocessing degassing did not have an overall negative effect on flavonol retention; rather, the small gains in flavonol retention from processing (about 1.5 mg/L) were reduced during storage (to about 0.2 mg/L after 6 weeks).

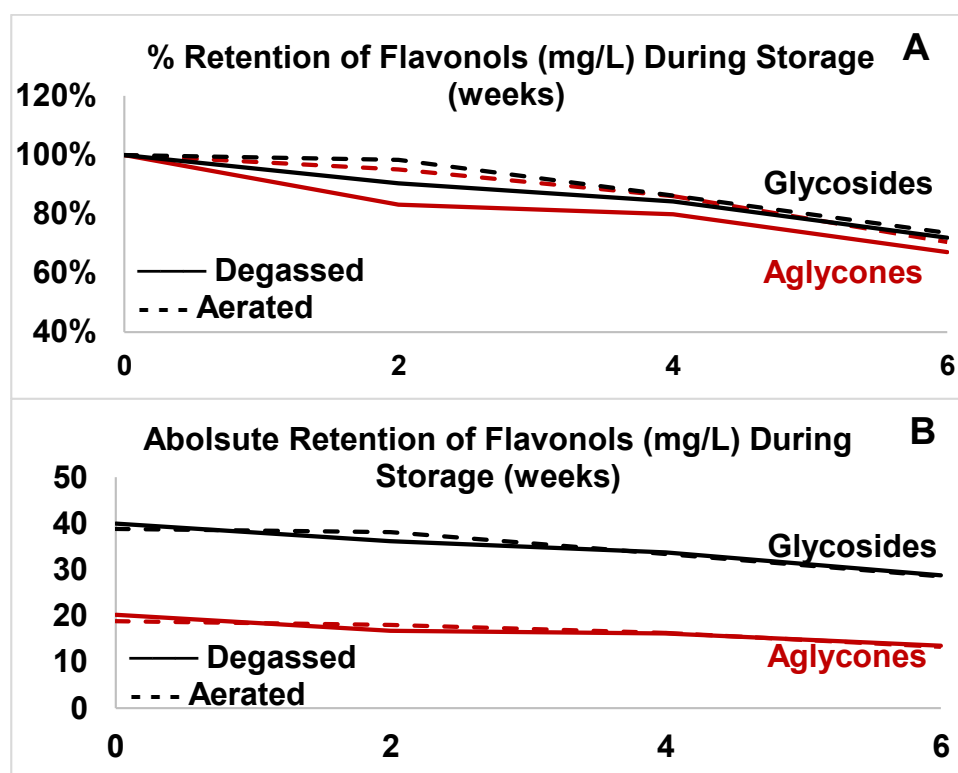


Figure 37: Effects of pre-processing degassing versus aeration on retention of flavonol aglycones and glycosides during six weeks storage of CJC under ambient conditions. Values expressed as A) a percent of post-processing concentration, and B) absolute concentration.

ANOVA indicates several interactions were present along with these main effects (Table 9). Among the most interesting interactions for aglycones, the damaging effect of light was observed only under conditions of no ascorbate fortification and 1% O₂.

Table 11: Significant sources of variance from ANOVA analysis of the retention of flavonol aglycones and glycosides during the 6 week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
<i>Aglycones</i>		
Time	<0.0001	
Ascorbate Fortification	0.0005	Ascorbate is protective
Storage Atmosphere	<0.0001	Oxygen exposure is damaging
Light Exposure	<0.0001	Light is damaging
Preprocessing Degassing	0.0003	Degassing is damaging
Time x Ascorbate Fortification	<0.0001	Effect of ascorbate greater at week 2
Time x Storage Atmosphere	<0.0001	Effect of atmosphere greater over time
Time x Light	0.0003	Effect of light more pronounced over time
Ascorbate Fortification x Storage Atmosphere	<0.0001	Combined effects more than additive
Ascorbate Fortification x Degassing	<0.0001	Effect of ascorbate greater when aerated
Storage Atmosphere x Light	<0.0001	Light only damaging with oxygen exposure
Time x Ascorbate x Degassing	0.0029	Effect of ascorbate greater when aerated → decreases over time
Time x Ascorbate x Light	0.0122	Combination of light exposure and no ascorbate more damaging over time
Time x Storage Atmosphere x Light	0.0061	Combination of light exposure and oxygen exposure more damaging over time
Ascorbate x Degassing x Light	0.0186	Effect of light greater when degassed and not fortified with ascorbate
Ascorbate x Storage Atmosphere x Light	<0.0001	Damaging effect of light only observed when exposed to oxygen and no ascorbate
Storage Atmosphere x Degassing x Light	0.0006	Effect of light x storage atmosphere more pronounced when degassed
<i>Glycosides</i>		
Time	<0.0001	
Ascorbate Fortification	<0.0001	Ascorbate is damaging
Storage Atmosphere	<0.0001	Oxygen exposure is damaging
Preprocessing Degassing	0.0004	Degassing effect is damaging
Time x Ascorbate	<0.0001	Effect is small in week 2
Time x Degassing	0.0156	Effect only prominent in week 2
Ascorbate x Degassing	<0.0001	Degassing effect is detrimental only when fortified with ascorbate; slightly protective when not fortified
Time x Ascorbate x Degassing	0.0352	Degassing effect is detrimental only when fortified with ascorbate → Decreases over time
Ascorbate x Storage Atmosphere x Degassing	0.0207	When protected from O ₂ , degassing is protective at week 6

5.3.4 Proanthocyanidins

Processing. PACs were more stable through processing than the other cranberry flavonoids, with no groups being significantly different than the preprocessed juice (Figure 38). However, taking the whole data set into account, ANOVA indicates that the degassing treatment significantly reduced PAC retention after processing ($p = 0.0414$) by ~10% compared to aeration treatments.

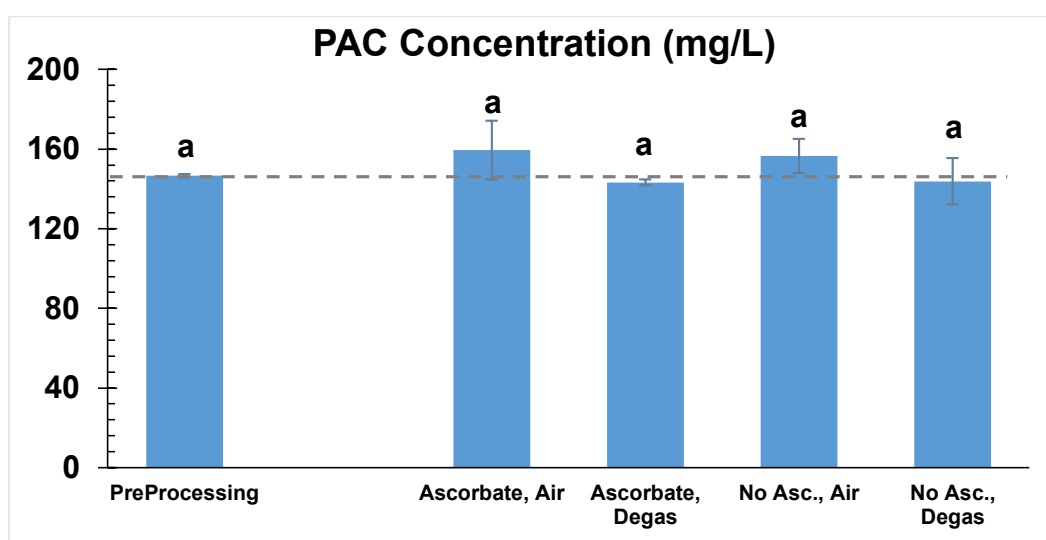


Figure 38: Proanthocyanidins before and after processing. Groups labeled with a common letter are statistically equivalent in total flavonols ($p < 0.05$). The dotted line indicates the initial, preprocessing PAC content.

Storage. For storage, all main effects were significant. Degassing before processing protected PACs during the early part of storage, retaining ~10% more PACs after the first week of storage than the aerated juice (Figure 39). However, over the long term, storage atmosphere had a greater impact: CJC retained ~30% more PACs after six weeks of storage under argon than 1% O₂ (Figure 40). Ascorbic acid fortification had an

opposite impact, reducing 25% more PACs than without AscA after six weeks storage. The damaging impact of light was small (~2% loss of PACs), but statistically significant.

Many interactions were also found in the ANOVA analysis (Table 12.) As observed for anthocyanins, effects of oxygen exposure and ascorbic acid were stronger in combination than the sum of the individual effects.

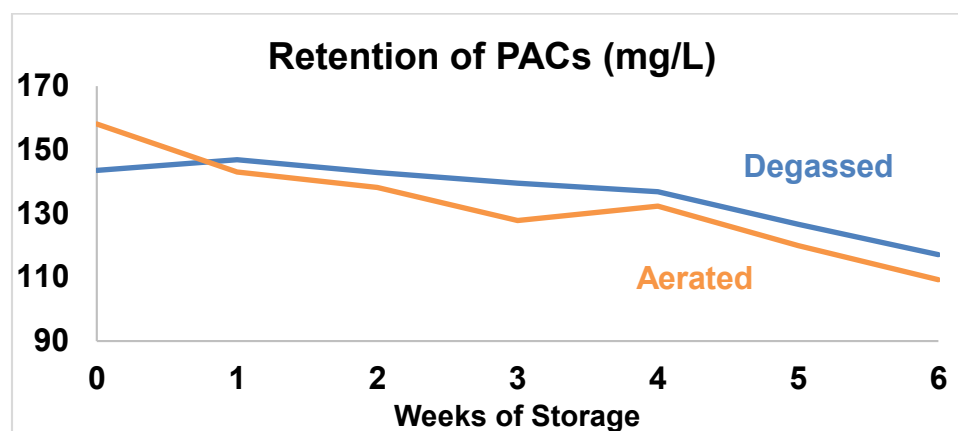


Figure 39: Effect of preprocessing degassing vs aeration on retention of proanthocyanins during storage of CJC. n=24 for each time point.

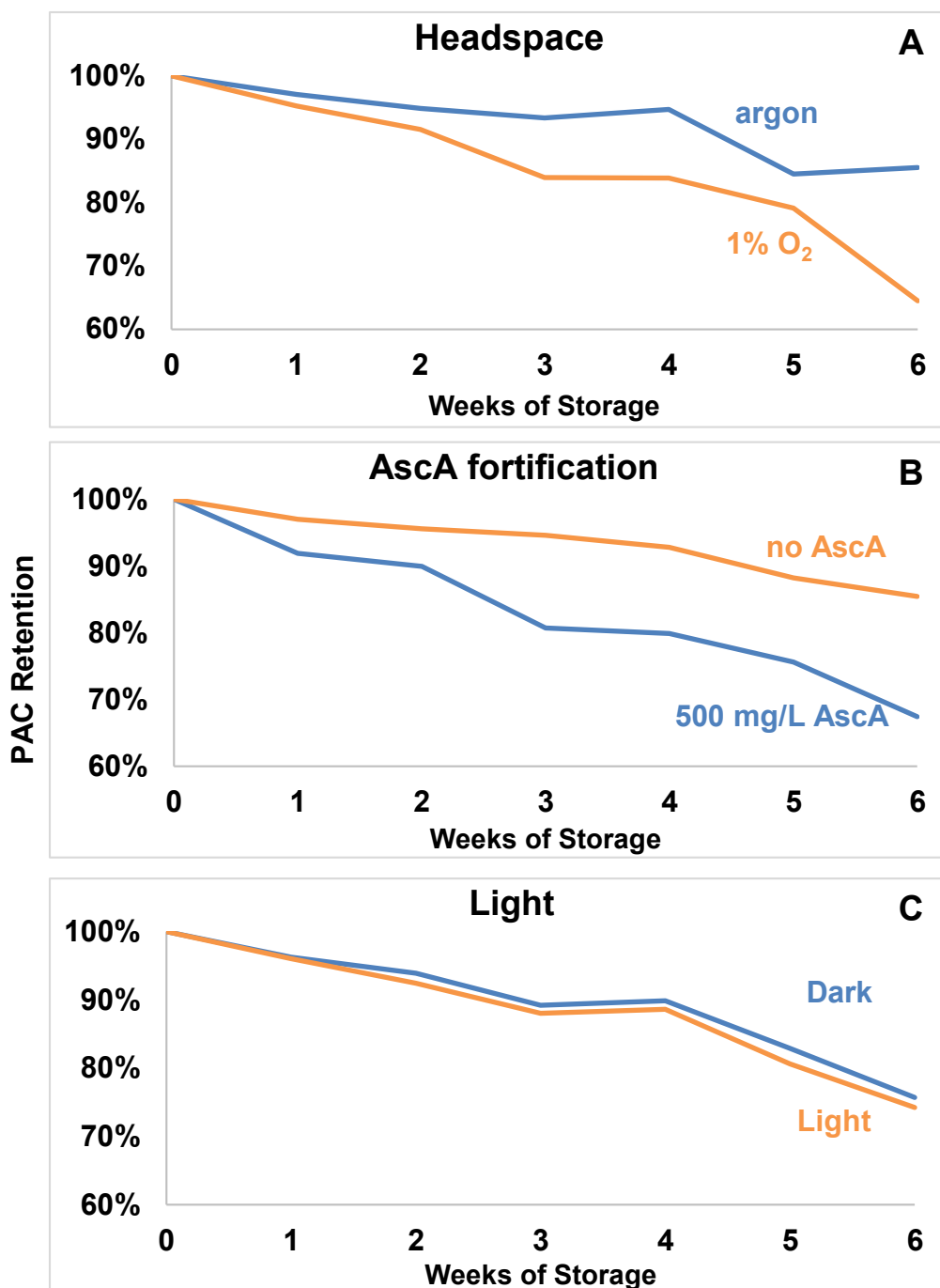


Figure 40: Effect of ascorbic acid, oxygen, and light on retention of proanthocyanidins in CJC during six weeks storage. A) argon vs 1% O₂; B) with and without ascorbic acid fortification; C) stored in the dark vs exposed to light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

Table 12: Significant sources of variance from ANOVA analysis of the retention of proanthocyanidins during the 6 week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbic Fortification	<0.0001	Ascorbate is damaging
Storage Atmosphere	<0.0001	Oxygen is damaging
Preprocessing Degassing	<0.0001	Degassing is protective
Light Exposure	<0.0001	Light is damaging
Time x Ascorbate Fortification	<0.0001	Effect of ascorbate increases over time
Time x Storage Atmosphere	<0.0001	Effect of storage atmosphere most prevalent at weeks 4 & 6
Time x Degassed	0.0138	Effect of storage atmosphere most prevalent at weeks 5
Ascorbate x Storage Atmosphere	0.0301	Combined effect is more than additive
Ascorbate x Degassed	<0.0001	Effect of ascorbate is more prominent when aerated
Degassed x Storage Atmosphere	<0.0001	Protective effect of degassing more prominent under argon
Time x Ascorbate x Degassed	<0.0001	Effect of ascorbate is more prominent when aerated→Increases over time
Time x Ascorbate x Storage Atmosphere	0.0041	Combined effect of ascorbate and oxygen is more than additive →Increases over time
Time x Degassed x Storage Atmosphere	<0.0001	Combined effect is more than additive→Increases over time
Time x Ascorbate x Light	0.0002	Combinations of light and ascorbate are more than additive at later time points
Time x Degassed x Light	<0.0001	Combinations of light and ascorbate are more than additive at later time points
Ascorbate x Degassed x Light	0.0194	Light has no effect when degassed and fortified with ascorbate
Ascorbate x Degassed x Storage Atmosphere	0.0056	Ascorbate fortification is less damaging when degassed and protected from oxygen

5.3.5 Ascorbic Acid

Processing. Ascorbic acid degraded more when juice was aerated (~30%) than when degassed (~15%) before processing (Figure 41).

Storage. Oxygen and light significantly reduced ascorbic acid retention during storage (Figure 42). After six weeks, nearly twice as much ascorbic acid remained when CJC was stored under argon than when under 1% oxygen and one-third more ASca remained when CJC was stored protected from light than when exposed to light. The degassing protection observed during processing did not carry through storage, where no significant differences from other treatments were observed.

Table 13 details sources of variances from the ANOVA. The most important observation here was that damage from light was mitigated by removal of oxygen (light-oxygen interaction).

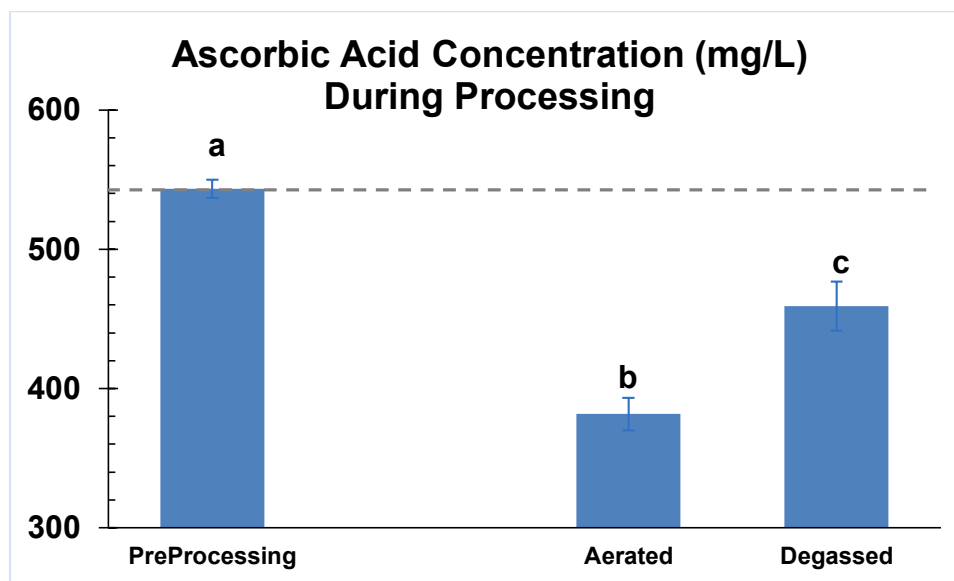


Figure 41: Effects of degassing on retention of ascorbic acid during processing of CJC. Groups labeled with different letters are statistically different ($p < 0.05$). The dotted line indicates the initial, preprocessing AscA content.

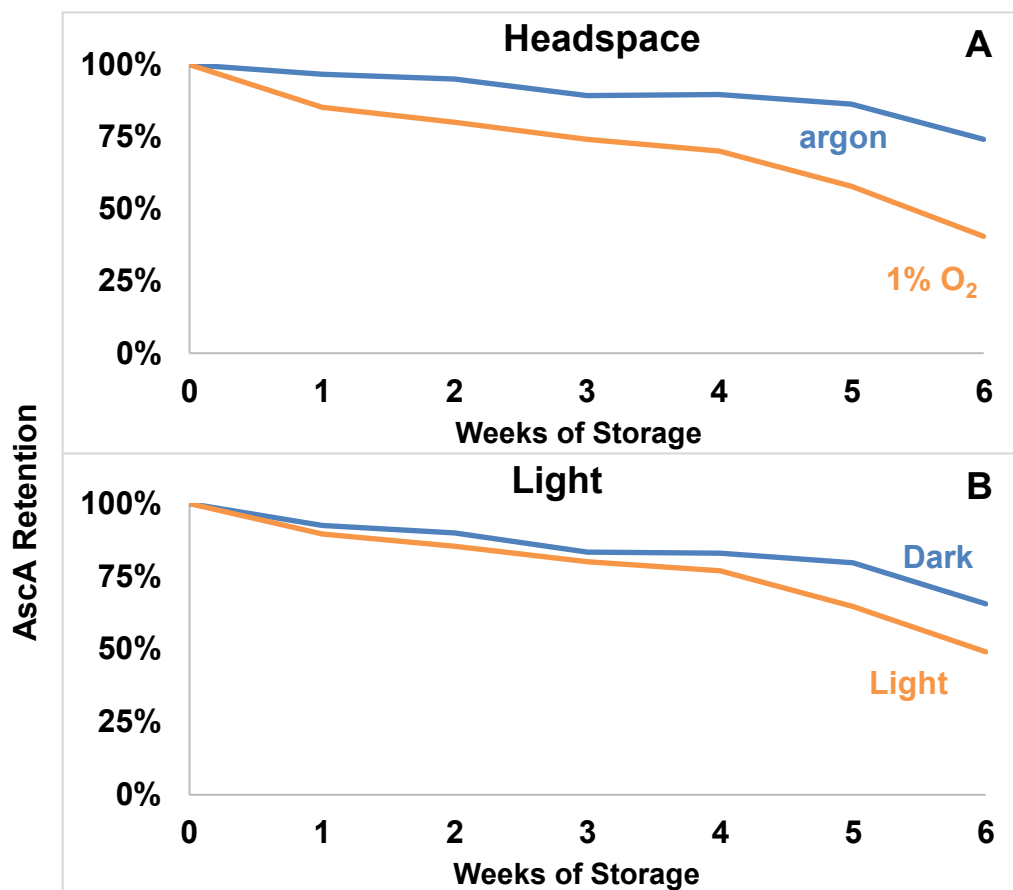


Figure 42: Effect of oxygen and light on retention of ascorbic acid in CJC during six weeks storage under ambient conditions. A) argon vs 1% O₂; B) dark vs exposed to light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

Table 13: Significant sources of variance from ANOVA analysis of the retention of proanthocyanidins during the 6 week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Storage Atmosphere	<0.0001	Oxygen is damaging
Light Exposure	<0.0001	Light is damaging
Time x Oxygen	0.0021	Effect of oxygen exposure increases over time
Oxygen x Light	0.0259	Effect of light more prominent when exposed to oxygen

5.3.6 Total Phenolics

Processing. Only subtle changes to corrected total phenolics (CTP) were observed. No single group was significantly different than the preprocessed juice by Duncan's MRT test. However, those groups fortified with ascorbate tended to accumulate CTP while non-fortified juice did the opposite (Figure 43). ANOVA of the dataset revealed that fortification with ascorbate correlated with higher retention of corrected total phenolics (~20%, $p=0.0307$.)

Storage. Atmosphere and light exposure were significant factors but none made large impacts on CTP (Figure 44 and Table 14). 3% more CTP remained after six weeks of storage under 1% O₂ than under argon. When exposed to light, 6.5% more remained than when CJC was stored in the dark. The main effect of ascorbate fortification was not significant ($p=0.1441$), but the interaction of time and ascorbate was ($p=0.0117$): ~5% higher CTP levels were observed at later time points when CJC was fortified with ascorbate. The light/storage atmosphere interaction was also noteworthy: the combination

of protection from light and protection from oxygen yielded lower CTP (88% retention) than other combinations involving these two variables (93-94% retention.)

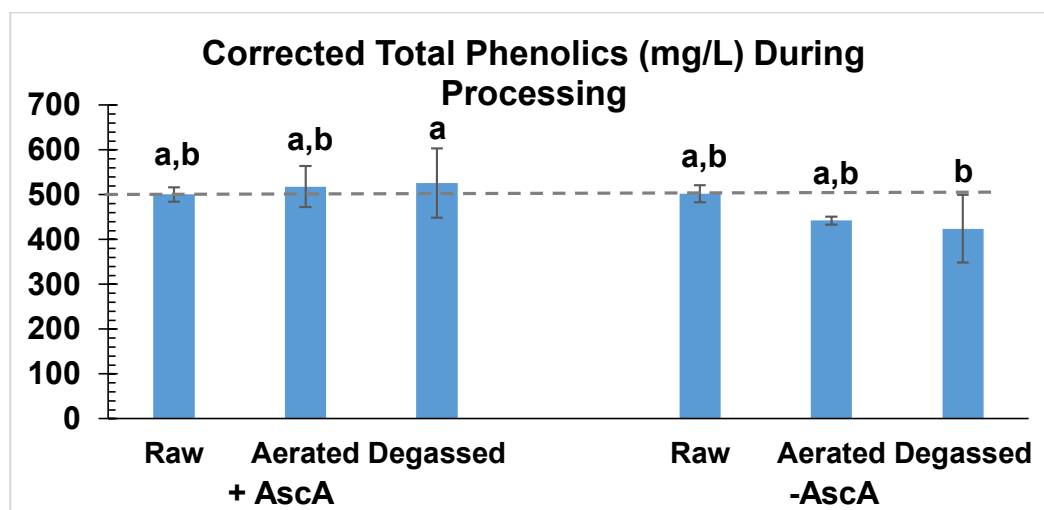


Figure 43: Effect of degassing and ascorbic acid on retention of corrected total phenolics in CJC during processing (Phase III). Total phenolics are expressed as gallic acid equivalents and corrected for interference from ascorbic acid. Groups labeled with a common letter are statistically equivalent ($p < 0.05$).

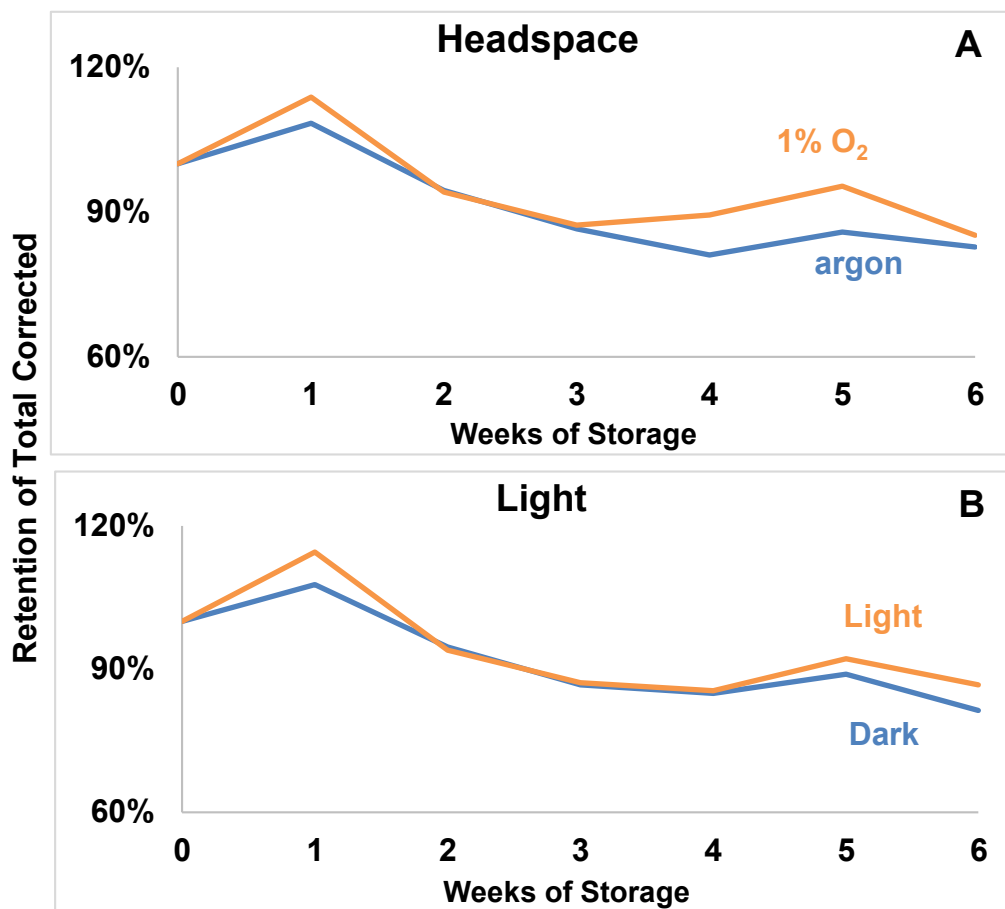


Figure 44: Effects of oxygen and light on retention of corrected total phenolics in CJC during six weeks storage under ambient conditions. A) argon versus 1% O₂; B) stored in the dark vs exposed to light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

Table 14: Significant sources of variance from ANOVA analysis of the retention of corrected total phenolics during the six week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Oxygen	0.0009	Oxygen exposure associated with higher CTP
Light	0.0199	Light exposure associated with higher CTP
Time x Ascorbate	0.0117	At weeks 5 and 6 ascorbate exposure associated with higher CTP
Time x Degassing	0.005	At week 1, degassing associated with higher CTP
Ascorbate x Degassing	0.0012	Degassing associated with higher CTP when not fortified with ascorbate, lower CTP when fortified
Ascorbate x Atmosphere	<.0001	Ascorbic acid fortification associated with lower CTP only when under 1% O ₂
Degassing x Atmosphere	0.0212	Impact of O ₂ exposure greater when aerated
Atmosphere x Light	0.0468	Combined effect is more than additive

5.3.7 Color

Processing. CJC exhibited two color changes during processing and storage – fading (increased L* values) and loss of red character (decreased a* value) probably due to browning. Ascorbic acid fortification led to lightening or fading of the color without changing the red hue in cranberry juice during processing (Figure 45 and Table 15.) This difference indicates that ascorbic acid radicals or oxygen radicals generated during its autoxidation attack anthocyanin pigments directly and destroy the conjugation required

for red color. No browning occurs in the process, probably because AscA reduced quinones before they can polymerized and did not simultaneously undergo browning itself. Degassing limited fading when AscA was present but had little effect on it in unfortified juice, verifying that oxidation of AscA mediated the fading. On the other hand, degassing reduced red color loss due to browning (decreased a^*) during processing by about 45% in both fortified and unfortified groups. This pattern supports phenolic oxidation and subsequent carbonyl condensation as a main source of browning and decreased a^* in CJC.

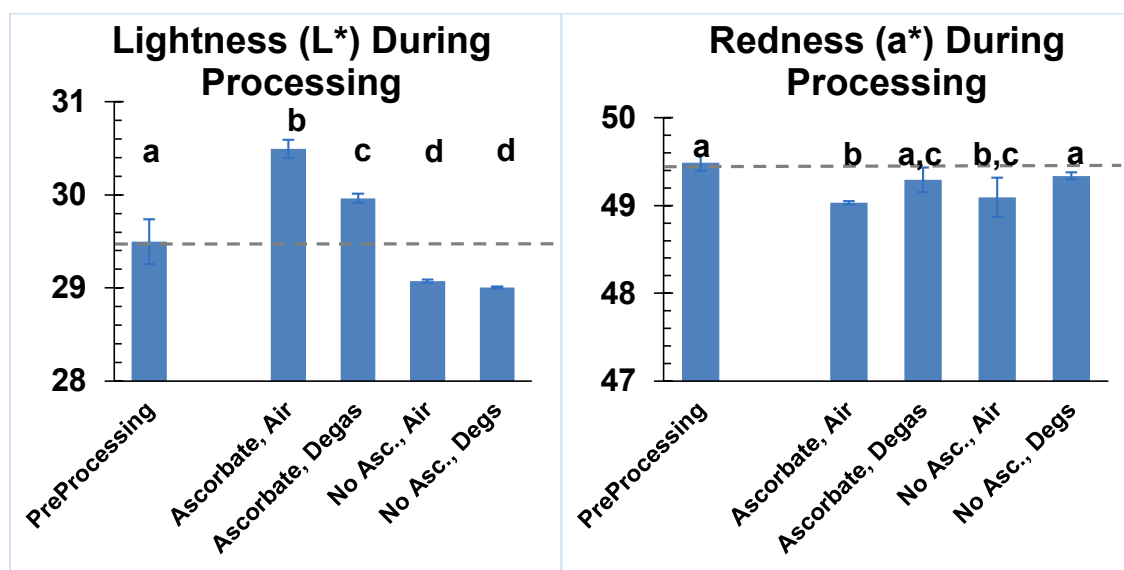


Figure 45: Effect of treatments on color change in CJC during processing. Colorimeter parameters L^* increases with lightness and a^* reflects redness of the hue. Different letters on the groups denote statistically significant differences ($p < 0.05$). Dotted lines indicate the initial, preprocessing L^* and a^* values.

Table 15: Significant sources of variance from ANOVA analysis of color parameters L* and a* for processing. Notes are interpretations of means plots generated by SAS (data not shown).

Source	P	Notes
<i>Lightness (L*)</i>		
Degassing	<0.0001	Degassing associated with darkening
Ascorbate Fortification	<0.0001	Ascorbate associated with lightening
Degassing x Ascorbate	<0.0001	Degassing has little/no effect unless ascorbate present
<i>Redness (b*)</i>		
Degassing	0.011	Degassing prevented red color loss

Storage. All factors and interactions were found to significantly increase color fading during storage, over and above processing effects (Figure 46 and Table 16). Ascorbic acid fortification had the largest impact, with ~50% increase in L* (lightness) after 6 weeks while unfortified groups barely registered a change over the same period. Storage atmosphere was similarly critical as groups stored under 1% O₂ gained ~40% in L* while in groups stored under argon had L* values rose only ~10%. The effect of light exposure was smaller with a ~35% increase in L* value when exposed to light and ~20% when protected from light. Preprocessing degassing led to less fading over the storage period (beyond that observed during processing): degassed groups lightened by ~7 L* units versus ~9 L* units in aerated CJC after 6 weeks. Meanwhile, groups that were protected from oxygen and not fortified darkened slightly, by ~1 L* (data not shown). The lightening observed for most groups can be attributed to loss of color intensity due to pigment degradation, which probably overshadows the formation of brown pigments

from browning reactions. The small amounts of darkening observed in the groups protected from oxygen and ascorbic acid are likely due to browning.

Similarly, there was greater loss of redness (color parameter a^*) during storage than during processing, and many factors and interactions contributed significantly (Figure 47 and Table 17.) Ascorbic acid fortification had the largest impact: fortified groups lost 42% of a^* while unfortified groups lost ~8% over 6 weeks. Storage atmosphere had a similarly large impact with 39% losses when exposed to oxygen and 12% losses when protected. Light exposure and degassing both had smaller but significant effects on red color with 20% and 26% more redness remaining in the protected groups after 6 weeks, respectively. Preprocessing degassing stabilized red color during storage (beyond the stabilization observed during processing). Redness (a^*) decreased by 22% after 6 weeks with degassing compared to 38% with aeration. Interactions indicate that light's damaging impact on red color requires oxygen exposure and is magnified if fortified with ascorbic acid and not degassed. Ascorbate fortification had many interactions, magnifying the damaging effects of the other variables in all instances. Interestingly, the protective effect of degassing increased after storage time, particularly when combined with protection from oxygen.

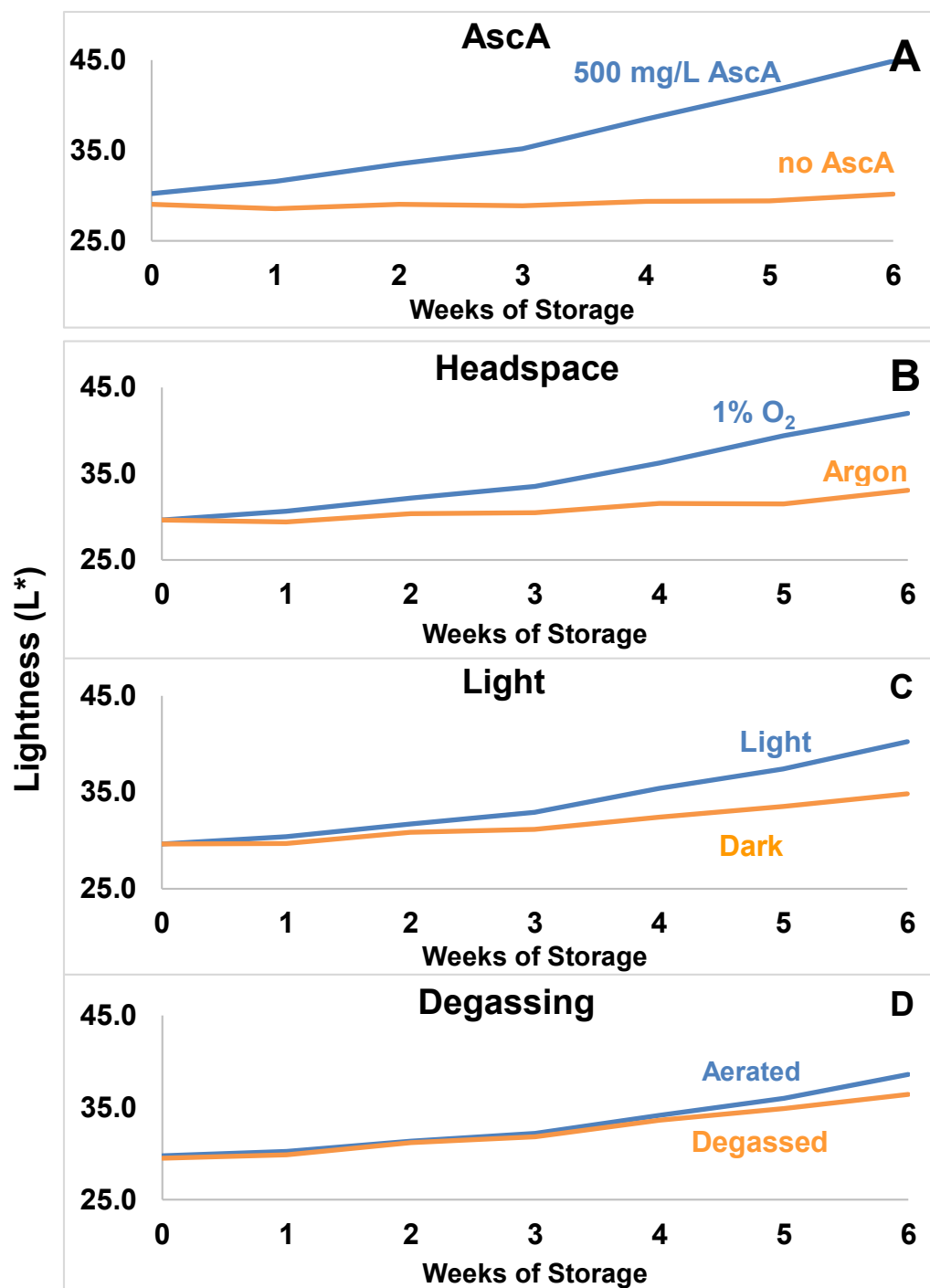


Figure 46: Effect of treatments on fading (colorimeter L* increases) in CJC stored for six weeks under ambient conditions. A) +/- fortification with 500 mg/L ascorbic acid; B) argon vs 1% O₂; C) dark vs light; D) preprocessing degassing. Values reported are averages of all conditions with and without each test factor. n=24 for each time point

Table 16: Significant sources of variance from ANOVA analysis of Lightness (L*) during the 6 week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbate fortification promotes lightening
Storage Atmosphere	<0.0001	1% O2 promotes lightening
Light Exposure	<0.0001	Light promotes darkening
Degassing	<0.0001	Aerating promotes darkening
Time x Ascorbate	<0.0001	Ascorbate effect increases over time
Time x Atmosphere	<0.0001	Atmosphere effect increases over time
Time x Light Exposure	<0.0001	Exposure effect increases over time
Time x Degassing	<0.0001	Degassing effect increases over time
Ascorbate x Atmosphere	<0.0001	Combination is more than additive
Ascorbate x Light	<0.0001	Combination is more than additive
Ascorbate x Degassing	<0.0001	No effect for degassing unless fortified with ascorbate
Atmosphere x Light	<0.0001	Combined effect more than additive
Atmosphere x Degassing	<0.0001	Degassing effect stronger when protected from O2
Light x Degassing	<0.0001	Effect of light stronger when aerated
Time x Ascorbate x Atmosphere	<0.0001	Combination is more than additive→Increases over time
Time x Ascorbate x Light	<0.0001	Combination is more than additive→Increases over time
Time x Ascorbate x Degassing	<0.0001	Combinations of ascorbate and degassing become more darkening over time
Time x Atmosphere x Light	<0.0001	Combination is more than additive→Increases over time
Time x Atmosphere x Degassing	<0.0001	At later time points, degassed samples under 1% O2 lighten less than aerated samples
Time x Light x Degassing	<0.0001	Effect of light stronger when aerated→Decreases over time
Ascorbate x Atmosphere x Light	<0.0001	With no ascorbate and under 1% O2, degassing more lightening than aeration
Ascorbate x Atmosphere x Degassing	<0.0001	Degassing has lightening effect when protected from exposed to oxygen and not fortified
Ascorbic x Light x Degassing	<0.0001	Degassing has lightening effect when exposed to light and not fortified
Atmosphere x Light x Degassing	<0.0001	Degassing has lightening effect when exposed to light and oxygen

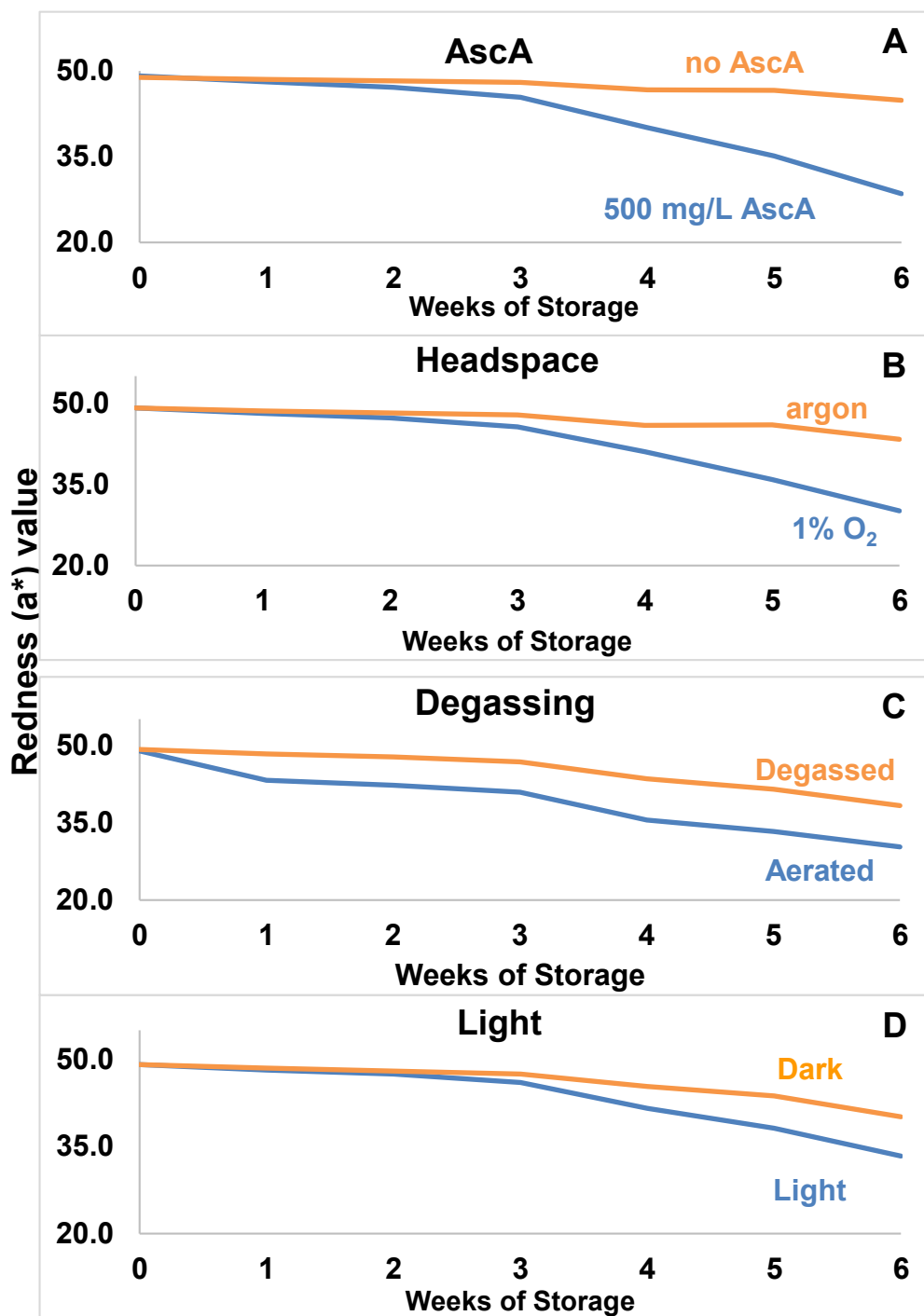


Figure 47: Effect of treatments on redness (colorimeter a*) in CJC stored for six weeks under ambient conditions. A) +/- fortification with 500 mg/L ascorbic acid; B) argon vs 1% O₂; C) preprocessing degassing; D) dark vs light. Values reported are averages of all conditions with and without each test factor. n=24 for each time point.

Table 17: Significant sources of variance from ANOVA analysis of Redness (a*) during the 6 week storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbate fortification promotes loss of red color
Storage Atmosphere	<0.0001	1% O ₂ promotes loss of red color
Light Exposure	<0.0001	Light promotes loss of red color
Degassing	0.0001	Aerating promotes loss of red color
Time x Ascorbate	<0.0001	Ascorbate effect increases over time
Time x Atmosphere	<0.0001	Atmosphere effect increases over time
Time x Light Exposure	0.0002	Light effect increases over time
Time x Degassing	0.0009	Degassing effect increases over time
Ascorbate x Atmosphere	<0.0001	Oxygen has little effect unless fortified with ascorbate
Ascorbate x Light	<0.0001	Combination is more than additive
Ascorbate x Degassing	<0.0001	No effect for degassing unless fortified with ascorbate
Atmosphere x Light	<0.0001	Light has no effect when protected from oxygen
Atmosphere x Degassing	<0.0001	Degassing protects red color only when under argon
Light x Degassing	0.0017	Effect of light stronger when aerated
Time x Ascorbate x Atmosphere	<0.0001	Combination is more than additive→Increases over time
Time x Ascorbate x Degas	0.0005	Combinations of aeration and ascorbate more degradative over time
Time x Ascorbate x Light	<0.0001	Combination of ascorbate and oxygen exposure more damaging over time
Time x Atmosphere x Light	0.0031	Combinations of light and oxygen exposure become more damaging over time
Time x Atmosphere x Degassing	<0.0001	Combination of aeration and oxygen exposure degrades red color→Effect increases over time
Ascorbate x Atmosphere x Light	<0.0001	Light exposure most impactful when both fortified with ascorbate and exposed to oxygen
Ascorbate x Atmosphere x Degassing	0.0241	Combinations of degassing and atmosphere have little/no effect unless fortified with ascorbic acid
Atmosphere x Light x Degassing	<0.0001	Light had no effect when degassed and protected from oxygen

5.4 Phase IV – Model Study

Results of the model study seeking to identify degradation products of purified flavonoids were disappointing (Table 18). Each of the flavonoids added to the model were detected but only few other peaks were found that might be degradation products. Perhaps the bulk of degradation products were not within the range of our detection with mass to charge ratios outside 100-2000 m/z. Another explanation is that the degradation products are so diverse that few single species were present at sufficient quantity to be detected.

Among samples including ascorbic acid (groups 5-8), two peaks not found in the other groups were observed with mass-to-charge ratios (m/z) of 1175 at 0.87 minutes and 625 at 1.02 minutes. The 625 m/z peak is possibly M^+ of an ascorbic acid conjugate of the anthocyanin minus hydrogen ($449+177-1 = 625$). No plausible identity for the 1175 m/z peak could be found, but the molecular weight suggests that it is condensation product of two or more flavonoids having undergone unknown rearrangements.

Other HPLC peaks detected by UV hint at the presence of additional compounds. Peaks absorbing at 280 nm, typical of PACs, were observed at 4.1, 6.1 and 9.1 minutes. A small peak with maximum absorbance near 520 nm, typical of anthocyanins, appeared at 1.3 minutes. Another peak with local maximum absorbance at 357 nm, typical of flavonols, was detected at 2.1 minutes. However, none of these peaks yielded sufficient mass spectral data for plausible identities to be postulated.

Table 18: Mass to charge (m/z) of ions detected and potential identities of peaks observed from HPLC-PDA-MS of extracts from model solution study. Odd number groups under 1% O₂, even groups under argon. Groups 1,2,5 and 6 were degassed prior to processing. Groups 5-8 are fortified with 500 mg/L vitamin C.

	Observed m/z (Positive Mode)									
Time	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Notes	Possible ID
0.76		431, 227	227		227	227	431, 227	431, 227		Cyanidin-3-glucoside minus water (431, [M+])
0.87					1175	1175	1175, 240	1175, 240		
0.89	577, 487, 425, 303	487, 240	487, 240	240			577, 487, 319			
0.92	449, 287	449, 287	449, 287	449, 287	449, 287	449, 287	287	577, 487, 449, 287	abs at 520nm	Cyandin-3-glucoside (449, [M+])→ Cyanidin (287, [M+])
0.94	487, 425, 303			487, 465, 303	487, 465, 303		303	425, 365, 303	abs at 365	Quercetin-3-galactoside (487 [M+Na], 465 [M+H])→ Quercetin ([M+H], 303)
0.97	577, 487, 425, 303, 287	577, 303	465, 425, 303	577	489, 465, 303	577, 425	425		abs at 280nm	Proanthocyanidin A2 (577, [M+H]); Quercetin-3-galactoside (465 [M+H])→ Quercetin ([M+H], 303)
1.02			577		625	625		625		Cyanidin-3-glucoside+ascorbic acid
1.14						609	489, 253,			
1.3	425	177		232, 177	589				small abs at 520nm	anthocyanin derivative?
1.49	905, 359, 303									dihydroxycoumarin glucoside? (359, [M+H])
1.87		531		640?, 531			669, 668			
2.05	581, 177							1238	abs at 237, 270, 357nm	flavonol deriviative?
2.5					488			338, 173		
2.7		214	214	214	214		214	214		
3.05			1524, 1479							
4.09	725, 223								abs at 280nm	PAC derivative?
6.9									abs at 280nm	PAC derivative?
9.13	653								abs at 280nm	PAC derivative?
13.2	641, 595	643, 641								

5.5 Phase V – Practical Experiments

In this final portion of the research, we evaluated several of the same variables as in Phase III, but under more practical conditions. The use of practical plastic packaging (10 oz bottles with and without oxygen barriers) and a three month storage period (as opposed to six weeks) were the main differences, but some more subtle differences were present as well. The CJC was prepared with a different batch of raw cranberry juice and with ground water rather than high purity water. The non-degassed groups were stirred vigorously to approximate the aeration that occurs during practical juice mixing (rather than being sparged with air). Also, taking into consideration results from Phase III, effects of preprocessing degassing were not evaluated independently, but only in combination with barrier bottles in order to reduce the number of groups for analysis from 16 to 12.

Overall, processing effects mirrored Phase III results for the most part (Figure 48A), but several notable differences in storage effects were documented, (compare Figure 30B with Figure 48B.) Most important for this study was that barrier bottles failed to provide the same universal protective effect as the more thorough exclusion of oxygen in Phase III.

Because of the unbalanced study design of this portion of research, charts exclude some groups in order to convey balanced data. For example, because the effect of degassing was only accessed in barrier bottles, non-barrier bottles groups are excluded in graphs showing degassed versus no treatment. The GLM statistical analysis used here accesses the whole dataset, taking the unbalanced design into account.

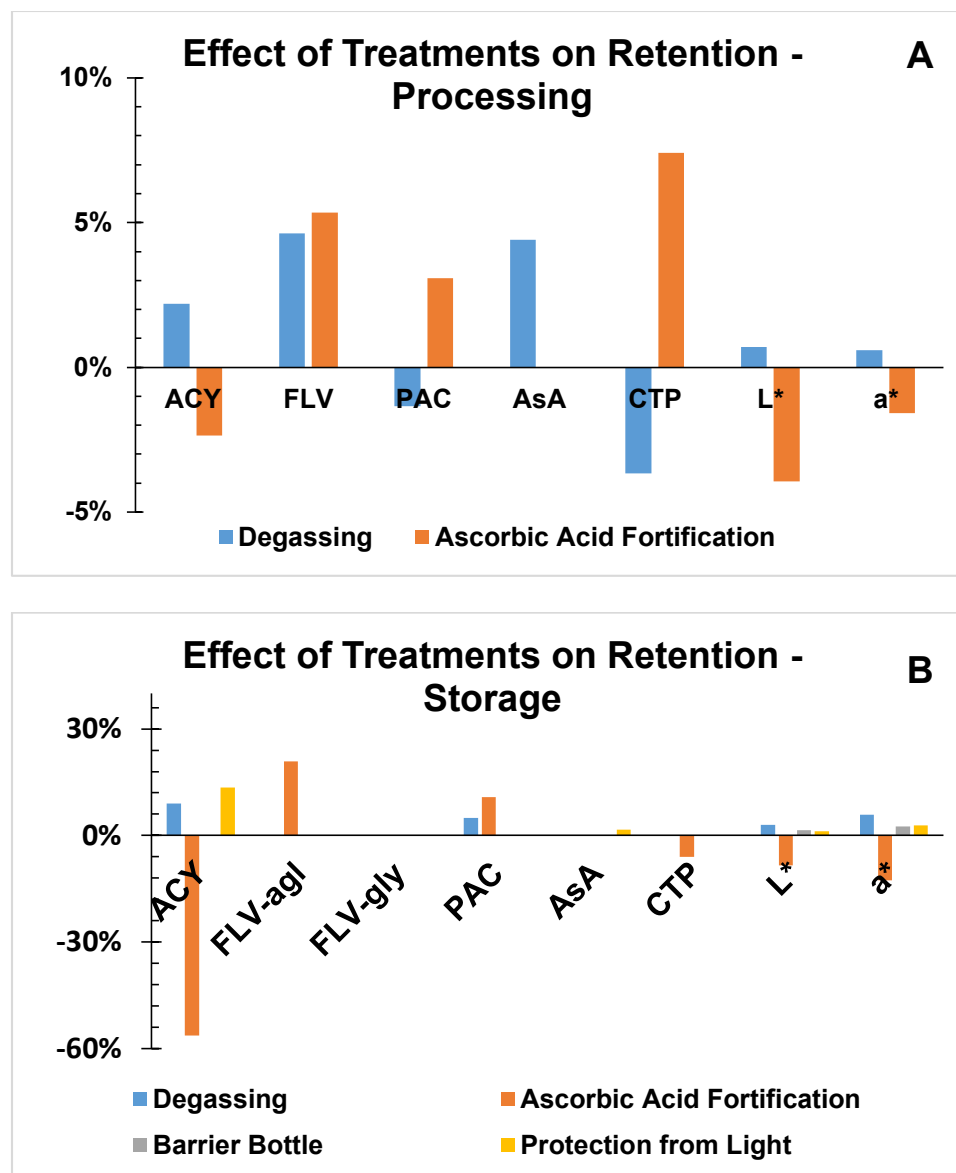


Figure 48: Overview of the main effects of treatments for Phase V experiments after processing (A) and three months of storage (B). Only statistically significant effects are shown ($p > 0.05$) and results are expressed relative to the average of all conditions tested.

5.5.1 Dissolved Oxygen (DO)

Before processing, non-treated juice had DO levels of ~5 mg/L. Degassing lowered DO content to 0.05-0.08 mg/L. For storage, DO results are markedly different

than in laboratory experiments presumably due to the dynamics of slow oxygen permeation through plastic packaging. Figure 49 shows each factor's effect on DO. Here, ascorbic acid fortification suppressed DO levels, limiting DO content by a factor of ~ 8.5 after three months storage. Similarly, light exposure suppressed DO levels but to a lesser degree, with ~ 5 times more oxygen accumulating in bottles stored in the dark. Storage in barrier bottles was less impactful on DO content than anticipated, resulting in only $\sim 20\%$ reduction in DO versus standard bottles after 3 months. The effect of degassing prior to processing partly persisted after storage with ~ 0.1 mg/L less DO in degassed bottles immediately after processing and ~ 0.05 mg/L less DO after three months. The average DO for all samples was 0.29 mg/L.

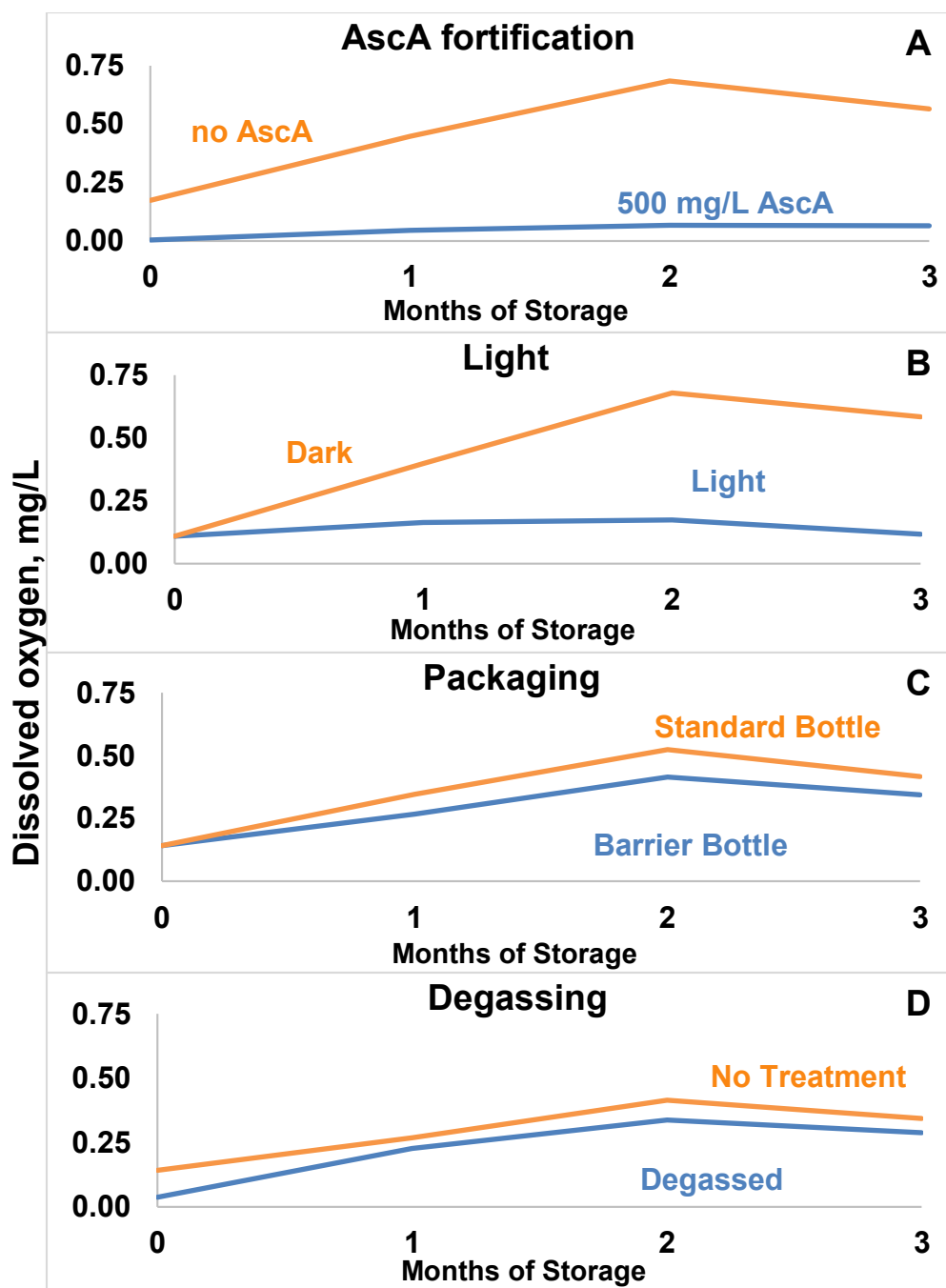


Figure 49: Effect of treatments on dissolved oxygen in CJC stored for three months under ambient conditions. A) +/- ascorbic acid fortification; B) light versus dark; C) barrier bottles versus standard bottles; D) +/- degassing before processing. Values reported are averages of all conditions with and without each test factor. n=12 for each data point.

5.5.2 Anthocyanins

Anthocyanin concentration of raw juice for this study was ~45 mg/L, comparable to the earlier experiments.

Processing. Loss of anthocyanins was less with degassing than without. With or without degassing, fortification of CJC with ascorbic acid increased anthocyanin degradation. Adding ascorbic acid without degassing led to 25% loss of anthocyanins while all other groups lost ~20% (Figure 50). General linear model (GLM) statistical analysis indicates both degassing ($p=0.0171$) and ascorbic acid fortification ($p=0.0432$) are independent significant factors without interaction. As in the laboratory phase, the individual ACY species degraded equally (data not shown).

Storage. After three months storage, ACYs had degraded 50-90% depending on conditions. Ascorbic acid fortification was again the most damaging main effect, with an average of nearly three times more ACYs remaining in unfortified groups. Protection from light and degassing decreased ACY loss by 7% and 3% respectively (Figure 51). Barrier bottles failed to provide any stabilization of ACYs after 3 months but did provide a modest 6% increased retention of ACYs at 2 months (data not shown)

Some interactions were also present (Table 19.) In contrast to the laboratory experiments, light had less damaging impact in CJC fortified with ascorbic acid than when not fortified.

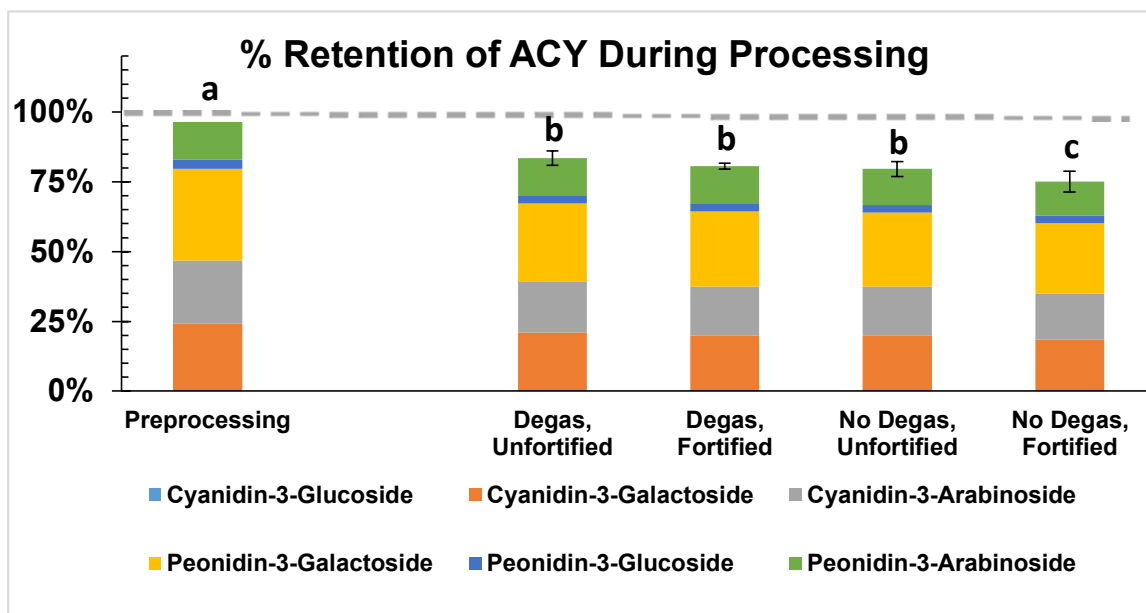


Figure 50: Effects of degassing and ascorbic acid fortification on retention of anthocyanins during processing. Error bars show standard deviation for total anthocyanins. Groups labeled with a common letter have statistically equivalent levels of total anthocyanins ($p < 0.05$). The dotted line indicates the initial, preprocessing ACY content.

Table 19: Significant sources of variance from GLM analysis of anthocyanin retention for the 3 month storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbic Acid promotes ACY degradation
Light	<0.0001	Light promotes ACY degradation
Preprocessing Degassing	<0.0001	Degassing promotes ACY retention
Time x Ascorbate	<0.0001	Effect of ascorbate increases over time
Time x Degassing	0.0072	Effect of degassing decreases over time
Time x Bottle	0.0149	Barrier bottle promotes retention at month 2
Ascorbate x Light	0.0037	Effect of light more pronounced when not fortified with ascorbic acid

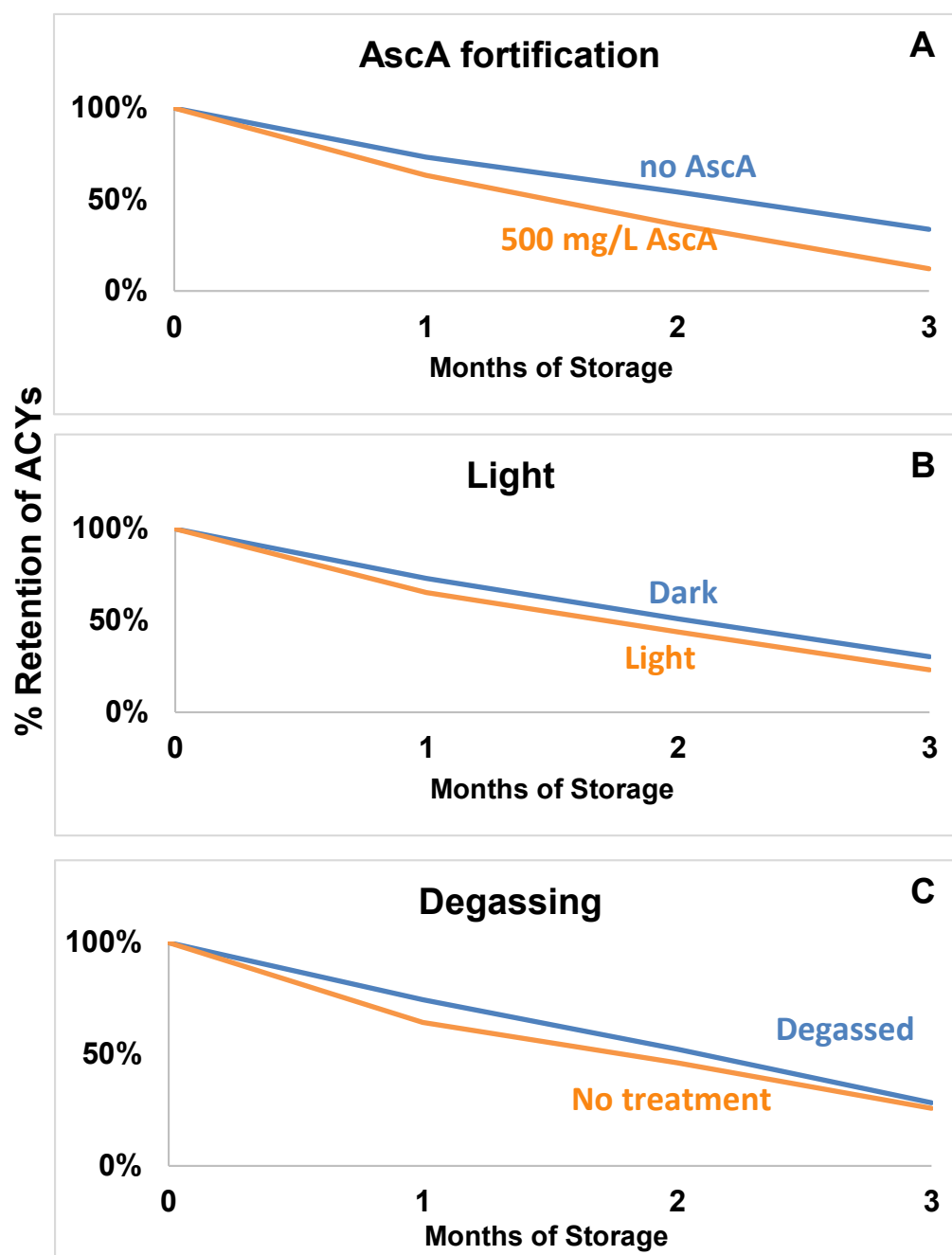


Figure 51: Effects of treatments on anthocyanin retention during three months storage under ambient conditions. A) +/- ascorbic acid fortification; B) light versus dark; C) +/- degassing prior to processing. Values reported are averages of all conditions with and without each test factor. n=18 in A and B; n=12 in C for each data point.

5.5.3 Flavonols

The flavonol composition of raw juice used here was different than in the laboratory experiment. Here, only ~40 mg/L total flavonols were present, about 30% less than the juice used in the laboratory experiments. Aglycones, in particular, were less concentrated: 1.15 mg/L myricetin and 10.62 mg/L quercetin were detected versus 5.63 mg/L and 14.23 mg/L earlier, respectively.

Processing. The group that was not degassed or fortified lost nearly 20% of total flavonols while other groups lost ~10% (Figure 52). Degassing ($p=0.0039$) and ascorbic acid fortification ($p=0.0017$) were both significantly protective towards flavonols during processing. Most of the flavonol losses for processing (60% of the total) were due to loss of aglycones; glycosides were less affected.

Storage. Different stability between aglycones and glycosides was observed, so these two groups were analyzed separately. The only significant main effect (other than time) for either aglycones or glycosides was promotion of aglycone stability by ascorbic acid (Table 20). This effect was large: there was 5x more retention of aglycones when fortified with ascorbic acid than not (Figure 53). In contrast, glycosides as a whole were slightly more stable at the end of three months with no ascorbic acid fortification (time x ascorbic acid interaction was significant at $p = 0.0005$). For individual flavonol glycoside retention, disparate effects for ascorbic acid were observed: ascorbic acid stabilized myricetin-3-galactoside (M3G) ($p<0.0001$) by 3%, did not affect quercetin-3-galactoside (Q3G), and destabilized quercetin-3-rhamnoside (Q3R) ($p=0.0002$) by 4%.

The effect of degassing closely approached significance for aglycones ($p=0.0508$) and glycosides ($p=0.0585$). In both, the increase in flavonol retention after processing

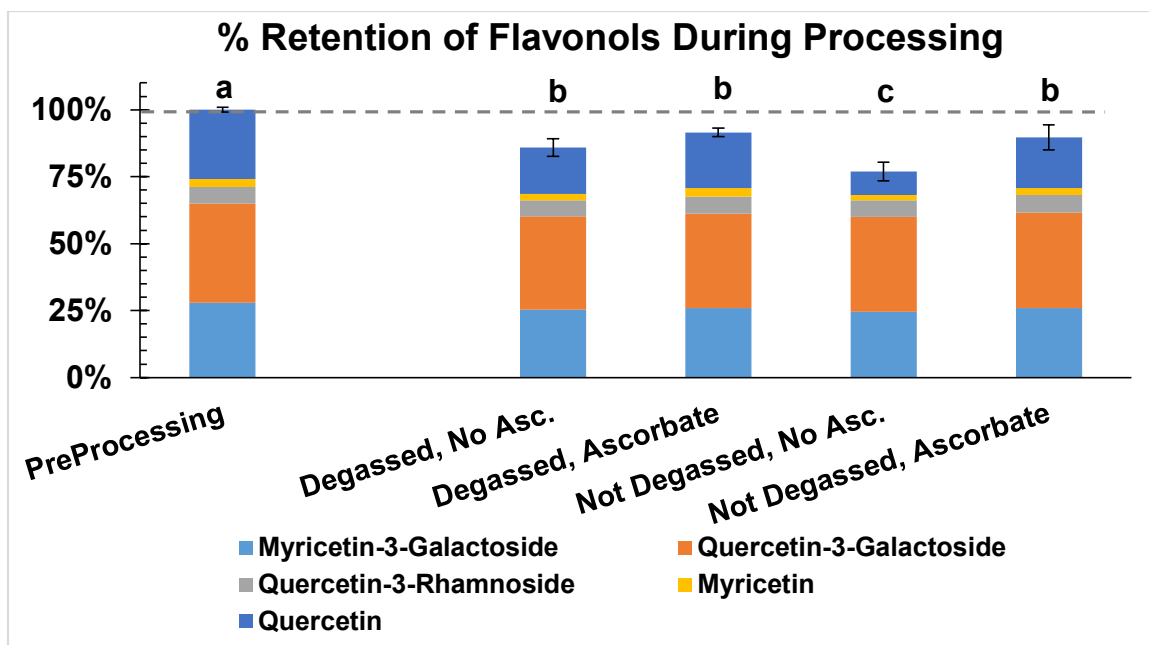


Figure 52: Effect of treatments on retention of flavonols during processing. Error bars show standard deviation for total flavonols. Groups that are not labelled with a common letter are statistically different for total flavonols ($p < 0.05$). The dotted line indicates the initial, preprocessing FLV content.

was partially lost over time in degassed groups. At three months, degassed CJC contained more aglycones than the juice not treated, ~7 mg/L vs 5.8 mg/L, but contained slightly lower levels of glycosides (21.7 mg/L vs. 22.9 mg/L). For M3G however, the effect of degassing was highly significant ($p < 0.0001$), providing an average of 8% increased retention, although the effect weakened over time.

Light and bottle type did not significantly affect retention of total flavonols, glycosides/aglycones, or for individual flavonol species. Light and time interacted for glycosides, whereas protection from light provided some stabilization at month 1 but not at later time points.

Table 20: Significant sources of variance from GLM analysis of flavonol aglycones and glycoside retention for the 3 month storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
<i>Aglycones</i>		
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbic Acid is protective
Time x Ascorbate	<0.0001	Effect increases over time
Time x Degassing	0.0057	Benefit of degassing decrease over time
Ascorbate x Degassing	0.0037	Ascorbate more protective when not degassed
<i>Glycosides</i>		
Time	<0.0001	
Time x Ascorbate	0.0005	At final timepoint, ascorbate is damaging; at others, ascorbate appears stabilizing
Time x Degassing	<0.0001	Benefit of degassing decrease over time
Time x Light	0.0068	Protection from light promotes retention only at month 1
Degassing x Ascorbate	<0.0001	Ascorbate is protective when not degassed, damaging when degassed

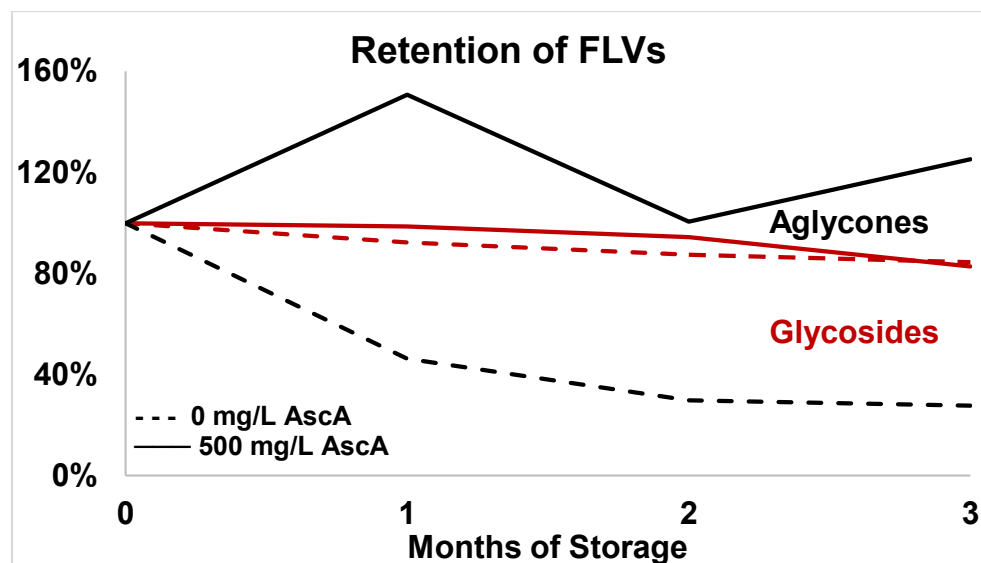


Figure 53: Effect of ascorbic acid on flavonol aglycone and glycoside retention during three months storage under ambient conditions. $n=18$ for each data point.

5.5.4 Proanthocyanidins

Processing. The PAC content of raw juice was much lower than in the laboratory trials with ~95 mg/L here and ~150 mg/L earlier. For processing, all groups gained PACs as measured by the DMAC assay (Figure 54). GLM analysis shows effects for degassing ($p=0.0076$), ascorbic acid addition ($p<0.0001$) and their interaction ($p=0.0457$). Degassing was associated with lower increases in PACs. Meanwhile, ascorbic acid addition led to larger increases in PACs, up to 22% when juice was not degassed.

Storage. Degassing and ascorbic acid addition were significant main effects and interactions (Table 21). Degassing and ascorbate fortification mildly protected PACs during storage, with both treatments resulting in 7-8% increased retention after three months (Figure 55).

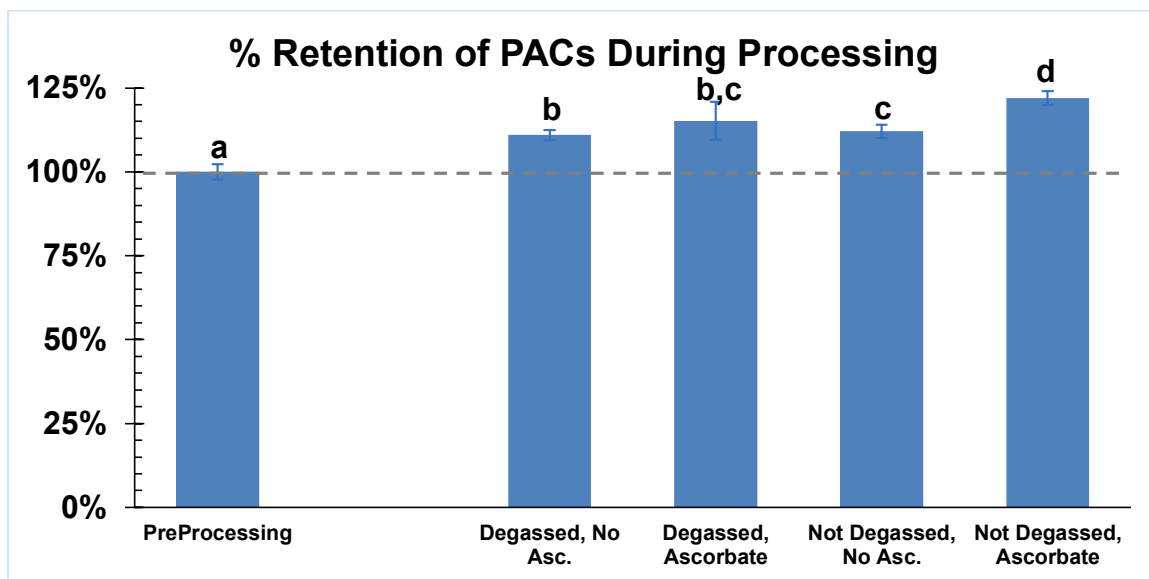


Figure 54: Percent retention of PACs for processing. Error bars show standard deviation for total flavonols. Groups that are not labelled with a common letter are statistically different for total flavonols ($p < 0.05$). The dotted line indicates the initial, preprocessing PAC content.

Table 21: Significant sources of variance from GLM analysis of PAC retention for the 3 month storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbic Acid promotes PAC retention
Preprocessing Degassing	<0.0001	Degassing promotes PAC retention
Time x Ascorbate	0.0003	Effect of ascorbate increases over time
Time x Degassing	0.0015	Effect of degassing less at month 2

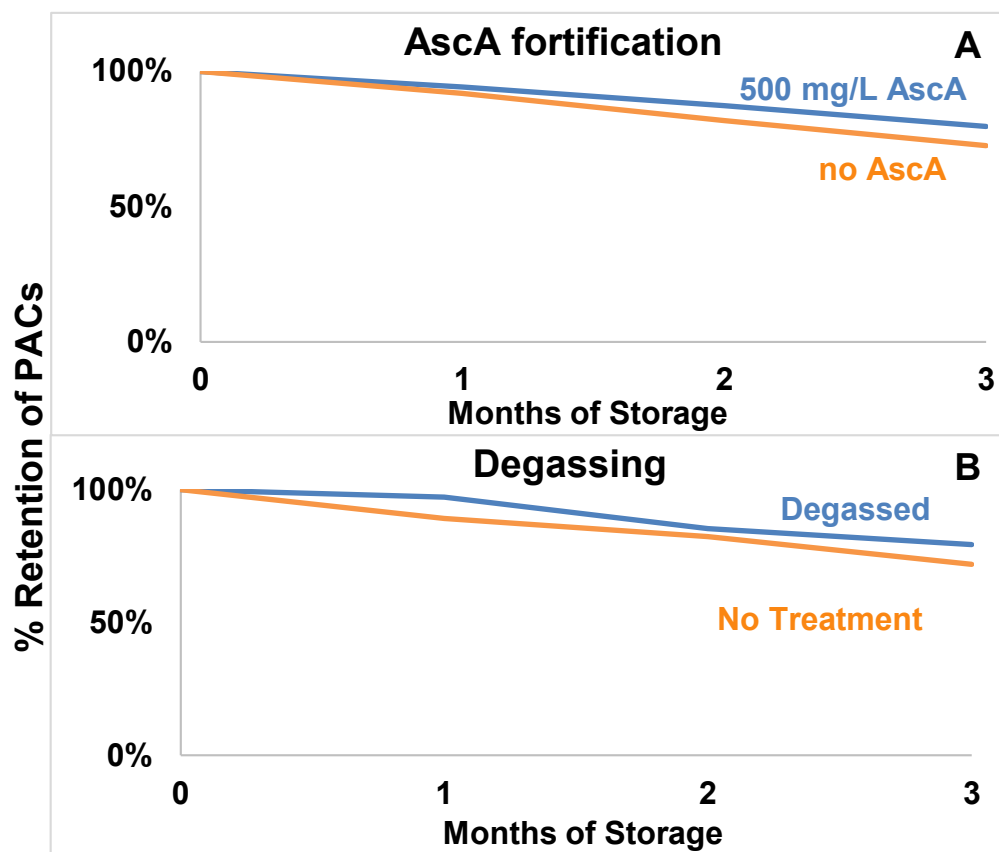


Figure 55: Effect of ascorbic acid and degassing on PAC retention during three months storage under ambient conditions. A) +/- ascorbic acid fortification; B) +/- degassing prior to processing. Values reported are averages of all conditions with and without each test factor. n=18 in A and n=12 in B for each data point.

5.5.5 Ascorbic Acid

Ascorbic acid was not measured prior to processing but juice was fortified with 500 mg/L. Degassing before processing reduced vitamin C losses to half those in untreated juice ($p < 0.05$, Figure 56).

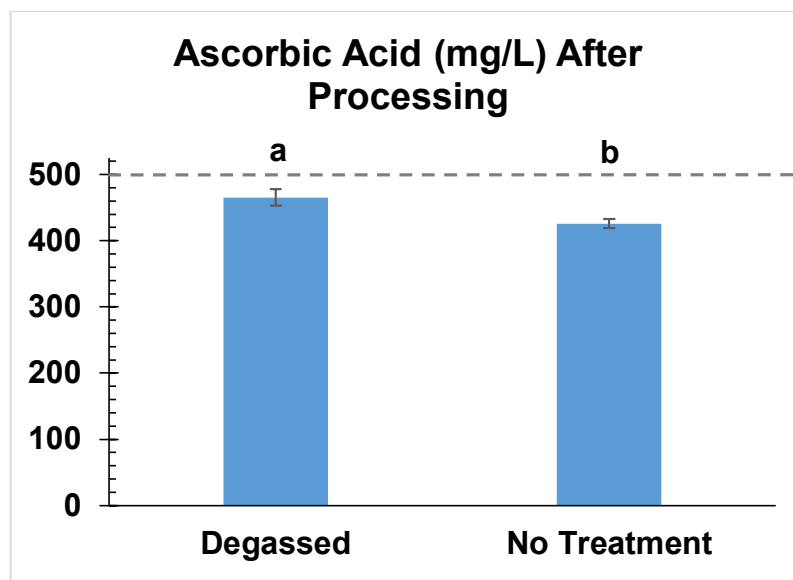


Figure 56: Ascorbic Acid concentrations after processing. The dotted line indicates 500 mg/L, the nominal AscA concentration before processing. Groups that are not labelled with a common letter are statistically different ($p < 0.05$). $n=6$ for each bar.

During storage, protection from light stabilized ascorbic acid by 3% after three months ($p=0.0159$) (Figure 57). Barrier bottles tended to provide a small protective effect (2%, $p=0.0812$). Degassed juice retained most of the additional ascorbic acid conserved during processing. No interactions were observed here.

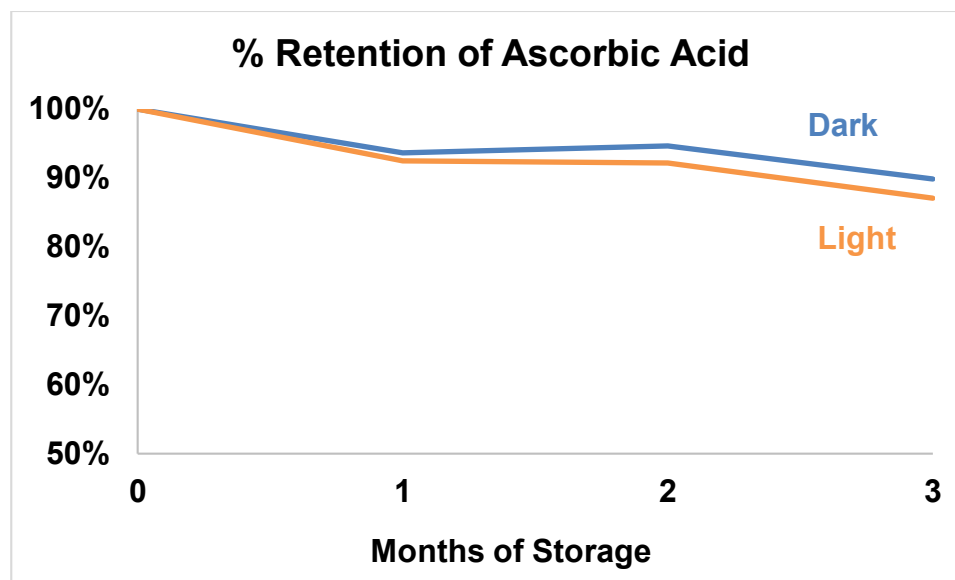


Figure 57: Effect of light on retention of ascorbic acid during three months storage under ambient conditions. n=18 for each data point.

5.5.6 Total Phenolics

Processing. All groups gained total corrected phenolics during processing, by an average of 16% (Figure 58). Degassing prior to processing resulted in 8% lower levels of CTP compared to untreated groups ($p < 0.0001$) while ascorbic acid addition resulted in 16% higher levels of CTP compared to unfortified groups ($p < 0.0001$). The degassing-ascorbic acid interaction was also significant ($p < 0.0042$) as the increase observed with ascorbic acid fortification was larger when not degassed. Factors associated with oxidation, it seems, cause increases in the CTP levels during processing, an unexpected result especially considering the degradation of phenolic compounds (ACYs and FLVs) observed under those conditions. Perhaps losses of those flavonoids resulted in the formation of other phenolic species that were more reactive to the Folin reagent, yielding higher results for CTP.

Storage. Ascorbic acid addition was the only significant main effect on CTP levels (Table 22). Ascorbic acid addition reduced retention of CTP by one-third after three months (Figure 59). Light exposure promoted higher CTPs only when ascorbic acid was present ($p < 0.0035$). Interactions are shown in Table 22.

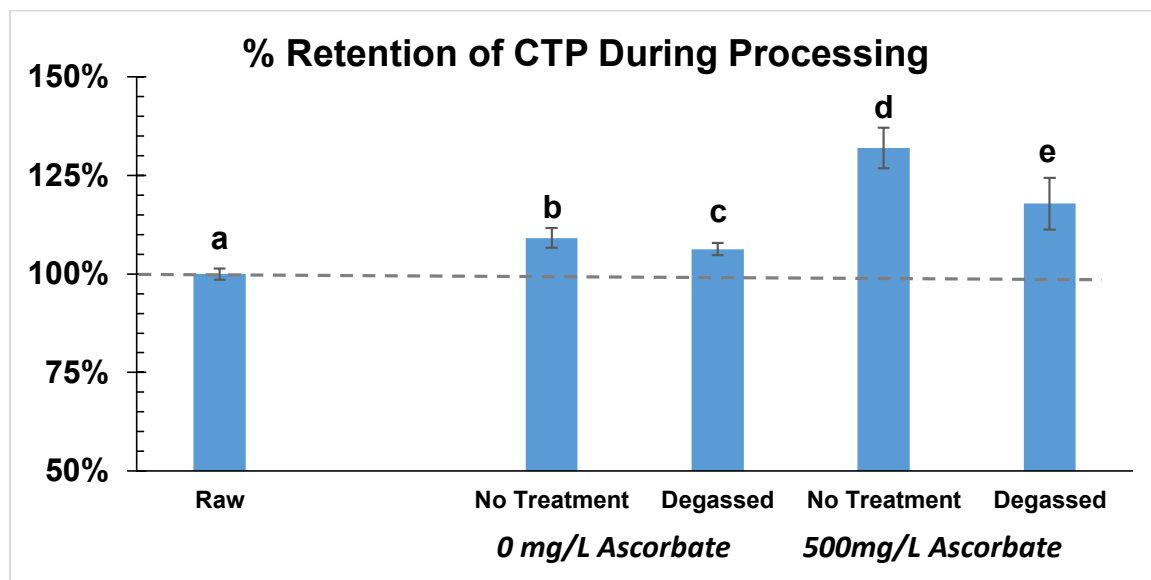


Figure 58: Effect of degassing on retention of total phenolics during processing, corrected for interference from vitamin C. Error bars show standard deviation of each group. $n=3$ for each bar. Groups labeled with a common letter have statistically equivalent levels of total flavonols ($p < 0.05$).

Table 22: Significant sources of variance from GLM analysis of corrected total phenolics retention during three months storage. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
Time	0.0438	CTP increase over time
Ascorbic Acid Fortification	<0.0001	Fortification lowered CTPs
Ascorbate x Light	0.0035	Light promoted higher CTPs only when fortified
Time x Bottle	0.0113	At 3 months, barrier bottles promoted higher CTPs

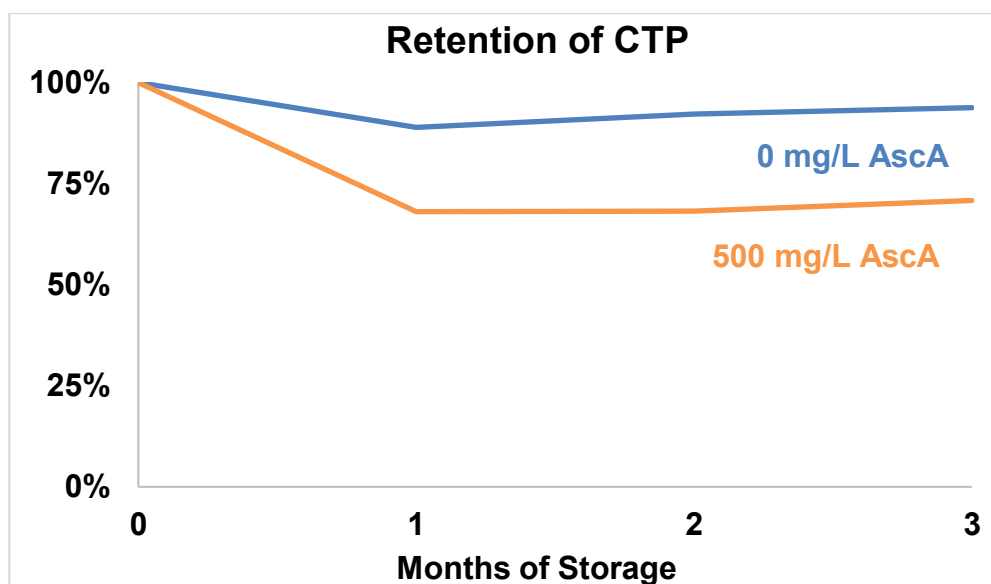


Figure 59: Effect of ascorbic acid fortification on retention of corrected total phenols in CJC during three months storage under ambient conditions. n=18 for each data point.

5.5.7 Color

Processing. After processing, all groups became lighter and less red (Figure 60). Ascorbic acid addition promoted both loss of redness and lightening ($p < 0.0001$ for both)

while degassing tended to promote less lightening and red color retention, compared to the opposite treatments ($p=0.0881$ and $p=0.0630$, respectively). No interactions were observed.

Storage. All main effects as well as several interactions were observed for lightness (Table 23). Ascorbate addition was the most impactful factor, with fortified CJC samples about 10 units L^* higher than unfortified samples at the end of three months. Degassing, barrier bottles and dark storage all prevent this lightening by smaller margins (~ 2 units L^*) versus their opposite treatments (Figure 61). Interactions reveal that light degraded juice color more when ascorbate was present and especially when CJC with ascorbate was not degassed. Direct effects of ascorbate were more prominent with no degassing treatment.

For color redness, all main effects were significant as were many interactions (Table 23). Ascorbic acid addition again was the single largest factor promoting about 3 times higher losses of color parameter a^* after three months. Degassing and protection from light prevented loss of redness to a lesser degree, by about 30% (Figure 62). The effect of the barrier bottle was small but significant, preventing about 20% of the color loss observed in standard bottles. Light exposure led to greater losses of red color in the presence of ascorbate. The effect of preprocessing degassing on red color increased over time and protected juice from red color loss due to ascorbic acid fortification.

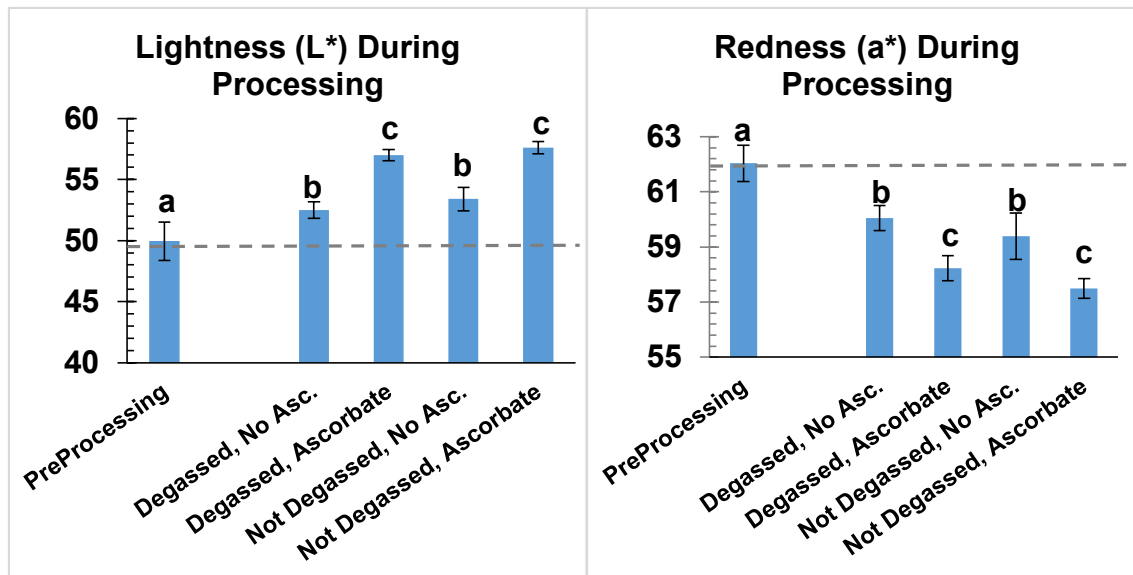


Figure 60: Effect of treatments on color parameters Lightness (L*) and Redness (a*) during processing. Groups labeled with a common letter are statistically equivalent ($p < 0.05$). $n=3$ for each bar.

Table 23: Significant sources of variances in GLM analysis of lightness (L^*) and redness (a^*) for the three months storage period. Notes are interpretations of means plots generated by SAS (data not shown).

Source	p	Notes
L^*		
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbate promotes lightening
Light	<0.0001	Light promotes lightening
Degassing	<0.0001	Degassing retards lightening
Bottle	<0.0001	Barrier bottle retards lightening
Time x Ascorbate	<0.0001	Effect increases over time
Ascorbate x Light	0.0064	Light promotes lightening only with ascorbate
Ascorbate x Degassing	0.0477	Ascorbate has larger effect when not degassed
Time x Ascorbate x Light	0.0171	Light promotes lightening only with ascorbate →effect increases over time
Time x Ascorbate x Degassing	0.0017	Ascorbate has larger effect when not degassed → effect increases over time
Degassing x Ascorbate x Light	0.0214	Combination of ascorbate, no degassing →light has larger effect
a^*		
Time	<0.0001	
Ascorbic Acid Fortification	<0.0001	Ascorbate promotes loss of red color
Light	<0.0001	Light exposure promotes loss of red color
Degassing	<0.0001	Degassing promotes red color retention
Bottle	<0.0001	Barrier bottle promotes red color retention
Time x Ascorbate	<0.0001	Effect increases over time
Time x Light	0.0028	Effect increases over time
Time x Degassing	<0.0001	Effect increases over time
Ascorbate x Light	0.0001	Effect of light much stronger with ascorbate fortification
Ascorbate x Degassing	<0.0001	Combined effect more than additive
Time x Ascorbic x Light	0.0014	Combined effect more than additive→ Increases over time
Time x Ascorbate x Degassing	<0.0001	Combination of no degassing and ascorbate fortification more damaging at later time points
Ascorbic Light x Degas	0.0381	Effect of light stronger with combined with no degassing and ascorbate fortification

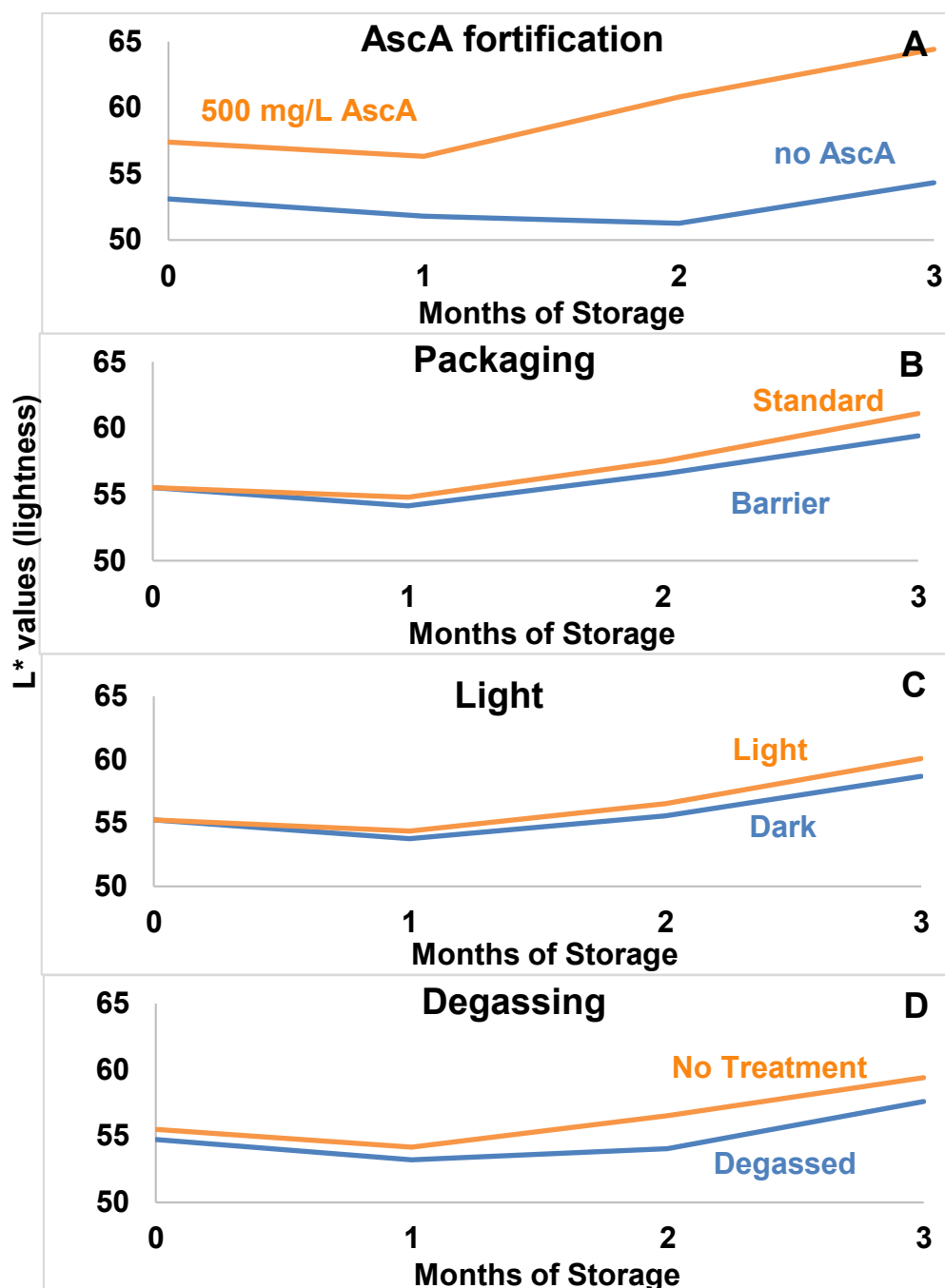


Figure 61: Effect of treatments on color Lightness (L*) of CJC during three months storage under ambient conditions. A) +/- ascorbic acid fortification; B) barrier versus standard bottles; C) light versus dark; D) +/- degassing prior to processing. Values reported are averages of all conditions with and without each test factor. n=18 in A and C; n=12 in B and D for each data point.

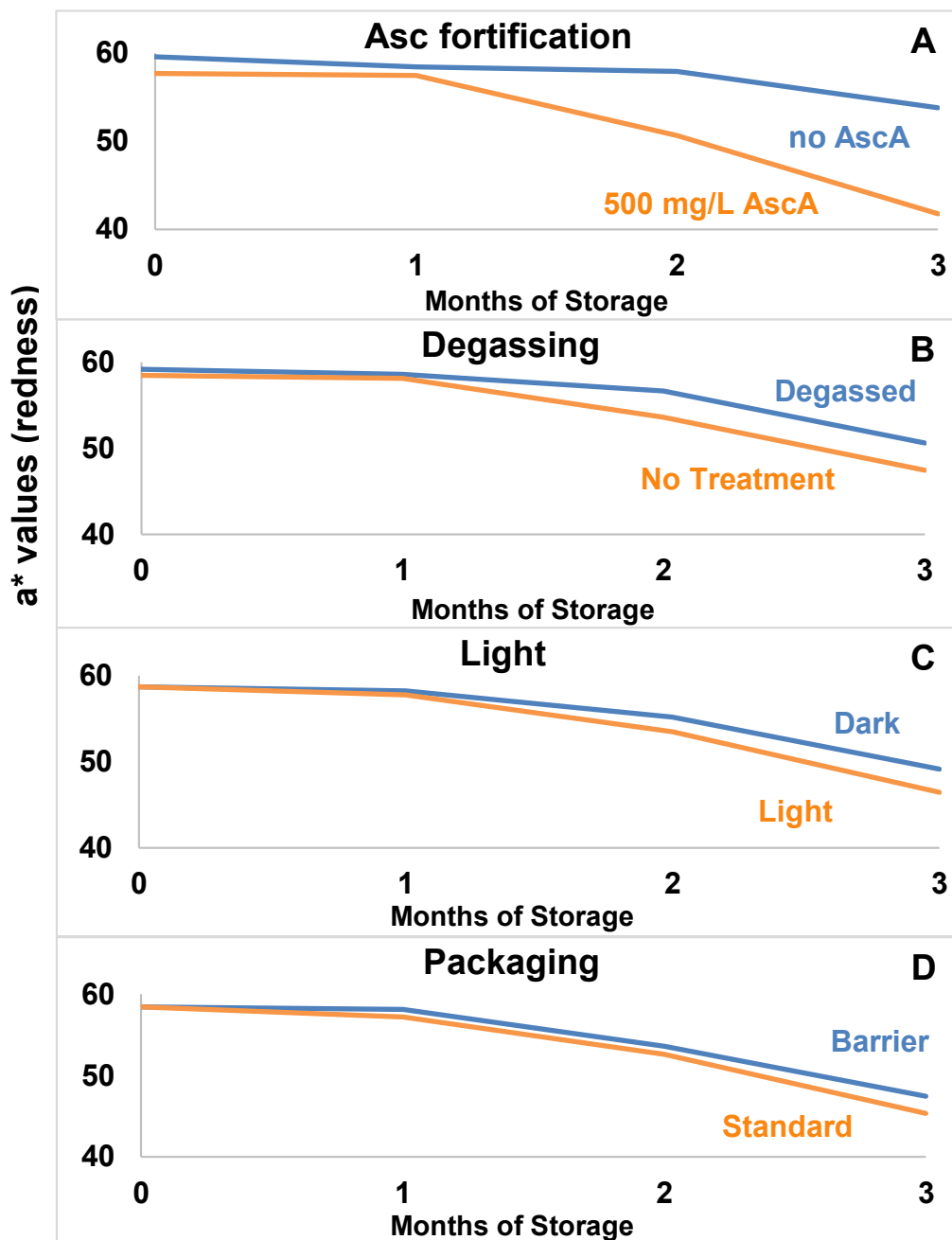


Figure 62: Effects of treatments on color redness (a^*) in CJC during three months storage under ambient conditions. A) +/- ascorbic acid fortification; B) +/- degassing prior to processing; C) light versus dark; D) barrier versus standard bottles. Values reported are averages of all conditions with and without each test factor. $n=18$ in A and C; $n=12$ in B and D for each data point.

6. DISCUSSION

Throughout this research, we evaluated several factors involved in oxidation -- oxygen exposure, light exposure, vitamin C fortification and degassing prior to thermal processing -- for their role in the stability of cranberry juice phytochemicals, ascorbic acid and color quality. In Phase I, we developed and validated the methodology to conduct high throughput analysis of cranberry flavonols. In Phases II-IV, cranberry juice was tested in tightly defined conditions in order to control oxygen exposure to better understand the interactions of the factors without regard for some real world practicalities, most notably the ingress of oxygen through packaging. In the final stage of the research, we used real packaging to better understand the interplay of test factors in practical conditions. In this discussion, we will highlight meaningful findings and provide explanations for the phenomena observed when possible. Exploration of the inconsistencies between the various experiments is necessary for clarity but also often gives valuable insights into the complicated interplay of factors at work.

Cranberry flavonoid classes reacted differently to the various treatments. While anthocyanins responded similarly to one another, individual flavonol species had differing responses that could mostly be grouped together for aglycones and glycosides. In terms of general stability for all the conditions, PACs > flavonol glycosides > flavonol aglycones > anthocyanins during storage (Figure 63).

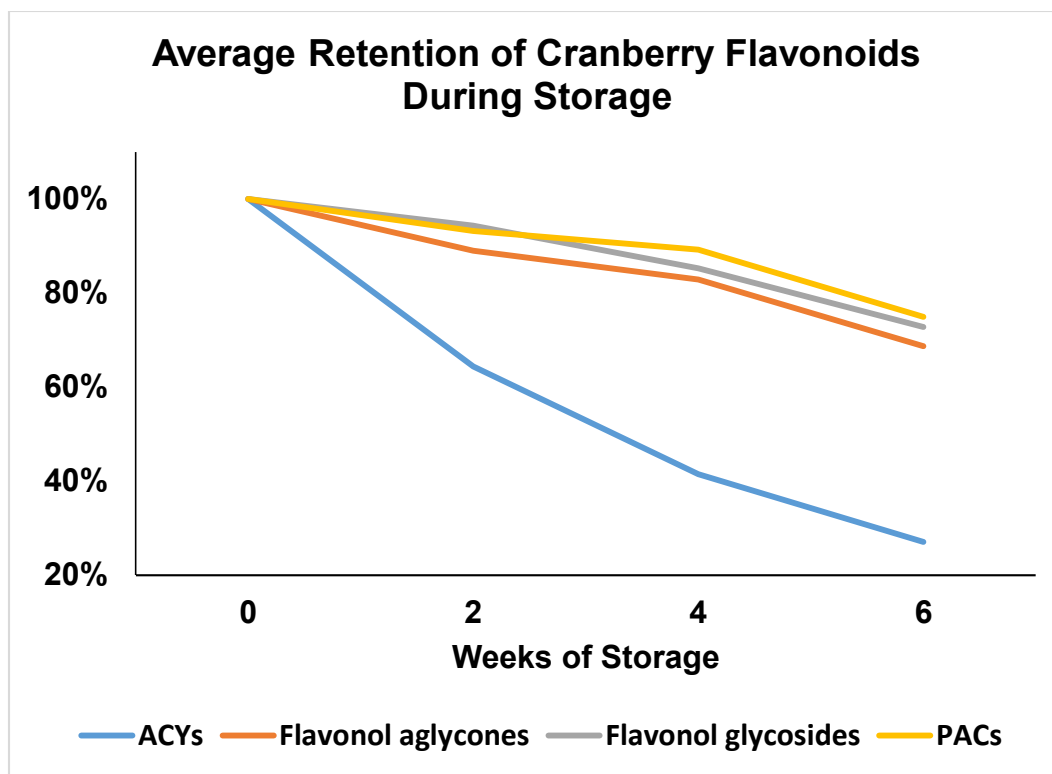


Figure 63: Stability of cranberry flavonols during storage over all conditions.

The share of degradation due to oxygen exposure can be estimated from the laboratory experiments (Figure 64A). Share of losses due to oxygen exposure is defined as the difference in average loss in groups with and without protection from oxygen divided by the average loss when exposed to oxygen. It was assumed that 50% of the flavonol glycosides lost were converted to flavonol aglycones due to deglycosylation. Without this adjustment, flavonol aglycone losses attributed to oxygen exposure were 73% for the entire dataset and 100% for conditions. As ascorbic acid fortification is not essential to cranberry juice and was often a destabilizing factor, the same analysis was performed excluding groups containing the fortification (Figure 64B). Oxygen exposure alone (without AscA) accounted for >50% of losses for all but flavonol glycosides. Interestingly, the glycosides (anthocyanins and flavonol glycosides) showed the least loss

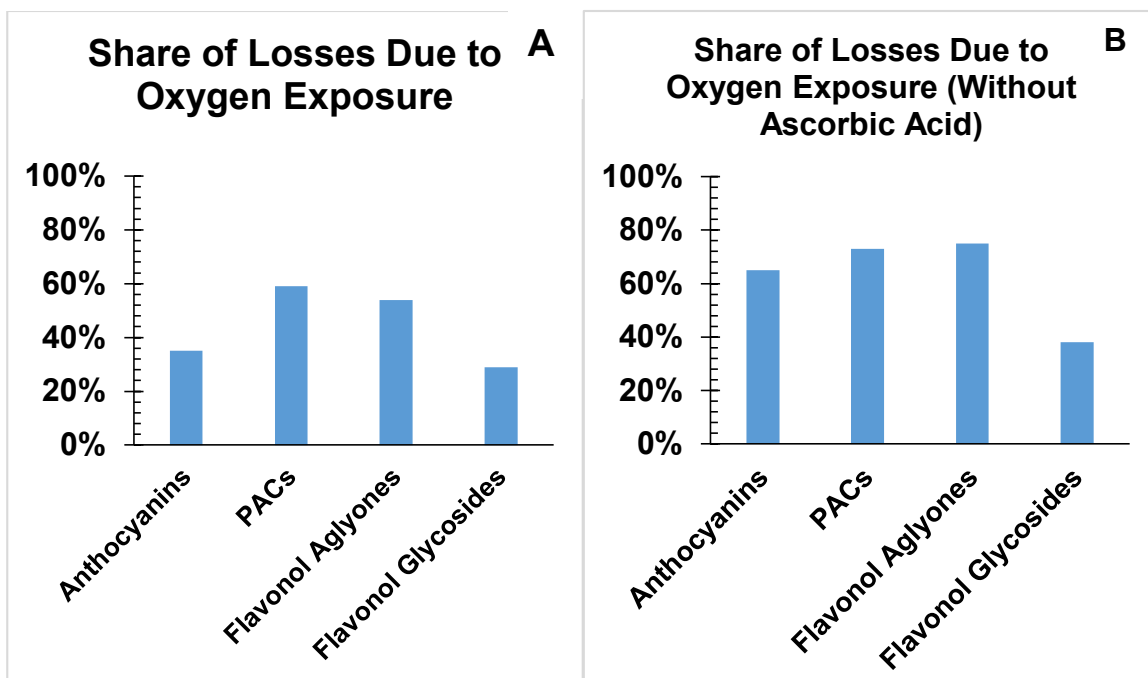


Figure 64: The share of degradation attributed to oxygen exposure after six weeks (A) for the entire Phase III dataset and (B) excluding groups containing ascorbic acid without ascorbic acid.

due to oxygen exposure, most likely because deglycosylation (the major source of losses in these species) is not an oxidative process. With or without ascorbic acid, the role of oxygen is paramount to the stability of flavonoids in cranberry juice and indeed, is the single most important factor for all but flavonol glycosides.

6.1 Dissolved Oxygen

Control of oxygen exposure proved more straightforward under the laboratory conditions of the Phase III trials while more complicated in the practical packaging used in Phase V. First, it is notable that even with rigorous controls to prevent oxygen

exposure, measurable dissolved oxygen persisted. This underscores the difficulty in excluding oxygen, even more difficult in practical conditions. In addition, even under controlled laboratory conditions, measured dissolved oxygen levels were not dependent simply on the storage atmosphere or dissolved oxygen in the CJC. An important chemical paradox was that autoxidation ascorbate fortification suppressed dissolved oxygen content by ~40% when under 1% oxygen atmosphere (0.20 vs 0.14 mg/L) and 20% when under argon (0.019 vs 0.016 mg/L). In this way, ascorbate helped to reduce direct oxygen effects but in the process created reactive oxygen species and ascorbyl radicals. Light and preprocessing degassing had no significant effects on dissolved oxygen during storage in the laboratory trials.

As anticipated, dissolved oxygen levels were more dynamic when we used practical plastic packaging in Phase V. Dissolved oxygen generally accumulated during the first two months of the storage period and remained at a steady level for the final month of storage. This trend was most pronounced in groups exposed to neither light nor ascorbic acid where dissolved oxygen levels rose as high as 1.45 mg/L because oxygen slowly permeating through the plastic packaging was minimally utilized in reactions. It was disappointing to find that barrier bottles limited oxygen ingress to only a small extent (~20% reduction in dissolved oxygen compared to plain bottles during three months storage). Thus, the particular barrier bottles used failed to significantly stabilize cranberry flavonoids and only modestly stabilized color, as discussed in the subsections below.

Dissolved oxygen reached much lower levels (maximum concentration of only 0.25 mg/L) when light or ascorbic acid were present. After 3 months storage, ascorbic acid fortified CJC had 8.5 times lower DO levels than unfortified CJC. Several of the

groups fortified with ascorbic acid remained particularly low in dissolved oxygen content throughout the three months of storage, with maximum readings at 0.01 mg/L. This occurred because the oxygen slowly diffusing into the bottle was consumed in autoxidation reactions with ascorbic acid.

AscA's reactivity with molecular oxygen, particularly when catalyzed by transition metals, is well documented¹⁸¹ and accounts for our observations of suppressed DO levels in fortified groups. One might assume that suppression of oxygen would stabilize cranberry phytochemicals and color, but we observed mostly destabilizing effects with AscA fortification, particularly for ACYs. Damage to polyphenols can be attributed to the chemistry of AscA oxidation and the reactive species that are generated therein (Figure 65). Transition metal ions catalyze the oxidation of AscA by molecular oxygen, forming dehydroascorbic acid and reactive O_2^- anions and hydrogen peroxide.^{181,182} Although not shown in Figure 65, critical intermediate compounds formed in this reaction are reactive ascorbyl radicals.¹⁸¹ Both ascorbyl radicals and dehydroascorbic acid co-oxidize phenolic compounds directly, forming phenolic radicals and regenerating ascorbic acid in the process.¹⁸³ Alternate reaction pathways utilize the hydrogen peroxide to oxidize the phenolic compound directly¹⁸⁴ or by reduction to highly unstable hydroxyl radicals via Fenton reactions with transition metals, particularly iron.¹⁸⁵ Hydroxyl radicals, in turn, may react with phenolic compounds.

As a consequence, while ascorbic acid may consume DO, it often does not disarm the reactivity of oxygen in the process. Rather, it transforms triplet molecular oxygen into numerous species that more readily react with phenolic compounds, including the flavonoids in CJC. With practical packaging, the ability of ascorbic acid suppress

dissolved oxygen content via autoxidation (metal-catalyzed) proves critical to understanding these results and many of the discrepancies between the laboratory and practical trials.

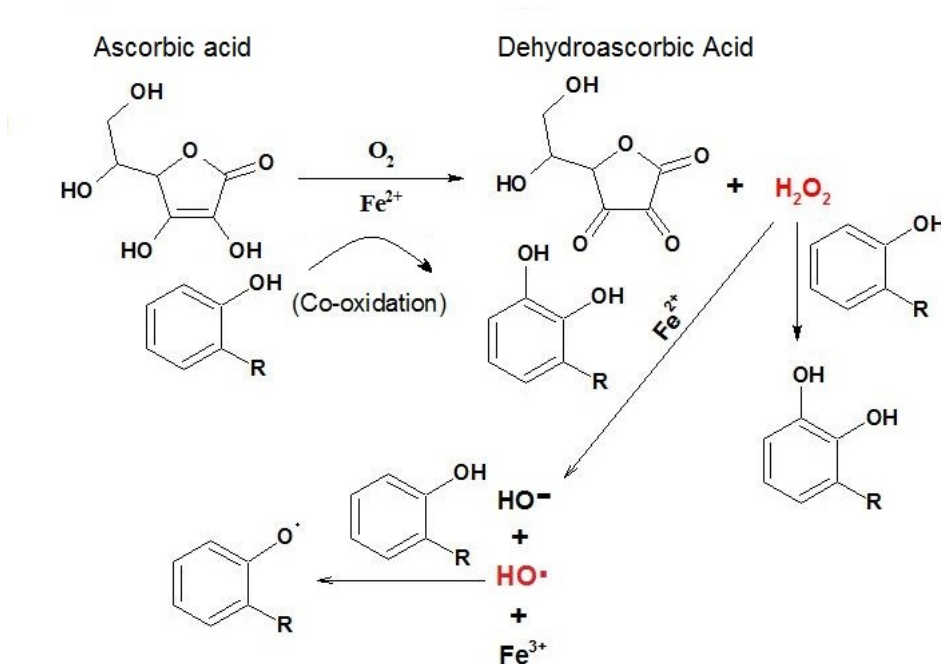


Figure 65: Pathways by which the metal-catalyzed oxidation of ascorbic acid by molecular oxygen may lead to coupled oxidation of phenolic compounds. Reactive intermediate oxygen species resulting from ascorbate oxidation are shown in red. Adapted from references ^{181,184,185}

Light exposure also suppressed DO levels (by five times compared to CJC stored in the dark) in Phase V trials but destabilized flavonoids by transforming oxygen in photo-oxidation reactions, as discussed in Section 2.4.4.2. In type II photo-oxidation reactions, light excites photosensitizers (such as riboflavin or perhaps anthocyanins in CJC) which in turn convert ground state triplet oxygen to highly reactive singlet oxygen. Singlet oxygen readily adds across double bonds (such as those found in cranberry

flavonoids) to form hydroperoxides. In type I photo-oxidation reactions, photosensitizers react with numerous other compounds (perhaps cranberry flavonoids), forming radicals that also react with molecular oxygen. Hence, as with ascorbic acid, light reduces dissolved oxygen levels by transforming molecular oxygen to reactive species that degrade phenolic compounds.

6.2 Anthocyanins

Processing effects on anthocyanins were somewhat inconsistent across the experiments. On average, ~25% anthocyanin losses were observed after processing in Phases II and V, consistent with expectations. In Phase III, however, average anthocyanin losses were much lower at ~2% on average with some groups appearing to gain small amounts of anthocyanin during processing. Gains in anthocyanins after processing of cranberry juice, although unusual, have been observed previously, attributed to cellular components that compartmentalize pigments and leave them unavailable for analysis/detection in incompletely filtered/clarified raw cranberry juice¹⁴². Heat disrupts such components/membranes, releasing the pigments for analysis/detection. A different batch of fresh raw juice was used for each phase of the research, and perhaps the batch used for Phase III may not have been fully filtered/clarified. Hence, the small gains in some groups and lower losses in others during processing may result from this phenomenon (any anthocyanins compartmentalized within cellular matrices were undetected).

Both processing factors influenced anthocyanin retention during our HTST treatments. In phases II, III and V, the use of preprocessing degassing resulted in 5-20% greater retention of anthocyanins after the HTST processing. In phases III and V,

ascorbic acid fortification decreased anthocyanin retention by 5-8% during processing. Indeed, preprocessing degassing and avoiding ascorbate fortification both appear to be effective means to limit anthocyanin losses from processing.

Heat accelerates chemical reactions and presumably the heat treatment applied here led to degradation of ACYs by numerous pathways. Deglycosylation of glycosides to unstable anthocyanidin aglycones is certainly accelerated by heat treatments and is likely to be a major route of anthocyanin degradation from heat treatments (Figure 9).¹³⁹ Indeed, previous research concluded that the stable products (sugar and monophenolic compounds) of heat-induced degradation — arise from deglycosylation reactions and subsequent scission.¹³⁹ Another investigation of the degradation products of anthocyanins exposed to heat identified coumarin glycosides and monophenolic compounds derived from scission between the B and C rings. Both of these pathways result in loss of the conjugated double bond structure and therefore loss of color.³⁷

That preprocessing degassing protected anthocyanins is evidence that oxidation reactions were also occurring during heat treatment. During pasteurization, CJC was enclosed in stainless steel vessels and not exposed to light, so photo-oxidation reactions are unlikely. Rather, metal catalyzed oxidation and oxidation via ascorbic acid degradation products are the most likely pathways. Indeed, the use of stainless steel cooking vessels and pasteurization systems (the industry standard) may promote metal catalyzed oxidation of anthocyanins and other flavonoids.

Ascorbic acid fortification was the most detrimental factor for anthocyanins during storage. Unfortified cranberry juice retained 6.7 times more anthocyanins after six weeks in laboratory conditions (Figure 33) and 2.8 times more anthocyanins in practical

packaging after three months (Figure 51) than groups fortified with ascorbic acid. Less anthocyanin damage in practical packaging may be explained by ascorbate suppressing dissolved oxygen levels far more efficiently in plastic containers than under laboratory conditions, so less oxygen was available to react via other oxidation pathways.

Indeed, oxygen exposure too had a large impact: we observed about four times more anthocyanins remaining after 6 weeks storage under argon than 1% O₂ (Figure 33.) The interaction between ascorbic acid fortification and oxygen exposure in storage was particularly devastating to anthocyanins: 38 times more anthocyanins remained when protected from oxygen and ascorbate versus than when exposed to both after 6 weeks, and the effect was highly significant ($p < 0.00001$, Figure 66). Such a large effect suggests that anthocyanins are particularly vulnerable to some aspect of ascorbic acid oxidation. One study of apple peel components found that anthocyanins were significantly more sensitive to degradation via hydrogen peroxide than other phenolic compounds.¹⁸⁶ Perhaps the same is true here for cranberry anthocyanins.

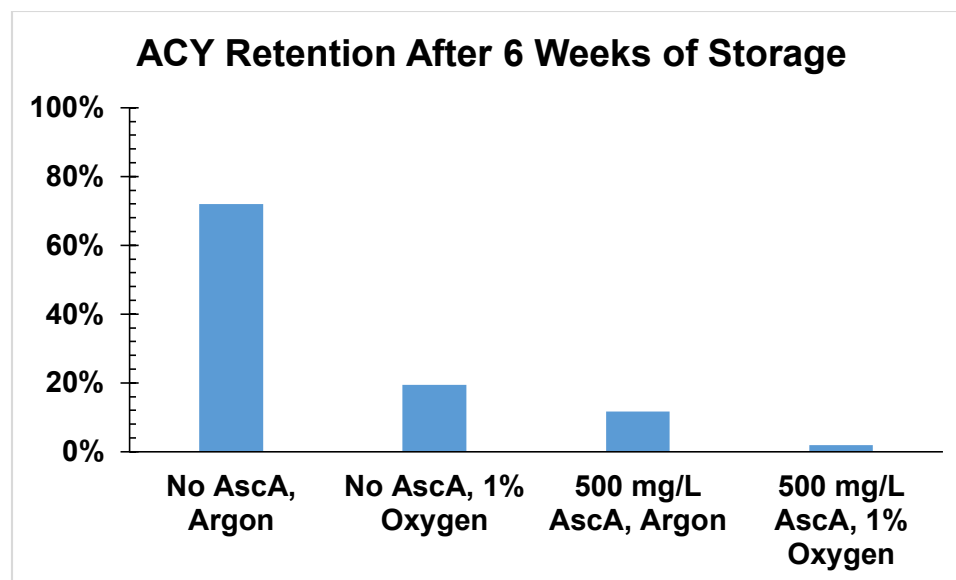


Figure 66: Percent retention of total anthocyanins after 6 weeks storage with ascorbic acid fortification and either argon or 1% oxygen atmospheres. Values reported are averages of the conditions indicated and include all other conditions. Because each bar represents the average of several conditions with a wide dispersion of outcomes, error bars are not shown.

Without the presence of ascorbate, oxygen was less damaging but still strongly destabilizing: 215% more anthocyanins remained under argon vs the 1% oxygen atmosphere. This observation can be attributed to metal catalyzed oxidation of anthocyanins. Further discussion of the reactions of oxygen, anthocyanins and ascorbic acid is presented in Section 6.8.

Light promoted degradation of anthocyanins during storage but to a far lesser extent than exposure to oxygen or ascorbic acid. On average, about 10% more anthocyanins remained when protected from light under lab conditions after six weeks and ~20% more in practical packaging after three months. In the laboratory trials, we

observed an important interaction -- that light had no impact on anthocyanins in juice stored under argon. This suggests that light only affects cranberry anthocyanins via a photo-oxidation mechanism. Whether anthocyanins themselves act as a photosensitizer is unclear, but combinations of light and oxygen exposure were more damaging to anthocyanins than to the other flavonoids, so their activity as photosensitizers cannot be ruled out. Further, considering its oxygen dependency, light-induced oxidation of anthocyanins likely proceeds via type II photo-oxidation reactions where photosensitizers activate oxygen to singlet oxygen, which in turn reacts with anthocyanins. The interaction between barrier bottle and light was not observed in later trials but this may be because the barrier bottles were not very effective at limiting oxygen exposure.

In both experiments, ANOVA indicated that ascorbate fortification interacted with light exposure but with opposite directionality. In laboratory trials, presence of ascorbate increased light destabilizing effect while conversely in practical conditions, the presence of ascorbate lessened damaging effects of light towards anthocyanins. A plausible explanation is that in the laboratory trials, light induced the degradation of ascorbate to form degradation products that destabilize cranberry anthocyanins. But in the practical trials, ascorbic acid reduced dissolved oxygen concentrations completely enough to prevent photo-oxidation of anthocyanins.

The effect of preprocessing degassing was not significant on anthocyanin retention during the storage period. However, our storage results are expressed as a retention percentage relative to the concentrations found in the post-processing (storage time 0) sample. When we examine anthocyanin concentrations on an absolute basis, we see that the increased retention of anthocyanins seen from processing due to

preprocessing degassing is durable and those gains are still mostly present at the end of storage. An average of 10.6 mg/L total anthocyanins remained from the degassed groups from Phase V while an average of 9.2 mg/L total anthocyanins remained from corresponding groups that did not receive the degassing treatment. Preprocessing degassing, therefore, protects anthocyanins during processing, a benefit that remains but does not increase after subsequent storage.

6.3 Flavonols

As with anthocyanins, processing effects on flavonols were inconsistent between laboratory and practical trials. In laboratory experiments, groups fortified with ascorbic acid gained up to 20% *total* flavonols while groups not fortified had losses up to 10%. Meanwhile, in the practical packaging experiments, 7-17% total flavonol losses were observed. Most of this effect was on the aglycones, which showed 50-70% increases compared to 2-3% for flavonol glycosides. Degassing similarly increased flavonol retention, by 10-40% for aglycones and by ~3% for glycosides, compared to aerated or untreated CJC (Figures 34 and 52).

Although increases in flavonol content from processing have not been observed in cranberry juice to our knowledge, a comparable effect from cooking has been observed in onions.¹⁸⁷ We attribute these gains mainly to the same compartmentalization phenomenon described for anthocyanins earlier, as did Nemeth *et al.* for their observations for onion flavonols. In addition, degassing plus reducing potential and reduction of DO from ascorbic acid may prevent oxidation of flavonols during heat treatment, or ascorbic acid may regenerate flavonols oxidized during pasteurization or subsequent cooling (see Section 6.8 for further discussion of this reaction). That

aglycones are strongly affected but glycosides only barely suggests that oxidation of the C-3 –OH is involved.

During storage, ascorbate had different effects on flavonol aglycones and glycosides. On average in the laboratory trials, vitamin C addition stabilized aglycones (~10%) but destabilized flavonol glycosides (~10%) compared to unfortified groups (Figure 36). In the practical packaging experiments, ascorbate's overall effect on flavonol glycosides was neutral while it strongly stabilized aglycones, with five times the amount aglycones remaining in fortified CJC compared to unfortified CJC after 3 months (Figure 53). Indeed, aglycone content in fortified groups increased over the storage period and exceeded the content at time 0 by about 20% after 3 months (Figure 53). This increase in aglycones is evidence that deglycosylation occurs here, converting glycosides to aglycones. The initial content of glycosides was higher than aglycones, so deglycosylation of even a small percentage of glycosides can amount to a large increase in aglycones. Apparently, ascorbic acid addition itself promotes deglycosylation of flavonol glycosides in CJC. As an acid (H donor) and reducing agent (electron donor), AscA may indeed catalyze this acid-mediated reaction. Deglycosylation likely occurs whether ascorbic acid is present or not, but in the presence of ascorbic acid, the resulting aglycones appear to be stabilized by ascorbate H donation to the C-3 position.

The ability of ascorbate to consume the slow trickle of oxygen into practical packaging explains its more stabilizing effects towards flavonol aglycones compared to laboratory conditions (500% more in practical trials vs 10% more in laboratory trials compared to unfortified groups). Ascorbic acid's consumption of nearly all the DO in

practical packaging, leaves little oxygen for type II photosensitization or metal catalyzed browning reactions.

Oxygen exposure by itself reduced the stability of cranberry flavonols during storage, with 6-93% higher retention under argon than 1% oxygen (Figure 67). Aglycones were more destabilized than glycosides by oxygen and indeed, each individual species behaved differently. Myricetin and its derivatives were more susceptible to oxidative damage than quercetin and its derivatives. Perhaps the B ring trihydroxy structure of myricetin oxidizes more easily than the dihydroxy B ring of quercetin.

The interaction of oxygen exposure and ascorbic acid addition is worthy of closer scrutiny (Figure 68). Particularly important, of all conditions, groups protected from oxygen without ascorbic acid fortification had the highest retention of flavonol aglycones (98%) and glycosides (80%). Ascorbate fortification stabilized flavonols only when combined with oxygen exposure. This pattern is strong evidence that the protective effect must be due to preventing or reversing the oxidation of flavonols. In the absence of oxygen, ascorbic acid was harmful to flavonol aglycones and glycosides alike. Ascorbic acid reacts quite rapidly with oxygen, but in the absence of oxygen flavonols become a competitive reaction target. As a critical consequence therefore, if practical packaging were to eliminate (or severely limit) oxygen exposure, we would expect ascorbic acid fortification to damage flavonols. This provides another argument against ascorbic acid fortification since elimination of oxygen otherwise stabilizes cranberry nutraceuticals.

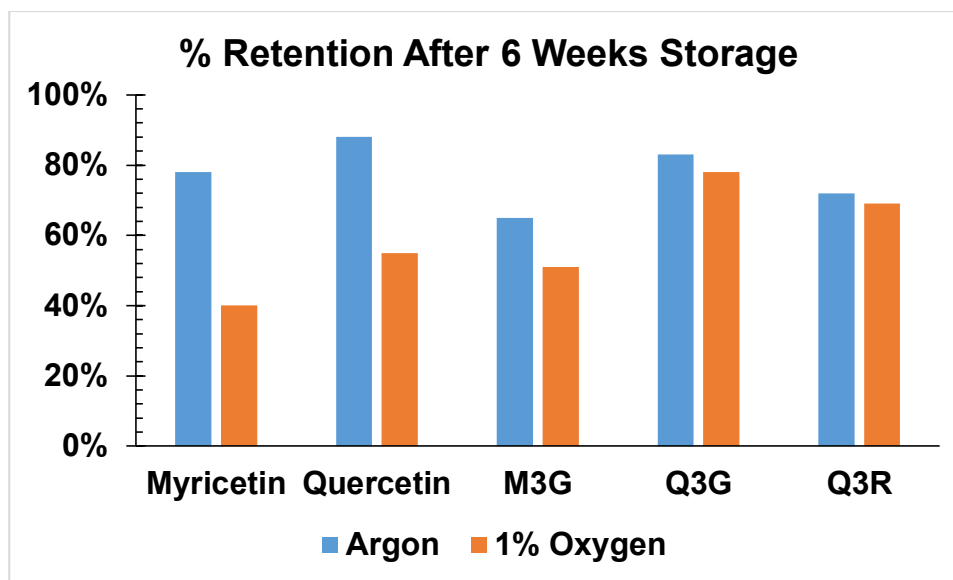


Figure 67: Percent retention of individual flavonol species exposed to either argon or 1% oxygen atmospheres after 6 weeks of storage. Values reported are averages of all conditions with and without each test factor. Because each bar represents the average of several conditions with a wide dispersion of outcomes, error bars are not shown.

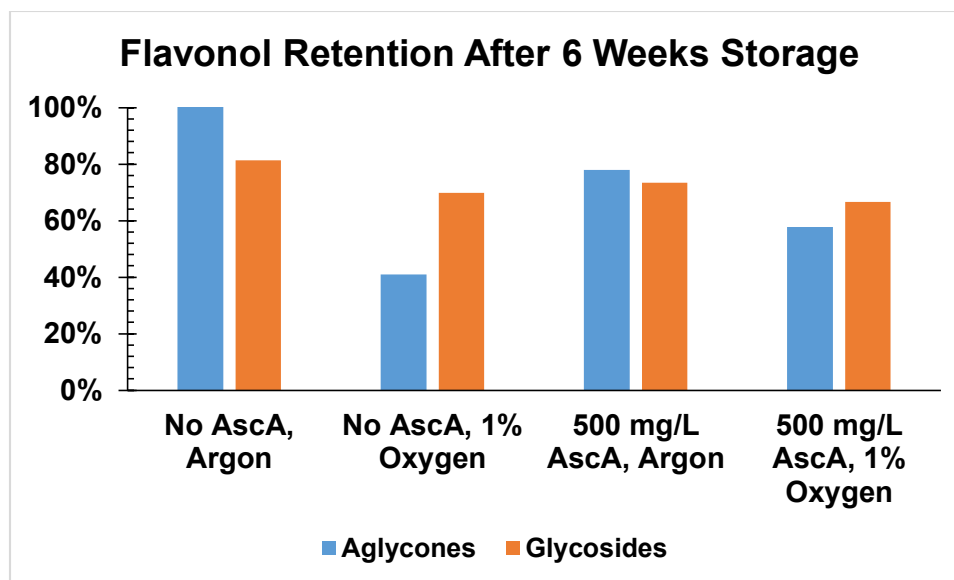


Figure 68: Retention of flavonol aglycones and glycosides after 6 weeks storage with ascorbic acid fortification and either argon or 1% oxygen atmospheres. Values reported are averages of the conditions indicated and include all other conditions. Because each bar represents the average of several conditions with a wide dispersion of outcomes, error bars are not shown.

In laboratory studies, light had no effect on flavonol glycosides during storage but destabilized aglycones. Exposure to light resulted in 10 and 25% lower retention of myricetin and quercetin aglycones, respectively, after 6 weeks compared to groups protected from light. That light affected aglycones and not glycosides is consistent with previous findings that substitutions at the 3 position of flavonols increases their sensitivity to light.¹³⁶ Furthermore, that increased damage was observed only when light was combined with oxygen exposure and was not observed for groups stored under argon certainly supports singlet oxygen from type II photo-oxidation mechanisms as the causative agent.

This effect could not be confirmed in practical packaging, unfortunately, because the higher levels of dissolved oxygen in these samples obscured effects of light. None of our packaging substantially reduced oxygen ingress. However, given the data from our laboratory experiments, if practical packaging was to eliminate or severely reduce oxygen exposure, light exposure would become a non-factor in flavonol stability.

Finally, degassing CJC before heating protected flavonols during processing, and these effects held during storage if oxygen and ascorbic acid were absent. Apparently, in the presence of oxygen, flavonol aglycone losses from oxidation during prolonged storage obscure the small gains from degassing during pasteurization. Similarly, the larger effects of ascorbic acid protection against oxidation or reduction of oxidized products minimizes contributions of degassing to stabilization. However, backwards it may seem, this is an important observation since both oxygen and ascorbic acid overall increase degradation of CJC. If these two factors are removed by abandoning fortification and by using oxygen barrier packaging, pre-pasteurization degassing assumes a much more significant role in long-term stabilization of CJC.

6.4 PACs

Thermal processing of cranberry juice resulted in no significant change in PACs for the laboratory trials and small increases in PAC content in practical trials compared to pre-processing levels. Here, the compartmentalization effect discussed earlier is unlikely to cause apparent increases in PAC content because the batch of juice used in the practical trials did not show gains in other flavonol classes during processing. It is more likely that smaller phenolic compounds condense or add to existing PACs in such a

manner during thermal treatments so that the DMAC assay detects them. The molecular mechanism of the DMAC assay is not understood and thus we cannot conclude such an explanation with certainty. However, that factors promoting oxidation and browning increased PAC content during processing in the practical trials does support this condensation effect. Degassing prior to processing decreased PAC levels in both practical trials (~5%) and laboratory trials (~10%). PACs increased more with ascorbic acid addition (19%) than in unfortified juice (12%). It appears PACs or PAC-like compounds are formed under oxidative conditions in cranberry juice during processing.

The phenolic condensations we propose are trademarks of phenolic browning reactions (discussed in Section 2.4.4.3). Here, metals catalyze oxidation of diphenols to form quinones, which in turn may react with other diphenols, eventually creating large and diverse structures of condensed phenolics (see Figures 13 and 14).³⁵ The initiation of such pathways is surely accelerated by heat, and condensation reactions may occur during subsequent cooling. Cranberry PACs with adjacent hydroxyl groups in the B ring (diphenol structure) are particularly prone to these reactions and likely have diverse phenolic compounds adding to those sites.

PACs were generally more stable than other cranberry flavonoids, retaining 50-97% after processing and after three months storage. Oxygen exposure proved to be the most damaging factor for PACs during storage. PAC retention after six weeks in the laboratory experiments improved by 30% when oxygen was removed. Barrier bottles failed to protect PACs in the practical trials because they were ineffective in preventing oxygen ingress. Ascorbic acid fortification was damaging during storage in laboratory trials (25% lower retention after six weeks), but protected PACs in practical packaging

(7% higher retention after three months) where it lowered dissolved oxygen levels within sealed bottles.

As with anthocyanins, the combination of ascorbic acid fortification and oxygen exposure was particularly damaging, causing up to 50% PAC losses after six weeks. With exposure to oxygen alone causing 23% losses and ascorbic acid alone causing 13% losses over the same period, it is clear that the combined effect is more than the sum of the two individually. PACs seem to be very sensitive to degradation from the reactive species generated by the oxidation of ascorbic acid, such as superoxide anion ($O_2^{\bullet-}$) that attacks double bonds and hydrogen peroxide that decomposes to reactive and highly oxidizing hydroxyl radicals, HO^{\bullet} . Indeed, previous observations in studies of apple peels suggest that two PAC species (procyanidin B1 and procyanidin B2) react with hydrogen peroxide even more readily than other phenolic compounds tested except for anthocyanins.¹⁸⁶

The effect of light on PAC stability during storage was minimal, with an average of 2% greater retention after 6 weeks in laboratory trials and no significant effect in practical packaging. When protected from oxygen and degassed prior to processing, light had no effect on PAC content, again supporting photosensitization and 1O_2 oxidation as the reactions responsible. However, light combined with ascorbate fortification was much more damaging, resulting in 8% fewer PACs after six weeks. As was discussed for anthocyanins, light induced degradation of ascorbic acid creates reactive species that may degrade PACs.

Strangely, degassing prior to pasteurization destabilized PACs during processing (Figures 38 and 54) but stabilized them during storage (Figures 39 and 55.) One plausible explanation is that degassing did indeed protect native PACs from degradation during

processing, but this effect was overshadowed by the formation of PAC-like compounds in non-degassed groups. Perhaps these postulated PAC-like compounds are unstable or undergo further condensations during storage to create large polymer compounds that no longer have reactivity with DMAC. This explanation would account for the sharp decline in PAC content observed after one week of storage in aerated but not in degassed CJC (Figure 39.) The overall effect of degassing on PACs after both pasteurization and storage was stabilizing – 7% more PACs remained with degassing versus opposite treatments in both trials ($p < 0.0001$).

Similarly, AscA fortification seemed to protect PACs during processing but destabilize them during storage. A similar explanation may account for this. During processing, ascorbic acid degradation products may spur condensation reactions of diverse phenolics to form PAC-like compounds. But during subsequent storage, perhaps those PAC-like compounds degrade or polymerize and no longer have reactivity with DMAC. Such an effect is consistent with the sharp decline in PACs observed between processing and 1 week of storage in fortified CJC (Figure 40.) Investigations using chromatographic separations of PACs before detection could test these explanations.

Nonetheless, the overall effect of ascorbic acid fortification on PACs was destabilizing in laboratory trials: ~25% less PACs remained after processing with fortification versus without. Conversely, in practical trials, the overall effect of AscA fortification was mildly stabilizing, perhaps because AscA limited DO levels in practical packaging, preventing metal catalyzed oxidation as well as type II photo-oxidation reactions. Once again, if practical packaging were to effectively limit oxygen exposure, we would expect ascorbic acid to damage PACs as we observed in the laboratory trials.

6.5 Ascorbic Acid

During processing, ascorbic acid losses were consistent between laboratory and practical trials with 10-30% losses from the heat treatment. In both, preprocessing degassing proved an effective treatment, reducing losses from pasteurization by ~50%.

During storage, oxygen exposure was most damaging to ascorbic acid, causing losses double those in groups protected from oxygen. (Refer to Section 6.1 and Figure 65 for a discussion of the reactions of ascorbic acid with oxygen.) In laboratory experiments, light was also an important factor, causing an average of 33% greater losses than storage in the dark. However, combinations of light and oxygen were most damaging to AscA in CJC, causing more degradation than the sum of each effect (Figure 69) due mostly to $^1\text{O}_2$ photo-oxidation reactions. Indeed, ascorbic acid is known to have very high rates of reactivity with singlet oxygen formed via photosensitization in numerous food systems.¹⁴⁵ Since light effects are not entirely dependent on the presence of oxygen, some type I (free radical) photo-oxidation also occurs, though to a lesser degree. In contrast, in practical trials light stabilized ascorbic acid by 3%. Perhaps the $^1\text{O}_2$ generated by light exposure preferentially reacts with phenolic compounds over ascorbic acid. Meanwhile, DO is consumed in that process and less oxygen is available to degrade AscA from metal catalyzed oxidation reactions.

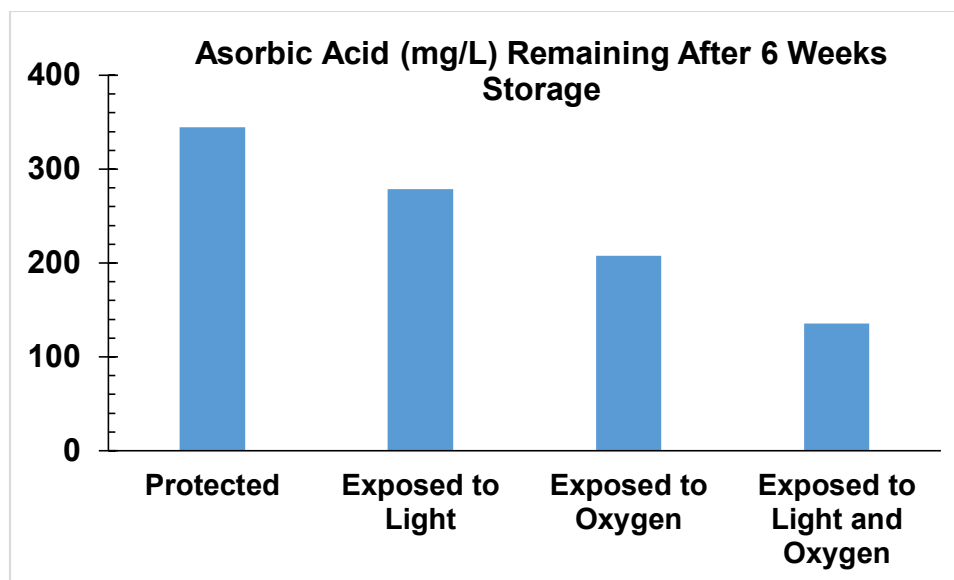


Figure 69: Ascorbic acid content remaining after six weeks of storage with combinations of light and oxygen exposure.

Degassing increased retention of ascorbic acid during pasteurization; most of this additional ascorbic acid was conserved during storage in both experiments but no additional impact was gained. In laboratory trials, ascorbic acid levels remaining after six weeks were 270.8 mg/L in degassed juice and 212.5 mg/L in aerated juice. A similar pattern was observed in practical trials where an average of 30 mg/L more ascorbic acid remained after three months in degassed groups than in untreated groups. This shows the importance of adding a degassing step when CJC is fortified with ascorbic acid.

It is important to note that the degradation rates of ascorbic acid in cranberry juice observed here are slower than one would expect in pure aqueous solutions. Aqueous solutions of ascorbic acid (330 mg/L) exposed to oxygen at pH 2.35 degraded completely after 9 days.¹⁸⁸ However, the phenolic compounds abundant in the juice appear to

stabilize ascorbic acid, most likely because dehydroascorbic acid can react with phenolic compounds to regenerate ascorbic acid.¹⁵³

6.6 Total Phenolics

Total phenolic levels by themselves are not very revealing or diagnostic of overall degradation, but they do account for non-flavonoid components of CJC that may also be degraded during processing and storage of CJC and marked shifts, up or down, in content can suggest when decompartmentalization, polymerization, or depolymerization may be occurring.

Some inconsistencies were observed for CTPs between the practical and laboratory trials. CTPs were not significantly altered in any group during processing in laboratory trials but in practical trials all groups showed an average of 16% increased CTP levels after processing. One difference between the two studies is that ground water was used to make the cranberry juice cocktail in practical trials while high purity water was used in the laboratory trials. Perhaps during processing, some polyphenolic compounds were split into smaller monophenolic compounds, increasing measurable CTP levels. Metal ions present in ground water (and thus in the cranberry cocktail used for practical trials) might promote such scission in polyphenolics, accounting for their higher CTP levels after processing.

Some parallels between the two experiments were also observed. In both trials, ascorbic fortification correlated with about 20% higher levels of CTPs after processing than in unfortified groups. Degassing resulted in 8% lower CTP levels after processing in the practical trials, but had no effect in laboratory experiments. In both cases, oxidative

factors resulted in higher levels of apparent CTPs. Similarly during storage, factors promoting oxidation coincide with higher levels of CTP: light and oxygen exposure correlated with slightly higher CTP levels in laboratory trials (7% and 3%, respectively). As for PACs, perhaps phenolic condensations after oxidation are an explanation here. If such condensation products have higher reactivity with the Folin reagent than native phenolics, this would explain the higher CTP levels we observed.

Given the lack of specificity of the Folin-Ciocalteu analysis and the disparate results observed, one must question the utility of these observations in drawing any firm, meaningful conclusions. Given the rise in CTP during processing in the laboratory trials and fall in CTP during storage, one could argue that these data support the notion that cranberry polyphenolics are more likely to split into smaller phenolics during processing. Meanwhile during storage, phenolics are more likely to condense into polymeric compounds. However, to make this assertion with confidence, one would need to conduct a more targeted experiment. Perhaps a chromatographic approach to monitor a few representative mono-phenolic compounds along with a colorimetric analysis to measure polymeric pigment compounds would yield more concrete information.

6.7 Color

Processing generally resulted in loss of red color and lightening (fading) of cranberry juice. In all experiments, ascorbic acid fortification caused notable loss of red color (lower a^*) and lightening (higher L^*) due to anthocyanin degradations (Figures 45 and 60). Degassing lessened these changes in the laboratory experiment but not in the practical trials.

For subsequent storage, as well, nearly all main effects were significant for both color parameters. Degassing, no fortification with ascorbate, dark storage, argon atmosphere and barrier bottles resulted in significantly less lightening of cranberry cocktail during the storage period. In both laboratory and practical experiments, ascorbic acid fortification had the largest impact by far, causing differences of approximately 9.0 and 15.0 L* units, respectively, between fortified and unfortified samples after six weeks storage in the laboratory and practical experiments, respectively (Figures 46 and 61). The effect of barrier bottles was significantly stabilizing but quite small resulting in an average difference of 1.8 L* units after 3 months compared to standard bottles (Figure 61). Interestingly, light exposure led to fading only in combination with oxygen exposure or vitamin C. Combinations of light, vitamin C and oxygen exposure were most damaging. The small effect of degassing, preventing lightening during storage, was more pronounced with exposure to ascorbic acid, oxygen and light. While most groups had substantial lightening over the storage period, several groups that were protected from oxygen and not fortified with ascorbic acid darkened slightly after 6 weeks in the laboratory trials. We attribute these changes in L* to two competing phenomenon. First and most prominent is the loss of color intensity due to pigment degradation. Second is the formation of brown pigments due to various browning reactions, which undoubtedly occurs in all groups but was only observed when protection from oxygen and ascorbic acid yielded smaller losses of pigment. The observed pattern of mostly lightening of the cranberry juice over the 6 week storage period is consistent with our earlier observations (Figure 17). This pattern, typical for commercially bottled CJC, consists of mostly fading

and lightening during the first several months before browning and darkening becomes prominent after several months.

All simple effects except degassing in the practical trials showed were significant loss of redness (decreased a^*) during storage in both experiments. Obviously, this color loss stemmed from degradation of anthocyanins. Again, vitamin C fortification had the largest impact by far, with ~2.5 times greater average loss of a^* when fortified in both experiments. Protection from oxygen stabilized red color by more than 40% while barrier bottles inhibited color loss by only about 5%. Light induced color loss only in the presence of oxygen; light effects were not influenced by bottle type. Degassing before processing improved retention of red color even during storage (in addition to the improved color retention observed during processing), but the effect was dependent on the presence of ascorbate and protection from oxygen.

6.8 Reactions and Mechanisms

Unfortunately, our LC-MS analysis of model solutions of purified flavonoids produced few useful results. However, some findings in that experiment and other trials give clues regarding the reactions involved in degradation of cranberry phenolics and their vitamin C interactions.

Available literature suggests that vitamin C reacts with anthocyanins via a direct reaction¹⁵⁰ or via vitamin C degradation products.¹⁸ Results from this study suggest both pathways occur in cranberry cocktail. MS data from the model solutions study shows the presence of a compound with m/z of 625 in three out of four groups that contained ascorbic acid and in none of the groups that did not contain ascorbic acid. This m/z corresponds with the molecular weight of the addition product of ascorbate ion and

cyanidin-3-glucoside; Figure 70 presents a proposed structure. Addition of ascorbate to the 4 position in the center flavylum ring is plausible as this site is known to be prone to attack from bisulfite, phenol and various other nucleophiles.¹⁸⁹ Such a reaction would result in loss of red color due to disruption of the system of conjugated double bonds. We must underscore that this proposed structure is speculation based on our observations – a firm conclusion would require isolation of the compound and additional structural analyses. We also saw indirect evidence for ascorbic acid degradation products having a detrimental effect on anthocyanins. In particular, the interaction of vitamin C fortification and storage atmosphere effect on anthocyanins was strong and synergistic; that is, the combined effect of these two factors was much more than additive. As oxygen exposure is highly destabilizing for ascorbic acid, it is logical to conclude that the ascorbyl radicals, dehydroascorbic acid, $O_2^{\bullet-}$, H_2O_2 , and HO^{\bullet} formed during the oxidation of vitamin C formed in the presence of oxygen also contributed to loss of anthocyanins beyond that of the losses expected from direct vitamin C exposure and oxygen exposure without interaction.

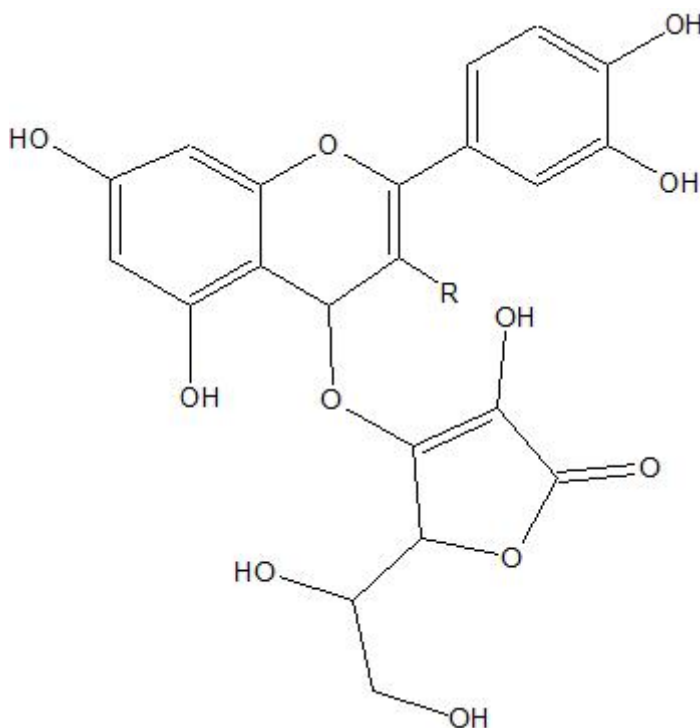


Figure 70: Proposed structure of an anthocyanin-ascorbic acid conjugate accounting for the 625 m/z peak observed in MS analysis of model solutions.

Ascorbic acid had the opposite effect for flavonol aglycones, protecting these species from losses during oxygen exposure. While ascorbic acid chemically reduces dissolved oxygen, leaving it unavailable for direct oxidation of flavonol aglycones, it is likely that ascorbic acid also reversed oxidation in flavonols, as has been shown for ascorbic acid reversing oxidation for other phenolics.¹⁹⁰ Figure 71 shows such a reaction scheme.

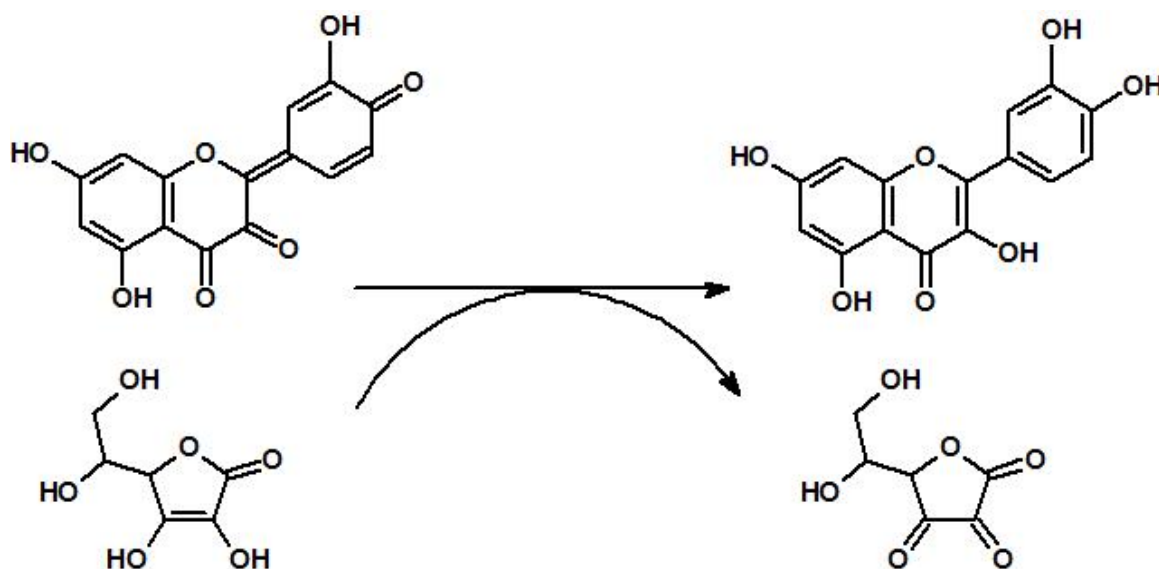


Figure 71: Reversal of flavonol oxidation by ascorbic acid.

Flavonol glycosides, on the other hand, were not stabilized by ascorbic acid with oxygen exposure. Previous research indicates that glycosyl substitutions at the 3 position substantially reduces the antioxidant potential of flavonols (and flavonoids in general).²⁷ The hydroxyl group at the 3 position (as occurs in aglycones), adjacent to the 2,3 double bond and 4-keto group (Figure 5), is a prime target for oxidation¹⁹¹. Meanwhile, glycosyl substitutions block such activity at the 3 position. This is supported by our own observations that aglycones are more damaged with oxygen exposure with and without ascorbic acid exposure compared to glycosides (Figure 67.) Perhaps this same feature facilitates its regeneration from oxidized forms by reaction with ascorbic acid (Figure 70), while glycosyl substitution at the 3 position blocks regeneration from reaction with ascorbic acid.

Without such a recycling in the initial stages of oxidation, cranberry flavonoids oxidize further. Much research has investigated phenolic oxidation (though none directly

for cranberry compounds.) Quinones form in the initial oxidation steps, then they condense to form larger, diverse polyphenolic polymers with brown color (see Section 2.2.4.3 and Figure 14). Perhaps the products of such condensations in our model system were so diverse that none predominate and therefore cannot be easily detected by HPLC-MS analysis. It is thought that as anthocyanins undergo such reactions, they similarly form polymeric pigments, characterized as having reddish-brown hues.²⁰ As we did not observe darkening in most groups of cranberry juice during the six week and three month time scales of our experiments, it is not likely that considerable amounts of polymeric pigments were formed during our experiments. However, we did see evidence of phenolic condensation. Accumulations of PACs, as measured by the DMAC assay, were observed in several groups during processing and storage in laboratory phase experiments. Whether this finding resulted from phenolic compounds adding to existing PAC structures or whether smaller phenolic compounds condensed into PAC-like structures detected by the DMAC assay remains to be determined. Interesting, however, is that such accumulations of PACs were observed only in low oxygen environments, during processing after degassing and during storage in groups protected from oxygen. Perhaps oxygen exposure promotes other routes of degradation simultaneously with condensation. It should be noted too that the condensation reactions in the later phases of phenolic condensation do not require the presence of molecular oxygen (see Section 2.2.4.3 and Figure 14).

Deglycosylation appears to be a major route of degradation for flavonoid glycosides in cranberry juice cocktail (Figure 72). Given the low pH of cranberry juice (pH of ~2.5), this is quite logical because hydrolysis is often acid-catalyzed. Direct

evidence for deglycosylation is seen in the accumulation of flavonol aglycones and corresponding losses of glycoside. Deglycosylation likely occurs with anthocyanins as well, but we saw no evidence for it, perhaps because anthocyanin aglycones are unstable and quickly degrade further into monophenolic compounds.^{37,139}

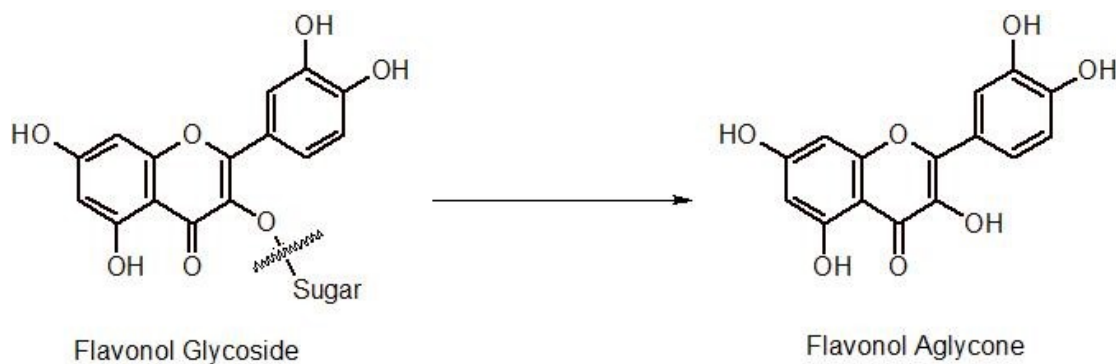


Figure 72: Deglycosylation of a flavonol glycoside.

7. SUMMARY AND CONCLUSIONS

This research investigated factors that affect flavonoid, ascorbic acid, and color stability of processed cranberry juice cocktail – heat, light, oxygen, ascorbic acid fortification and packaging. Results verified flavonoid destabilization by individual factors, but most importantly, identified key interactions that drive flavonoid degradation and showed that some previous assumptions about cranberry juice were erroneous.

Critical roles of dissolved oxygen in degradation of cranberry flavonoids, alone and in combination with ascorbic acid, light and high heat treatments, were clearly demonstrated. Oxygen mediates direct oxidation of molecules, but its interactions with other factors make additional, more highly degrading reactions possible. Alone, oxygen (oxidant) and ascorbic acid (reducing agent) react with molecules directly, and targets of oxygen are limited due to its triplet spin state. However, together their effects are greatly amplified. Ascorbic acid reduces oxygen with opposing and competing consequences – diminished dissolved molecular oxygen contents of solutions but also enhanced levels of reactive ascorbyl radicals, dehydroascorbic acid, superoxide anion ($^1\text{O}_2$), H_2O_2 , and hydroxyl radicals (HO^\bullet) in the process. Variation in which of these actions dominates under different conditions and with different flavonoids accounts for widely varying effects of ascorbic acid in cranberry juice.

Net effects of ascorbic acid fortification were destructive to both ACY and PACS that appear to be particularly sensitive to degradation by one or more products from ascorbic acid oxidation. Loss of anthocyanins was 38 times greater when exposed to oxygen and ascorbic acid together than when protected from both. For PACs, the

combined effect of ascorbic acid fortification and oxygen exposure was also larger than additive, but less extreme than for anthocyanins.

In contrast, ascorbate-oxygen interactions stabilized flavonol aglycones but did not affect flavonol glycosides, even though both factors were damaging individually. Here, the reducing capacity of ascorbic acid was paramount, recycling oxidized aglycones to parent compounds probably by reducing quinones and carbonyls ($C=O$) to $C-OH$. Ascorbic acid stabilized several classes of cranberry flavonoids in practical trials where scavenging of oxygen slowly permeating into the sealed bottles played a dominant role. Paradoxically, better control of oxygen ingress through packaging improvements would eliminate this benefit and ascorbic acid fortification was universally destabilizing when oxygen was excluded.

Light has always been considered as a major factor responsible for fading and browning of cranberry juice color. In this study, PACs were indeed destabilized by light alone, but light did not degrade anthocyanins, flavonol aglycones, and ascorbic acid except in the presence of oxygen. Both of these actions can be attributed to photosensitized oxidation utilizing visible light rather than direct degradation by ultraviolet light. PAC degradation in the absence of oxygen proceeds by type I free radical photosensitization while the perhaps more important anthocyanin and flavonol degradation requiring oxygen involves type II singlet oxygen photosensitization as the active mechanism.

All these results show that limiting oxygen is probably the most important intervention to improve juice stabilization, but accomplishing this is a distinct challenge. Degassing before HTST processing did indeed reduced losses of anthocyanins, flavonols

(particularly aglycones) and ascorbic acid. Degassing effects, though generally small (<10%), were mostly retained throughout storage. Clearly, removing oxygen before heat treatments improved the nutraceutical content in cranberry cocktail and is worthy of further exploration for hot filled cranberry juice operations.

Oxygen barrier bottles were less stabilizing than anticipated. The bottles we utilized reduced dissolved oxygen levels by only ~20% and resulted in no significant impact on flavonoid retention. Barrier bottles did reduce changes in color, but only slightly. Other packaging solutions need to be investigated to provide more effective control of oxygen ingress into cranberry juice.

Taken together, these results verify the first part of our hypothesis that dissolved oxygen is involved in most degradation pathways of cranberry nutraceuticals. Destabilizing impacts of light, heat and ascorbic acid fortification were generally tempered by removal or limitation of oxygen, except for deglycosylation of anthocyanins and flavonol glycosides which is a hydrolytic reaction.

The second hypothesis – that oxygen exposure could be controlled through processing and packaging changes to extend the life of cranberry cocktail color and nutraceuticals – was supported but not fully verified. We have documented that degassing juice prior to pasteurization results in lower dissolved oxygen content before and after bottling, so it is a beneficial processing treatment for hot filled cranberry juice, stabilizing PACs, flavonol aglycones, anthocyanins and color. However, the particular oxygen barrier packaging tested was only marginally effective at reducing oxygen ingress during storage and failed to significantly extend the stability of cranberry flavonoids. It did, however, extend color stability somewhat. These results do not negate the concept but

show clearly that not all “oxygen barrier” or “oxygen scavenging” packaging efficiently eliminates oxygen. Further testing of other packaging will be necessary to find a version that can protect flavonoids and color of cranberry juice.

Based on these results, the primary importance of anthocyanins in both nutritional value and color of cranberry juice, the very strong effects of oxygen, and the complications introduced by ascorbic acid fortification, we make the following recommendations to the cranberry industry for maximum stabilization of cranberry juice products: 1) Since the direct effects of light are minimal and most effects can be eliminated by removal of oxygen, protection from light need not be a requirement in package design of cranberry juice products. 2) Abandon the use of ascorbic acid fortification altogether and educate consumers on the anti-oxidant quality of natural cranberry components instead. 3) Focus instead on limiting oxygen by pre-pasteurization degassing and packaging.

8. FUTURE WORK

Our practical experiments with barrier bottles resulted in only minor decreases in oxygen exposure of the CJC. Future studies should focus on other practical packaging solutions and their potential stabilizing effects on cranberry flavonoids. Perhaps oxygen scavenging packaging systems would better limit ingress of oxygen more than the barrier package evaluated here.

So many of the oxidation reactions in CJC are metal catalyzed. Therefore, it would be worthwhile to determine to what extent removing trace metals (like iron and copper) from cranberry juice cocktail would stabilize the flavonoids. Perhaps this could be accomplished using chelating agents.

The methodology employed here for monitoring PAC content and total phenolic content lacked specificity. Employing HPLC analysis to track the stability of individual species of PACs and smaller phenolic compounds may give more valuable information on their stability and mechanisms of degradation in cranberry juice.

Degassing proved to be a successful treatment in stabilizing several cranberry nutraceuticals. However, the physical, batch-type means of degassing juice utilized here is energy intensive and time consuming. As such, it is not practical for application on commercial scales. Investigating the effectiveness and utility of more practical means of degassing for HTST pasteurized, hot-filled CJC – perhaps by a rapid, in-line degassing system or degassing by chemical means (perhaps enzymes) – might prove valuable. Further, degassing might be employed in concert with emerging processing to increase flavonoid stability. For example, high pressure pasteurization showed no overall benefit compared to traditional heat treatment in terms of the stability of cranberry anthocyanins

and PACs,¹⁴² but perhaps if juice was carefully degassed before being packaged, the treatment might prove beneficial compared to traditional processing. Likewise, ohmic heating, UV radiation and other emerging juice processing methods might benefit from this same treatment.

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