Seasonal and Postharvest Changes in Amino Acid Composition in ‘Crimson Crisp’ Apple (*Malus domestica* Borkh.) in Response to Summer Foliar Urea Applications

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Abstract. Insufficient biologically available nitrogen (N) for yeast is a persistent issue facing cidemakers, whose apple juice base usually does not provide adequate nutrition for a complete fermentation. Cidemakers often supplement their juice with additional yeast assimilable nitrogen (YAN) in the cellar to aid fermentation. The development of biologically available N in apple juice is not well understood. In this study, juice samples from ‘Crimson Crisp®’ apples were taken at several sampling dates in the 2016, 2017, and 2018 growing seasons and analyzed for YAN using formol titration and high-performance liquid chromatography. It was observed that while the total YAN concentration in these apples drops from the period shortly after fruit set to the end of summer, YAN remains stable from several weeks before harvest until the date of harvest. The total YAN did not change after a 6-week postharvest storage period. By contrast, the individual amino acid components of YAN do change during this period. This experiment shows that foliar urea sprays in ‘Crimson Crisp®’ produce an increase in organic N in the juice, mostly in the form of asparagine. Increased organic N impacts yeast growth and sensory characteristics of cider and may be seen as desirable by cider producers.

One of the perennial obstacles for cider producers is nitrogen (N) as a limited resource in apple juice. For a complete fermentation to take place without producing off-flavors, the juice must have an adequate N supply that is biologically available to yeast (Bisson, 1999). This available N, known as YAN, consists of primary amino nitrogen (PAN) and ammonium and is an important factor in controlling fermentation kinetics (Kelkar and Dolan, 2012). Proteins and small polypeptides, in addition to proline (Pro), are not easily assimilable by yeast and are thus not included in the YAN quantity

(Bell and Henschke, 2005). Proline uptake, specifically, is often inhibited by N catabolite repression by the yeast (Bell and Henschke, 2005). Recommendations for ideal YAN concentrations in apple juice range widely, with some suggesting similar concentrations to those found in grape juice. Neilsen et al. (2010) suggested 140 mg·L⁻¹ as sufficient for YAN in wine production, which has a higher initial sugar concentration than cider production and therefore should require a longer fermentation based on fermentation models (Kelkar and Dolan, 2012).

Amino acid components of YAN are alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), γ -aminobutyric acid (GABA), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val). Proline, a nonassimilable amino acid, is only present in apples in small quantities (Blanco Gomis et al., 1990).

Studies of apple juices made from both culinary and cider cultivars show variation in YAN concentrations among cultivars (Cline et al., 2021; Plotkowski and Cline, 2021). Some studies found that the greatest fraction of amino acids was generally composed of Ala followed by Asn, Ser, Gln, and Thr (Wu et al., 2007), whereas others observed that the

major amino acids were Asn, Asp, Glu, and Ser (Burroughs, 1957). This variation among cultivars was also seen in past experiments in cider apples, which tested the amino acid composition of the entire fruit, rather than just the juice (Blanco Gomis et al., 1990). The major amino acids in ‘Collaos’, ‘Raxao’, and ‘Meana’ apples include Asn and Asp, with ‘Collaos’ also having similar relative proportions of Gln, Glu, Ser, and Phe (Blanco Gomis et al., 1990). In addition to Asn and Asp, Ma et al. (2018) found that many cultivars, such as Northern Spy, Winesap, and Rome, had relatively high concentrations of Phe.

Excess N fertilization can reduce fruit firmness and color while increasing storage disorders in apples (Neilsen and Neilsen, 2002). Nitrogen from soil-applied fertilizer in the spring has been demonstrated to make up a greater percentage of fruit N than N from soil-applied fertilizer in the summer, with N accumulation increasing consistently in the fruit until about a month before harvest (Toselli et al., 2000). During fermentation, the amino acids in the must are depleted due to consumption by yeast, but they are eventually returned to the must due to yeast autolysis (Blanco Gomis et al., 1990; Suárez Valles et al., 2005). Changes in amino acid composition continue to occur after primary fermentation and during the aging process of cider (Suárez Valles et al., 2005). The relationship between nutrient availability and the production of desirable aroma compounds is important for the selection of yeast strains (Carrau et al., 2008; Dukes and Butzke, 1998), but studies describing yeast-produced aromas do not usually consider N composition. Fewer differences are found among strains when initial N is high than when nutrient availability is an issue (Carrau et al., 2008).

YAN in apple juice is principally composed of amino acid N, with a small quantity of ammonium (Boudreau et al., 2018). The quantity and composition of YAN will change based on the apple cultivar and year-to-year variations (Blanco Gomis et al., 1990). Many cidemakers choose to supplement their base juice with ammonium or a complex N source to ensure a complete fermentation without hydrogen sulfide (H₂S) production, which has an undesirable odor. Other methods for manipulating YAN in the cidery include N extraction by keeving, in which yeast and nutrients are physically removed, or the addition of N sources like wine lees (Nichols and Proulx, 2003). To measure YAN before additions, samples of nonfermented juice can be analyzed using methods like formol titration or by using commercial testing kits with spectrophotometric assays. Individual amino acids can be measured using high-performance liquid chromatography (HPLC) with orthophthalaldehyde (OPA) derivatization of amino acids (Blanco Gomis et al., 1990; Dukes and Butzke, 1998).

Previous work in Spain measured seasonal changes in amino N concentration in apples using whole fruit samples including seeds, skin, and mesocarp (Blanco Gomis et al., 1990). The amino acids extracted from the

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pureed fruit may differ from what cidemakers access using standard juice extraction methods. Still, a significant seasonal variation in specific amino acids among different apple cultivars exist, with ‘Collaos’, ‘Raxao’, and ‘Meana’ apples all having different relative proportions of amino acids (Blanco Gomis et al., 1990).

Many of the considerations for cidemaking are similar to winemaking; the changes in amino acids in grapes throughout the growing season and in response to N fertilization treatments have been studied, including the evaluation of YAN for the fermentation process. In grapes, there are common N development trends across many cultivars between véraison and harvest. In most grape cultivars, total N, amino acid N, and Pro increase while ammonium steadily decreases, whereas Arg decreases after a rapid increase (Bell and Henschke, 2005). It is believed that the change in Arg concentration in grape juice is due its use as a transport molecule in the remobilization of the N to storage organs for future use. Other horticultural factors have been shown to change amino acid concentration, including cluster shading, elicitors, N fertilization, cultivar, and weather protection (Guan et al., 2017; Gutiérrez-Gamboa et al., 2018; Meng et al., 2018).

Hannam et al. (2016) conducted an N-fertilization experiment based on grapes, which demonstrated that the application of foliar N was most effective for increasing YAN in grapes when the fruit has its greatest sink strength at ripening. After determining that a series of three foliar urea applications of 3.8 g N/vine resulted in the vine incorporating more amino acids and ammonium into the juice than the same series of urea application to the soil, the researchers applied foliar urea to grapes in three different treatments. Late-season applications of urea were the most effective at improving YAN in grape must. In the first year of the experiment, there was no treatment difference in fruit in Merlot, but in the following year there was a greater yield in the fertilized Merlot vines. In separate years, fertilization led to a decrease in titratable acidity in Pinot Gris fruit. In the fertilized vines, the juices produced has a lower proportion of Pro, which is nonasimilable, therefore increasing the total YAN (Hannam et al., 2016). Past research has shown, however, that N application should only be made when factors indicate that the vineyard is lacking in N (Bell and Henschke, 2005). Nitrogen controls vine growth, but its misuse can negatively affect the environment,

such as through leaching nitrates into groundwater (Hannam et al., 2016; Neilsen and Neilsen, 2002). A similar study was done in ‘Red Spy’ apples, where late-season foliar N applications of a 1% urea solution were conducted 6 weeks before harvest (Karl et al., 2020a). The researchers found that apple YAN increased by 200% to 400%, mostly as PAN with asparagine having the greatest increase in apple juice in response to fertilization. Other juice attributes did not seem to be affected by the late-season applications and the cider produced from the juice did not have any residual H₂S production. The same group conducted spring applications of calcium nitrate to soil in ‘Golden Russet’ and ‘Medaille d’Or’ apples and found that the fertilization resulted in YAN increases in both cultivars, again with a pronounced increase in asparagine (Karl et al., 2020b).

There are potential additive effects of N fertilization on YAN based on application methods (Hannam et al., 2016). Increasing N fertilization at any stage leads to higher N concentrations in the apples (Khemira et al., 1998), while in grapes, postharvest N supplementation doesn’t affect fruit composition or YAN the following year, but can increase yield (Neilsen et al., 2010). Nitrogen

Table 1. Primary amino nitrogen concentrations in ‘Crimson Crisp’[®] apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

		2016		2017		2018	
Treatment	Sampling date	Primary amino nitrogen (mg·L ⁻¹ N) ^z		Primary amino nitrogen (mg·L ⁻¹ N)		Primary amino nitrogen (mg·L ⁻¹ N)	
Main effects							
Number of sprays							
0		104 b		83 b		42 b	
3		119 b		91 b		51 b	
6		155 a		116 a		64 a	
P value		0.0003		<0.0001		0.0013	
Harvest date							
			5 July	186 a			
			25 July	116 b			
			16 Aug.	84 c			
			5 Sept.	66 d			
	4 Oct.	126	25 Sept.	63 d	1 Oct.	54	
			3 Oct. ^y	66 d	1 Oct. ^x	50	
P value				<0.0001		0.1124	
Simple effects							
0 Sprays			5 July	181 ab			
3 Sprays				178 ab			
6 Sprays				198 a			
0 Sprays			25 July	78 ef			
3 Sprays				122 cd			
6 Sprays				148 bc			
0 Sprays			16 Aug.	67 f			
3 Sprays				79 ef			
6 Sprays				106 de			
0 Sprays			5 Sept.	64 f			
3 Sprays				57 f			
6 Sprays				76 ef			
0 Sprays	Mature fruit	4 Oct.	25 Sept.	51 f	1 Oct.	48	
3 Sprays				54 f		49	
6 Sprays				85 ef		66	
0 Sprays	Stored fruit		3 Oct. ^y	57 f	1 Oct. ^x	36	
3 Sprays				56 f		52	
6 Sprays				84 ef		62	
P value				0.009		0.0654	

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1 °C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1 °C and pressed on 28 Nov. 2018.

treatments in grapevines consistently affect YAN concentrations in grape juice every year, but the response varies with application rate and timing (Neilsen et al., 2010). Based on recommended YAN concentrations for wine, though, YAN only exceeds deficiency in grape juice when the vineyard receives applications of N fertilizer at a rate that exceeds twice the recommendation (Neilsen et al., 2010). This may prove true for apples as well, which could mean that N fertilization in the orchard has limited use as a means to increase prefermentation YAN for cider production.

To better understand how YAN develops in apples and specifically in the juice, an experiment was conducted to evaluate how YAN changes during a growing season in addition to how YAN composition changes in response to foliar N fertilization. ‘Crimson Crisp’[®] apples show promise for cider production in Ontario, and are a good candidate for this study due to its low concentration of YAN in typical years, which may require amelioration (Plotkowski and Cline, 2021). Transport amino acids, such as Asn and Glu (Pate, 1980), are likely to make up a large portion of additional amino nitrogen, while the concentrations of sulfur-containing amino acids may affect H₂S concentrations in fermentation.

The research objectives of this study were to develop a profile of the concentration of individual amino acids and total YAN in apples at different stages throughout the growing season and to understand the effect of orchard foliar N fertilization on the YAN concentration of apple juice/must. The hypotheses tested to achieve the research objective were that fertilizing apple trees with a foliar urea spray would increase YAN in apple juice and that amino nitrogen would decrease in the fruit juice during the growing season and after storage.

Materials and Methods

Orchard work. The trees used in the experiment were from two adjacent mature orchard plots of ‘Crimson Crisp’[®] apple trees on M.9 rootstock planted in 2009 and 2012 at the Simcoe Research Station in Simcoe, ON (lat. 42°51′40″ N, long. 80°16′8″ W) at a spacing of 1.0 m × 4 m (2500 trees/ha) and trained to a super spindle orchard system. The orchard soil consisted of a Brady sandy loam (Brunisolic Grey Brown Luvisol) (Presant and Acton, 1984) with imperfect drainage and soil textures consisting of mainly lacustrine sand and sandy loam over glaciolacustrine clays at depths greater than 1.5 m (Hohner and Presant, 1985). Trees were trickle-irrigated daily with an equivalent of ≈2.5 cm of water weekly (adjusted for natural rainfall) on a schedule of six irrigation run-times per day every 4 h (20 min per event). Irrigation was delivered using 2 L·h⁻¹ pressure-compensating emitters spaced 45 cm apart. Standard cultural and pest management practices for Ontario were followed (OMAFRA, 2016). Weeds were controlled within a 1-m strip on each side of the tree row using 1% (v/v) glyphosate applications made mid-May, June, and July. A permanent sod culture was established at the time of

planting in the row middle using a mixture of 40% perennial rye and 60% red fescue (Vineland Growers, Vineland, ON).

In 2016, beginning 1 week after petal fall, ‘Crimson Crisp’[®] apple trees were treated with one of three treatments: 1) untreated, 2) three foliar urea sprays applied weekly after petal fall, and 3) six foliar urea sprays applied weekly after petal fall. Foliar sprays of 5.1 g·L⁻¹ N (46% N, urea; PotashCorp, Saskatoon, SK) with Regulaid (KALO, Overland Park, KS) were sprayed to drip (≈0.5 L per tree) using a hand wand and a 12V DC diaphragm pump (SHURflo, Minneapolis, MN). Sprays were applied on 10 June, 17 June, and 29 June for treatments 2 and 3. Treatment 3 also received sprays on 6 July, 15 July, and 26 July. Treatments were applied in a randomized complete block design with 6 replications, 3 treatments, and 10 trees per replication. A single buffer or “guard” tree was maintained between blocks to prevent spray drift.

Apple fruits in 2016 were sampled after the third spray, and every 3 weeks thereafter until harvest. Sampling consisted of taking 1 apple (free of defects) selected randomly from each of the 10 trees, for a total of 10 apples per sampling date. In 2016, apples were labeled and stored at -20 °C until they were processed. Fruits collected on the sampling dates 30 Aug., 14 Sept., and 4 Oct. were analyzed for YAN. Before processing, fruit were thawed at 4 °C for 12–18 h, and then pressed. Ten thawed apples were wrapped in cheesecloth (Grade #50; Fisher Scientific, Whitby, ON), and placed on a custom-made steel rack (Allingham Machining

Inc., Stoney Creek, ON). Another stainless-steel rack was placed on top and the fruit was pressed with a PowerFist hydraulic press to a pressure of 17,000 kPa (Princess Auto, Hamilton, ON). This method of collecting juice is used as an alternative to milling apples in home cider production as well as in some ice cider production (Jolicoeur, 2013). The juice from each sample was collected, mixed, and placed into a 50-mL centrifuge tube and stored at -80 °C.

In 2017, the same treatment regime for spraying and sampling was followed. Sprays were applied on 8 June, 14 June, and 21 June for treatments 2 and 3, while treatment 3 also received sprays on 6 July, 14 July, and 20 July. Sampling dates were 5 July, 25 July, 16 Aug., 5 Sept., 25 Sept., and 3 Oct. In 2017, apples were pressed within a day of harvesting, except for the final harvest, which was left for 6 weeks at 4 °C to investigate the effect of storage. The freshly sampled apples were sectioned to fit into the feed tube of a commercial juicer (Model 8006; Omega, Harrisburg, PA), which ground the apples using the grinding attachment. The pomace was then wrapped in cheesecloth before being pressed using the hydraulic press. The juice from each sample was collected, mixed, and placed into a 50-mL centrifuge tube and stored at -80 °C.

In late Fall 2017, samples from 2016 and 2017 were thawed in batches in a water bath to 15 °C. Each 5-mL sample was analyzed using an autotitrator (G20 Compact Titrator; Mettler Toledo, Mississauga, ON) set to a pH endpoint of 7.0. An equivalent amount of 0.1 N NaOH was added to 5 mL of thawed

Fig. 1. Changes in primary amino nitrogen (PAN) concentration in ‘Crimson Crisp’[®] juice from mid-season through harvest and storage in ‘Crimson Crisp’[®] apple juice based on treatment with 5.1 g·L⁻¹ N solution foliar urea sprays. University of Guelph, Simcoe, Ontario, 2017.

juice, mixed, and stored in 15-mL centrifuge tubes at -80°C until analyzed by HPLC.

In 2018, the same treatments were applied as in 2016 and 2017, on 11 June, 19 June, and 26 June for treatments 2 and 3, while treatment 3 also received sprays on 3 July, 10 July, and 17 July. Due to the light fruit set, samples were only taken at maturity on 1 Oct., with 10 fruit per block being pressed immediately and 10 fruit being left for 8 weeks in storage at 4°C . The juice samples pressed in 2018 underwent HPLC analysis the same week they were pressed.

Amino acid analysis. Samples were analyzed for Ala, Arg, Asn, Asp, Cys, GABA, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, and Val by HPLC using the methods for amino acid detection using the Agilent Zorbax Eclipse AAA column (Agilent Technologies, Waldbronn, Germany; Henderson et al., 2000). Proline was not analyzed because it is not assimilable by yeast. Sodium phosphate monobasic buffer was mixed for mobile phase A, whereas methanol/acetonitrile buffer was mixed and filtered using a vacuum flask for mobile phase B. The Agilent 1100 Series HPLC instrument (Agilent Technologies) and ChemStation software (Agilent Technologies) were

programmed with the Agilent amino acid analysis protocol using reverse-phase chromatography and OPA derivatization. Tubes of titrated juice samples were thawed in cool tap water and vortexed to homogenize the sample. In a 1-mL Eppendorf tube, 250 μL of sample was mixed with 250 μL of sodium phosphate monobasic buffer and centrifuged. The supernatant was then passed through a sterile syringe (Fisher Scientific Company 14-823-30) with a filter tip (Mandel Scientific RSK-26143, Guelph, ON). A 50- μL aliquot of the filtered solution was then loaded into a vial insert and placed into a vial. The samples were passed through a Zorbax Eclipse AAA column and then detected using a diode array detector at 262 nm. A standard sample of the amino acids was run after every five samples for calibration using authentic standards in the form of a commercially available standard mixture (Agilent Technologies). The chromatograms were analyzed for consistency within 10% for the calibration samples, with repeats being run if necessary. Fluorescence response factors were calculated as the proportion of the average integrated area of the standard chromatogram peaks to the concentrations of the amino acids found in the authentic standards. For analysis, the area under the peaks on the

chromatograms of the samples were integrated using ChemStation and multiplied by the fluorescence response and dilution factors to calculate the amino acid concentrations in the samples. Samples of juice collected from mature fruit were analyzed for the 2016 and 2018 seasons. Samples of juice collected at various points from the season up until maturity were analyzed for the 2017 season.

Statistical analysis. Data were analyzed using the GLIMMIX procedure in SAS 9.4 (The SAS Institute, Cary, NC). Significance was evaluated at a P value of 0.05 and residuals were analyzed for normality and outliers. Post-hoc means separation was analyzed using Tukey–Kramer grouping for least square means ($\alpha = 0.05$). The factors analyzed were sampling date, which included a storage period postmaturity in 2017 and 2018, foliar urea spray treatment, and the interaction between sampling date and treatment.

Results

The results describe the changes in amino acid concentrations in response to foliar urea fertilization treatments, sampling date, and their interaction.

Table 2. Aspartic acid concentrations in ‘Crimson Crisp’ apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	2016		2017		2018	
	Sampling date	Aspartic acid ($\mu\text{mol}\cdot\text{L}^{-1}$) ^z	Sampling date	Aspartic acid ($\mu\text{mol}\cdot\text{L}^{-1}$) ^z	Sampling date	Aspartic acid ($\mu\text{mol}\cdot\text{L}^{-1}$) ^z
Main effects						
Number of sprays						
0		2,361 b		1,083 b		1,312
3		2,769 b		1,056 b		1,587
6		3,264 a		1,316 a		1,531
P value		0.001		<0.0001		0.0508
Harvest date						
			5 July	627 d		
			25 July	619 d		
			16 Aug.	628 d		
			5 Sept.	869 c		
	4 Oct.	2,798	25 Sept.	1,903 b	1 Oct.	1,450
			3 Oct. ^y	2,265 a	1 Oct. ^x	1,504
P value				<0.0001		0.376
Simple effects						
0 Sprays			5 July	617 de		
3 Sprays				586 e		
6 Sprays				678 de		
0 Sprays			25 July	469 e		
3 Sprays				653 de		
6 Sprays				734 de		
0 Sprays			16 Aug.	569 e		
3 Sprays				614 de		
6 Sprays				702 de		
0 Sprays			5 Sept.	810 de		
3 Sprays				723 de		
6 Sprays				1,074 de		
0 Sprays	Mature fruit	2,361	25 Sept.	1,686 c	1 Oct.	1,372
3 Sprays		2,769		1,733 c		1,253
6 Sprays		3,264		2,290 ab		1,498
0 Sprays	Stored fruit		3 Oct. ^y	2,084 bc	1 Oct. ^x	1,677
3 Sprays				2,029 bc		1,481
6 Sprays				2,681 a		1,581
P value				0.0001		0.1264

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1°C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1°C and pressed on 28 Nov. 2018.

Primary amino nitrogen. Total PAN of juice was consistently affected by the number of foliar urea sprays in the six-spray treatment each year of the study (Table 1; Fig. 1). There was a significant interaction ($P = 0.009$) between the number of sprays and the sampling date; trees that received six sprays produced apples that consistently had higher PAN concentrations than those that received zero or three sprays, 36% to 62% higher in each year from 2016 to 2018 at maturity. PAN concentration decreased as the season progressed in 2017, starting at $186 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ on 5 July 2017, and dropping to $63 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ at maturity on 25 Sept. Storage did not have a significant effect on total PAN

concentration in 2017 or 2018 (Table 1). The data across different years could not be directly compared due to differences in methods and typical seasonal variation in YAN (Plotkowski and Cline, 2021). PAN at fruit maturity ranged from 104 to $155 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ in 2016, 51 to $85 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ in 2017, and 48 to $66 \text{ mg} \cdot \text{L}^{-1} \text{ N}$ in 2018.

Aspartic acid. There was a significant treatment effect on juice Asp concentrations in 2016 ($P = 0.001$), but not in 2018, with Asp concentrations 27% higher in the apple juice from the trees that had received six sprays over those that received zero or three sprays (Table 2). There was a significant ($P = 0.0134$) interaction between the number of sprays and the sampling

date in 2017. Generally, trees that received six sprays produced apples that had juice Asp concentrations that were 12% to 40% higher than those that received zero or three sprays. Asp concentration increased as the season progressed in 2017, ranging from $627 \mu\text{mol} \cdot \text{L}^{-1}$ on 5 July to $1903 \mu\text{mol} \cdot \text{L}^{-1}$ at maturity and $2265 \mu\text{mol} \cdot \text{L}^{-1}$ after storage (Fig. 2). In 2018, treatments did not affect Asp concentration, with Asp ranging from $1253 \mu\text{mol} \cdot \text{L}^{-1}$ to $1677 \mu\text{mol} \cdot \text{L}^{-1}$ after storage; however, the interaction of the spray treatments and storage were not significant.

Asparagine. The Asn concentration in the juice was significantly affected by the number of foliar urea sprays applied to the block in 2016 ($P = 0.0015$) and 2018 ($P = 0.0001$)

Fig. 2. Seasonal development of aspartic acid (Asp), glutamic acid (Glu), Asparagine (Asn), and serine (Ser) in 'Crimson Crisp' apple juice in response to $5.1 \text{ g} \cdot \text{L}^{-1} \text{ N}$ foliar urea sprays. University of Guelph, Simcoe, Ontario, 2017.

Table 3. Asparagine concentrations in 'Crimson Crisp'® apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	2016		2017		2018	
	Sampling date	Asparagine (μmol·L ⁻¹) ^z	Sampling date	Asparagine (μmol·L ⁻¹) ^z	Sampling date	Asparagine (μmol·L ⁻¹) ^z
Main effects						
Number of sprays						
0		3,867 b		3,676 c		1,226 b
3		4,429 b		4,270 b		1,535 b
6		6,382 a		5,601 a		2,452 a
<i>P</i> value		0.0015		<0.0001		0.0001
Harvest date						
			5 July	10,656 a		
			25 July	6,159 b		
			16 Aug.	4,012 c		
			5 Sept.	2,794 d		
	4 Oct.	4,893	25 Sept.	1,812 e	1 Oct.	1,838
			3 Oct. ^y	1,661 e	1 Oct. ^x	1,637
<i>P</i> value				<0.0001		0.125
Simple effects						
0 Sprays			5 July	10,339 a		
3 Sprays				10,254 a		
6 Sprays				11,375 a		
0 Sprays			25 July	3,960 de		
3 Sprays				6,510 bc		
6 Sprays				8,006 b		
0 Sprays			16 Aug.	2,892 ef		
3 Sprays				3,722 de		
6 Sprays				5,423 cd		
0 Sprays			5 Sept.	2,408 ef		
3 Sprays				2,423 ef		
6 Sprays				3,550 de		
0 Sprays	Mature fruit	4 Oct.	25 Sept.	1,219 f	1 Oct.	1,526
3 Sprays				1,400 f		926
6 Sprays				2,817 ef		1,453
0 Sprays	Stored fruit		3 Oct. ^y	1,236 f	1 Oct. ^x	1,617
3 Sprays				1,313 f		2,534
6 Sprays				2,433 ef		2,369
<i>P</i> value				0.0134		0.0626

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey's test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1 °C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1 °C and pressed on 28 Nov. 2018.

(Table 3), with Asn concentrations 54% and 78% higher, respectively by year, in the apple juice from the trees that had received six sprays than in those that had received zero or three sprays. The interaction between the sampling date and the spray treatment in 2017 was significant ($P = 0.0134$), with Asn concentration decreasing as the season progressed from 10,656 μmol·L⁻¹ on 5 July to 1812 μmol·L⁻¹ at maturity while staying elevated in the samples that received the six-spray treatment (Fig. 2). Storage did not affect the Asn concentration in 2017 or 2018 (Table 3).

Serine. The Ser concentration in the juice was affected by the number of foliar urea sprays applied to the block in 2017 ($P < 0.0001$) and 2018 ($P = 0.0005$), with Ser concentrations being 87% and 33% higher, respectively by year, in the samples that received six urea sprays than in those that received zero or three sprays, but the treatment was not significant in 2016 (Table 4). In 2017, the sampling date was significant ($P < 0.0001$), with Ser first decreasing and then increasing toward harvest (Fig. 2). The sampling date and the treatment did not have a significant interaction for Ser, nor did storage in 2017. In 2018, the Ser concentration

decreased significantly ($P < 0.0001$) by 27% after storage.

Glutamic acid. The Glu concentration in the samples was significantly affected by the foliar urea spray treatment in 2016 ($P = 0.0182$), with the Glu concentration being 21% higher in the samples that had received the six-spray treatment than in those that had received zero or three sprays (Table 5). The interaction between spray treatment and sampling date was significant in 2017 ($P = 0.0005$), with Glu concentration decreasing as the season progressed from 701 μmol·L⁻¹ on 5 July to 247 μmol·L⁻¹ at maturity, but rising to 282 μmol·L⁻¹ after storage (Fig. 2). During the 2017 season, Glu concentration was normally highest in those samples that received six urea sprays. In 2018, there was a significant ($P = 0.0003$) 17% decrease in Glu concentration in the samples after storage.

Other amino acids. The other amino acids that were detected in measurement include Gln, His, Gly, Arg, Ala, GABA, Val, Cys, and Met. Their concentrations were much lower than the previously listed amino acids and they are detailed in Figs. 3 and 4.

Total YAN concentration by formol number. The total YAN concentration as determined by formol titration was significantly

affected by the foliar urea spray treatment in 2016 ($P < 0.0001$) and 2018 ($P = 0.0007$), with YAN concentration being 38% and 29% higher, respectively by year, in those samples that had received the six-spray treatment than those that had received zero or three sprays (Table 6). In 2016, there was no significant difference between samples taken at the end of August and the mature harvest in October, nor was there a significant interaction between the spray treatment and the sampling date in 2016. In 2017, there was a significant ($P = 0.0395$) interaction between spray treatment and sampling date, with a general decrease in YAN concentration as the season progressed from 242 mg·L⁻¹ N on 5 July to 85 mg·L⁻¹ N at maturity on 25 Sept. On each date, the six-spray treatment samples had the highest YAN concentration. The YAN concentration stabilized early in September through harvest and storage. There was a significant ($P < 0.0001$) 25% decrease in YAN concentration after storage in 2018.

Discussion

These experiments demonstrated that without late-season N additions, total YAN in 'Crimson Crisp'® juice does not change in

Table 4. Serine concentrations in ‘Crimson Crisp’[®] apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	2016		2017		2018	
	Sampling date	Serine (μmol·L ⁻¹) ^z	Sampling date	Serine (μmol·L ⁻¹) ^z	Sampling date	Serine (μmol·L ⁻¹) ^z
Main effects						
Number of sprays						
0		276		151 b		111 b
3		308		142 b		119 b
6		340		275 a		153 a
<i>P</i> value		0.095		<0.0001		0.0005
Harvest date						
			5 July	273 b		
			25 July	224 c		
			16 Aug.	222 c		
			5 Sept.	262 b		
	4 Oct.	308	25 Sept.	287 ab	1 Oct.	147 a
			3 Oct. ^y	322 a	1 Oct. ^x	108 b
<i>P</i> value				<0.0001		<0.0001
Simple effects						
0 Sprays			5 July	258		
3 Sprays				245		
6 Sprays				282		
0 Sprays			25 July	224		
3 Sprays				283		
6 Sprays				353		
0 Sprays			16 Aug.	302		
3 Sprays				307		
6 Sprays				356		
0 Sprays			5 Sept.	269		
3 Sprays				252		
6 Sprays				300		
0 Sprays	Mature fruit	4 Oct.	25 Sept.	118	1 Oct.	135
3 Sprays				129		87
6 Sprays				180		129
0 Sprays	Stored fruit		3 Oct. ^y	158	1 Oct. ^x	109
3 Sprays				125		177
6 Sprays				170		128
<i>P</i> value				0.0859		0.1074

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1 °C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1 °C and pressed on 28 Nov. 2018.

the last few weeks before harvest, nor does it change after a short period of storage, such as one that a cider producer may use. This is consistent with the results from Toselli et al. (2000), where N accumulation increased consistently until about a month before harvest in the fruit without late-season N additions in ‘Mutsu’ apples. On the contrary, total fruit amino N concentrations fluctuated in the final month leading to harvest in ‘Meana’, ‘Raxao’, and ‘Collaos’ apples, which may be due to changes in the flesh, seeds, or skin rather than the juice, which were included in the analyses in this study. The composition of YAN in extracted juice, however, changes after storage and in response to treatment with foliar urea sprays.

The first research objective of this study was to develop a profile of the concentration of individual amino acids and total YAN in apples at different stages throughout the growing season. Based on data from the 2017 study, summarized in Figs. 1 and 5, the main changes are that total amino acids decrease over the course of the growing season. This change in amino acid concentrations in the growing season in ‘Crimson Crisp’[®] comes primarily from the loss of Asn while the

relative concentration of Asp increases (Fig. 5). This pattern is the same regardless of treatment, though both residual Asn and additional Asp are the highest in the six-spray treatment. (Fig. 2). This change appears to depend on the cultivar, as Asp has been shown to decrease in ‘Meana’ and fluctuate in ‘Raxao’ and ‘Collaos’ apples as the fruit approaches maturity (Blanco Gomis et al., 1990). Similarly, the overall profile of amino acids at maturity is different among dessert cultivars (Wu et al., 2007); this study did not differentiate between Asn and Asp or Glu and Gln, but in the results found here and in other literature, the three highest proportions of the amino acid fraction belonged to Asp/Asn, Glu/Gln, and Ser in every cultivar (Blanco Gomis et al., 1990; Burroughs, 1957; Wu et al., 2007). In this study, the proportion of Asn and Asp as parts of the total varied significantly based on year and by treatment, with the six-spray treatment being associated with a higher proportion of Asn and a lower proportion of Asp and other amino acids relative to the zero- and three-spray treatments, which were the same (Table 7). The higher proportion of asparagine at the highest fertilization rate is consistent with the findings

conducted by Karl et al. (2020a, 2020b). Both Asn and Gln are used as transport molecules for N in plant vascular tissue, which could explain their high concentrations early in the growing season as the apples are expanding (Taiz and Zeiger, 1991). Both sulfur-containing amino acids, Cys and Met, decrease for the first part of the season and then increase before harvest and maturity. These amino acid concentrations, while not reported in Blanco Gomis et al. (1990), were observed in low concentrations at maturity in Wu et al. (2007) and Burroughs (1957). If the concentration of Met continues to increase as fruit ripens in other cultivars, then H₂S production should be reduced during fermentation due to a reduced need for Met production by the yeast (Jiranek et al., 1995). Similarly, it means that harvesting immature or underripe apples could lead to higher H₂S production if other cultivars have similar amino acid development patterns.

The second research objective of this study was to observe the effect of orchard foliar N fertilization on the YAN concentration of apple juice/must. Only the highest spray treatment of six foliar urea sprays consistently had an effect on the YAN

Table 5. Glutamic acid concentrations in mature ‘Crimson Crisp’[®] apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	2016		2017		2018	
	Sampling date	Glutamic acid (μmol·L ⁻¹) ^z	Sampling date	Glutamic acid (μmol·L ⁻¹) ^z	Sampling date	Glutamic acid (μmol·L ⁻¹) ^z
Main effects						
Number of sprays						
0		46 b		417 b		146
3		57 ab		431 ab		161
6		62 a		461 a		157
<i>P</i> value		0.0182		0.0071		0.2622
Harvest date						
			5 July	701 a		
			25 July	551 b		
			16 Aug.	506 b		
			5 Sept.	332 c		
	4 Oct.	55	25 Sept.	247 d	1 Oct.	169 a
			3 Oct. ^y	282 cd	1 Oct. ^x	141 b
<i>P</i> value				<0.0001		0.0003
Simple effects						
0 Sprays			5 July	715 ab		
3 Sprays				658 ab		
6 Sprays				731 a		
0 Sprays			25 July	434 ef		
3 Sprays				594 bcd		
6 Sprays				626 abc		
0 Sprays			16 Aug.	499 de		
3 Sprays				504 cde		
6 Sprays				516 cde		
0 Sprays			5 Sept.	353 fg		
3 Sprays				316 fg		
6 Sprays				325 fg		
0 Sprays	Mature fruit	4 Oct	25 Sept.	235 g	1 Oct.	166
3 Sprays				249 g		126
6 Sprays				257 g		164
0 Sprays	Stored fruit		3 Oct. ^y	268 g	1 Oct. ^x	158
3 Sprays				264 g		177
6 Sprays				314 fg		138
<i>P</i> value				0.0005		0.0546

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1 °C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1 °C and pressed on 28 Nov. 2018.

composition, primarily by increasing the relative concentration of Asn, which is a common amino acid for transport in the vascular tissue of plants along with Glu (Pate, 1980). Within the plant cells, urea is degraded into ammonia and carbon dioxide by urease, which is the only method of urea N assimilation into plants (Polacco and Holland, 1993).

The first hypothesis tested in this study was that, similar to the grapes studied by Hannam et al. (2016), apple trees fertilized with a foliar urea spray will produce fruit with a higher total YAN concentration in the juice than those that did not receive foliar urea sprays. Although this was observed at nearly every sampling date, it was only found for the treatment groups that received six sprays of urea, which was four times as much N as recommended by local agricultural authorities for correcting N deficiency (OMAFRA, 2016). The treatment groups that received three foliar urea sprays, also with a concentration higher than recommended for correcting tree N deficiency, usually did not produce juice with individual amino acid concentrations significantly different from those that received no foliar urea sprays, which is consistent with research by Khemira et al. (1998) that

showed low percentage of N derived from foliar spray in plant tissue. Additionally, Khemira et al. observed that N responses are less pronounced in orchards with high amounts of reserved N from years of fertilization management, which may also explain why only the largest fertilization dosage produced a measurable effect. These results indicate that orchard manipulation of YAN is possible as a concept, but is not practical using the same methods used in this study.

The second hypothesis tested in this study was that amino acid N concentration in juice will decrease as the fruit matures. This prediction was supported by the data, where total PAN concentration decreased as the season progressed (Fig. 1), regardless of treatment. Amino acid content per fruit may have stayed stable, though, as the increased size of the fruit may have diluted the amino acids that were present. This could be tested in a future experiment by sorting fruit from the same tree by size and comparing the concentrations of amino acids in the juice of the differently sized fruit.

The third hypothesis tested in this study was that after harvest, amino acid N in juice decreases. The data from this experiment did

not support the hypothesis. Rather, total PAN did not change significantly after storage in either 2017 or 2018. Similar work where stored apples were tested for PAN concentrations showed inconsistent results among cultivars, with some cultivars, like ‘Dabinett’ decreasing in PAN concentration after storage in 1 year and increasing the following year (Ewing et al., 2019). These inconsistencies could be related to the PAN composition on an amino acid level, as our research showed stability in some amino acids after storage, like Ser, and changes in others, like Asp.

Conclusions

By analyzing the composition of YAN in apple juice both during the growing season and after a postharvest storage period, we were able to better understand how nitrogenous compounds in apple juice change within ‘Crimson Crisp’[®] juice. Unlike winemakers monitoring the changes in N concentration in grape juice as harvest approaches, cidemakers may be able to sample fruit juice well before harvest and plan accordingly without having to worry about late-season changes in total YAN or the accumulation of nonassimilable Pro. This would

Fig. 3. Seasonal development of glutamic acid (Glu), histidine (His), glycine (Gly), and arginine (Arg) in 'Crimson Crisp'® apple juice in response to 5.1 g·L⁻¹ N foliar urea sprays. University of Guelph, Simcoe, Ontario, 2017.

allow cidemakers time to choose if they would like to manipulate the N concentration of the juice and if they would like to use a complex N source or diammonium phosphate. As this study only analyzed one cultivar, other patterns of changes in amino acids in other apple cultivars would be needed to test the range of application of this information. Additional factors to consider would include foliar N status, crop yield, vegetative growth, and disease incidence.

Two extraction methods were used in this study, which resulted in different raw

numbers. Higher concentrations of amino acids were measured in 2016, where juice was extracted from thawed fruit, than in 2017 and 2018, where juice was extracted from fresh fruit. The freezing-thawing process may increase extraction of amino acids due to the rupture of cell walls and membranes from expanding ice, as well as potentially increasing extraction from skin and seeds. Similarly, the freezing process may lead to the degradation of particular amino acids. It cannot be said from this experiment whether the

difference in amino acid concentration was due to extraction method or due to year-to-year variations in YAN concentrations. As such, there was not a direct comparison of YAN or amino acid concentrations among years. The response to foliar fertilization in each year was similar regardless of extraction method.

Future research pertaining to the effects of fertilization in apple orchards on apple juice N should look at the timing of fertilizer application, the concentrations of N used, the effects on N

Fig. 4. Seasonal development of alanine (Ala), γ -aminobutyric acid (GABA), cysteine (Cys), valine (Val), and methionine (Met) in 'Crimson Crisp' apple juice in response to $5.1 \text{ g L}^{-1} \text{ N}$ foliar urea sprays. University of Guelph, Simcoe, Ontario, 2017.

fertilization on vegetative and yield parameters, and the formulations and application methods of N fertilizer. Research pertaining to YAN in apple juice should investigate the variation in

amino acid composition among different apple cultivars, particularly if the effects of storage are consistent across amino acids, and the effects of different maceration and extraction times and

techniques, which could also contribute to the composition of YAN in the juice of mature fruit.

This work demonstrated that urea, an inorganic N source, could be used in the

Fig. 5. Amino acid concentrations of 'Crimson Crisp' juice in the 2017 season arranged by date and $5.1 \text{ g L}^{-1} \text{ N}$ solution foliar urea spray treatment. University of Guelph, Simcoe, Ontario, 2017.

Table 6. Yeast assimilable nitrogen (YAN) in juice of mature ‘Crimson Crisp’[®] apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2016, 2017, and 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	2016		2017		2018	
	Sampling date	YAN (mg·L ⁻¹ N) ^z	Sampling date	YAN (mg·L ⁻¹ N) ^z	Sampling date	YAN (mg·L ⁻¹ N) ^z
Main effects						
Number of sprays						
0		141 b		115 c		99 b
3		149 b		124 b		100 b
6		200 a		157 a		128 a
<i>P</i> value		<0.0001		<0.0001		0.0007
Harvest date						
			5 July	242 a		
			25 July	166 b		
	30 Aug.	167	16 Aug.	121 c		
	14 Sept.	162	5 Sept.	95 d		
	4 Oct.	161	25 Sept.	85 d	1 Oct.	125 a
			3 Oct. ^y	82 d	1 Oct. ^x	94 b
<i>P</i> value		0.5374		<0.0001		<0.0001
Simple effects						
0 Sprays			5 July	223 bc		
3 Sprays				247 ab		
6 Sprays				256 a		
0 Sprays			25 July	141 de		
3 Sprays				155 d		
6 Sprays				203 c		
0 Sprays	30 Aug.	16 May	16 Aug.	100 fgh		
3 Sprays		29 May		111 fg		
6 Sprays		29 July		151 d		
0 Sprays	14 Sept.	19 May	5 Sept.	83 gh		
3 Sprays		29 May		87 gh		
6 Sprays		12 July		116 ef		
0 Sprays	Mature fruit	4 Oct.	25 Sept.	66 h	1 Oct.	119
3 Sprays		145		71 h		109
6 Sprays		193		109 fg		145
0 Sprays	Stored fruit		3 Oct. ^y	74 h	1 Oct. ^x	78
3 Sprays				73 h		91
6 Sprays				107 fg		111
<i>P</i> value		0.3171		0.0395		0.098

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

^yFruit harvested on 3 Oct. 2017 was stored at 1 °C and pressed on 14 Nov. 2017.

^xFruit harvested on 1 Oct. 2018 was stored at 1 °C and pressed on 28 Nov. 2018.

orchard to increase organic N found in juice. Although there are practical limitations to the specific methods used here, learning how to use intentional measures in the orchard to address needs for cider production can reduce the need for interventions during the fermentation process and ultimately simplify and improve cidermaking methods.

Table 7. Relative concentrations of amino acids in juice of mature ‘Crimson Crisp’[®] apple fruit treated with 5.1 g·L⁻¹ N solution foliar urea sprays harvested in 2018. University of Guelph, Simcoe, Ontario, 2018.

Treatment	Proportion of aspartic acid	Proportion of asparagine	Proportion of other amino acids
Year			
2016	32% c	54% a	15% c
2017	43% a	39% c	18% a
2018	38% b	46% b	16% b
<i>P</i> value	<0.0001	<0.0001	<0.0001
Number of sprays			
0 Sprays	40% a	43% b	17% a
3 Sprays	40% a	43% b	17% a
6 Sprays	33% b	52% a	15% b
<i>P</i> value	<0.0001	<0.0001	<0.0001
Simple effects			
2016	0 Sprays	33%	16% bc
2016	3 Sprays	33%	15% bc
2016	6 Sprays	30%	13% d
2017	0 Sprays	47%	20% a
2017	3 Sprays	45%	19% a
2017	6 Sprays	38%	16% bc
2018	0 Sprays	40%	16% bc
2018	3 Sprays	43%	16% bc
2018	6 Sprays	32%	15% cd
<i>P</i> value	0.0706	0.1596	0.0318

^zValues within columns not followed by common letters differ at the 5% level of significance, by Tukey’s test of least square means.

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