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Hydrogen sulphide production during cider fermentation is moderated by pre-fermentation methionine addition

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Yeast assimilable nitrogen (YAN) concentration and composition impact hydrogen sulphide (H_2S) production and fermentation kinetics during wine fermentation, but this phenomenon has not been extensively studied in cider fermentation. Our hypothesis was that H_2S production during cider fermentation could be decreased through pre-fermentation modification of concentrations of individual amino acids. Apple juice (53 mg L^{-1} YAN) was supplemented with asparagine, arginine, methionine or ammonium and fermented with EC1118 and UCD522 yeast strains. No difference in H_2S production among fermentations was observed with addition of asparagine, arginine or ammonium. Methionine addition of 5 mg L^{-1} decreased H_2S production by yeast strain EC1118 at 53 mg L^{-1} YAN. With 153 mg L^{-1} initial YAN, only methionine addition of 50 mg L^{-1} decreased H_2S production, and no tested methionine rates decreased H_2S production with 253 mg L^{-1} initial YAN. Supplementation to 153 mg L^{-1} YAN resulted in increased H_2S production at all methionine concentrations tested. Sensory differences in aroma were detected in samples supplemented with ammonium and methionine, and these differences were correlated with observed differences in H_2S production. Our results indicate that supplementing cider fermentations with methionine leads to lower H_2S formation, especially in apple juice containing low YAN. © 2017 The Authors Journal of the Institute of Brewing published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling

⌨ Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords: amino acids; methionine; cider fermentation; *Saccharomyces cerevisiae*; *Malus × domestica*

Introduction

Hydrogen sulphide (H_2S) is an undesirable aroma compound frequently present in grape (*Vitis vinifera L.*) wines and apple (*Malus × domestica* Borkh.) ciders. In grape fermentation, yeast assimilable nitrogen (YAN) deficiency can contribute to increased hydrogen sulphide (H_2S) production [1–4]. However, yeast strain [5,6], temperature and deficiencies in other yeast nutrients such as biotin and pantothenic acid [7] also contribute to H_2S formation. Therefore, it is reasonable to expect that differences in chemistry between apple and grape juice matrices may affect fermentation performance. While a considerable body of research has been conducted in grape fermentation (i.e. wine production), cider fermentation systems have been the subject of far fewer studies. Since differences in juice chemistry may result in differences in fermentation performance and final product quality, focused study on yeast metabolism during cider fermentation is needed. YAN comprises primary amino nitrogen (PAN) and ammonium, both of which affect fermentation rate, such that lower concentrations of YAN can lead to slow or arrested ('stuck') fermentations [8,9]. Ammonium is preferentially assimilated by yeasts during fermentation [10]. Despite this, greater PAN concentration increases yeast growth rates [11], more so than ammonium concentration alone. Mixtures of ammonium and PAN have been shown to increase yeast growth rates as compared with either component alone [12]. Similarly, the impact of PAN on yeast growth rate has been demonstrated in apple juice fermentation [13].

The interaction among YAN concentration, yeast growth rate and total H_2S production has been demonstrated in grape wine fermentation [14], and several studies have shown that the addition of ammonium and most amino acids leads to a decrease in H_2S formation during fermentation [1,2]. Additionally, a recent study found that nitrogen sources are assimilated differentially owing to nitrogen catabolite repression, and probably impact H_2S production as well [15]. In apple juice, asparagine is often the most prevalent PAN component [16,17], while arginine is generally the most prevalent PAN component of wine grapes [18]. Differences in amino acid composition in juice can result in differences in the production of volatile aroma compounds [19] and H_2S during fermentation [1]. Furthermore, asparagine may proportionally increase H_2S production as compared with arginine [1].

Methionine acts as an inhibitor in the sulphur reduction sequence (SRS) which would otherwise produce free sulphur ions during normal yeast metabolism, causing H_2S to be produced

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(20). When methionine is present in juice during fermentation, H_2S production is lower than in fermentation with no methionine present [1,21]. A threshold methionine concentration that would inhibit H_2S production has not been determined, but Eschenbruch [21] suggested that the concentration must be at least 20 mg L^{-1}. While concentrations of methionine exceeding 20 mg L^{-1} are often found in grape juice, methionine concentrations are typically far lower than 20 mg L^{-1} in apple juice [16,17]. In general, grapes contain a higher concentration of total PAN than apples, although relatively little data is available on YAN in apples [18,22].

The objective of this study was to determine the impact of composition and concentration of YAN on the production of H_2S and on fermentation kinetics during cider fermentation. This study evaluated the impact of the nitrogen sources asparagine, arginine and ammonium on H_2S production and fermentation kinetics, as well as the effect of increasing concentrations of methionine on H_2S production and fermentation kinetics during cider fermentation.

Materials and methods

Apple juice

Pasteurized apple juice, WhiteHouse Fresh Pressed Natural Apple Juice (National Fruit Product Co., Winchester, VA, USA) was acquired in 1.9 L containers from a local retailer. This juice was used to ensure the consistency of juice across samples. It was homogenized into a single uniform lot, aliquoted into 1 L containers and then stored at −20°C until use. Juice was thawed to 22°C prior to inoculating with yeast. Juice contained a soluble solid concentration of 12.9° Brix, pH 3.7, titratable acidity 3.4 g L^{-1} malic acid equivalent by titration to 8.2 pH with 0.1 M NaOH [23] and 53 mg L^{-1} YAN. YAN was quantified using commercially available Megazyme (Wicklow, Ireland) kits for PAN (K-PANOPA) and ammonium (Ammonia-Rapid).

Nitrogen additions

Amino acids were obtained from Sigma-Aldrich (St Louis, MO, USA). Diammonium phosphate (DAP) was obtained from Scott Laboratories, Inc. (Petaluma, CA, USA). L-Arginine, L-asparagine and DAP were added in sufficient amounts to contribute 140 mg nitrogen L^{-1} to each respective fermentation. L-Methionine was added at concentrations of 5, 20 and 50 mg L^{-1} to respective fermentation treatments. Juice with no nitrogen addition served as a control. Interactive effects of YAN and methionine were examined by supplementing YAN in the apple juice to 153 and 253 mg nitrogen L^{-1} by adding DAP at 100 and 200 mg nitrogen L^{-1}. Each YAN treatment level was then further supplemented with 0 (control), 5, 20 and 50 mg L^{-1} L-methionine.

Fermentations

Two experimental fermentation treatments were conducted using either Prise de Mousse *Saccharomyces bayanus* EC1118 (Lallemand, Montreal, Canada) to represent a low-H_2S-producing strain or Montrachet *Saccharomyces cerevisiae* UCD522 (Lallemand, Montreal, Canada) to represent a high-H_2S-producing strain. Fermentations comparing the interactive effect of total YAN and methionine were only conducted with EC1118. Juice was inoculated with 0.05 g of active dry yeast rehydrated in 35°C water for 20 min. Fermentations were conducted in 200 mL aliquots in a method described by Ugliano and Henschke for instant determination of H_2S formation during alcoholic fermentation [24]. These were conducted in 250 mL Erlenmeyer flasks fitted with a one-hole rubber stopper into which an H_2S detector tube was inserted (as described below). Fermentations were stirred twice daily at 800 rpm for 5 min to prevent yeast settling and to encourage aeration. Each fermentation treatment was carried out in triplicate to quantify variability, in a temperature-controlled chamber at 18°C. Fermentation rate was monitored by measuring the mass of the fermentation vessel twice daily. Changes in mass correspond to CO_2 evolution. Fermentations were ended when CO_2 production slowed to 0.2 g change within a 24 h period. Upon completion, triplicate fermentations for each treatment level were combined and transferred to 500 mL screw-cap bottles. Headspace was purged with N_2 gas for 30 s prior to bottling to prevent oxidation. Bottles were cooled to 4°C to inhibit further fermentation. Finished cider was analysed to determine pH, titratable acidity, residual YAN and residual sugar (RS). Residual sugar was analysed using the D-fructose/D-glucose (K-FRUGL) enzymatic kit (Megazyme, Wicklow, Ireland).

Hydrogen sulphide detector tubes

The H_2S detection and quantification method was described by Ugliano and Henschke [24]. Detector tubes were obtained from Komyo Kitagawa (Tokyo, Japan). Multiple silica tube models were used corresponding to their maximum range of H_2S quantification. CO_2 produced during fermentation carried H_2S through the detector tube, which reacted with the lead acetate (Tube 120SB, 120SD) or silver nitrate (Tube 120SF) contained in the tube, leading to discolouration of the silica in the tube. Both compounds used in the detector tubes were found not to be significantly impacted by other volatile sulphur compounds such as mercaptans [24]. The length of the band was proportional to the amount of purged H_2S. During fermentation, tubes were monitored regularly and replaced with a new tube prior to reaching their saturation point.

Quantification of amino acids

Amino acids were quantified with an Acquity UPLC (Waters Corporation, Milford, MA, USA) and the AccQ•Tag Ultra Derivitization Kit method adapted for cell culture analysis. Standards for L-Glutamine, γ-aminobutyric acid and L-asparagine (Sigma Aldrich, St Louis, MO, USA) were added to the standard mix to improve the free amino acids method for apple and grape juice analysis (Waters Corporation, Milford, MA, USA). According to the manufacturer's method, samples were subjected to precolumn derivatization and injected at 1 μL. Amino acids were separated using an AccQ•Tag Ultra C_{18} Column 100 mm in length (Waters Corporation, Milford, MA, USA) with an Acquity UPLC BEH C_{18} VanGuard pre-column as a guard column (Waters Corporation, Milford, MA, USA). Analytes were eluted at 43°C with a flow rate of 0.7 mL min^{-1} following the gradient described in Table S2 in the Supporting Information. Individual amino acids were quantified by UV detection using a photodiode array detector at the wavelength of 260 nm. Norvaline (Sigma-Aldrich, St Louis, MO, USA) was used as an internal standard.

Determination of maximum fermentation rate, duration and H_2S production rate

Maximum fermentation rate was determined by taking the slope of the fermentation curve during the exponential phase of yeast

growth corresponding to the highest constant rate of CO_2 production, as reported by Sablayrolles *et al.* (25). Steeper slopes correspond to a faster fermentation rate. Fermentation duration was expressed as the hours between inoculation and fermentation termination. Total H_2S production was determined by calculating the sum of H_2S produced over the time course of fermentation as measured by the discolouration of the silica in the tube. Fermentation duration was divided into four equal quartiles for each fermentation to determine the relative rate of H_2S production over the time course of fermentation. The percentage of H_2S produced in each quartile out of the total H_2S produced for a given fermentation was compared to determine whether there was a significant difference in the H_2S production over the time course of fermentation across treatments.

Sensory evaluation

Sensory analyses were conducted at the Virginia Tech Sensory Evaluation Laboratory with prior approval from the Virginia Tech Institutional Review Board (VT IRB #15–559) with informed consent obtained from panelists prior to beginning testing. Cider samples were stored at 4°C for 78 days prior to evaluation. Samples were compared for sensory differences in cider aroma using a triangle test, with an $\alpha = 0.05$, $\beta = 0.20$ and $P_d = 30\%$ (26). Cider samples were presented in 5 mL aliquots at 22°C in 21.5 cL ISO wine tasting glasses covered with Petri dishes to prevent the loss of volatiles. Panelists were seated in partitioned booths in a dedicated sensory evaluation laboratory setting, samples were coded with three-digit codes and served blind, and the presentation order of samples was randomized. Each panelist performed four consecutive triangle tests examining only the aroma of each cider sample. A total of $n = 40$ untrained panelists conducted the sensory evaluation. No demographic information was collected for the panelists.

Statistical analyses

Values were compared using a one-way analysis of variance (ANOVA) at a significance of $p < 0.05$ followed by parametric mean testing using Tukey's honest significant difference (HSD) using GraphPad Prism v.6 (La Jolla, CA, USA). Analyses comparing the interaction among yeast strain, YAN concentration and methionine concentration were analysed using a two-way ANOVA at a significance of $p < 0.05$ and *post-hoc* testing by Tukey's HSD. Statistical analyses for sensory tests were conducted using SIMS Sensory Software (Sensory Computer Systems, Berkeley Heights, NJ, USA) Figure 1.

Results

Hydrogen sulphide production

There was no difference in H_2S production among fermentations when asparagine, arginine or ammonium was added to a low nitrogen content apple juice in EC1118 fermentations (Table 1). However, all methionine concentrations decreased H_2S production as compared with the unsupplemented control juice in the EC1118 fermentation ($p < 0.01$; Table 2). A nearly 40-fold decrease in H_2S production was found when methionine was added at a concentration of 20 mg L^{-1} in the EC1118 fermentation. There was no detectable methionine present in the base apple juice, thus the added concentrations are expected to be equal to the total concentration of methionine (Table S1, Supporting Information). There
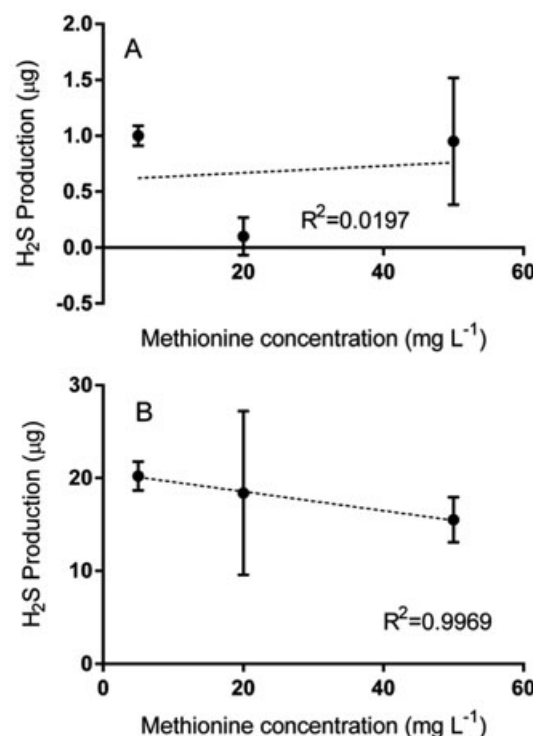

Figure 1. Linear regression of H_2S production and methionine concentration. (A, B) Fermentations using strains EC1118 and UCD522, respectively. There was no correlation of H_2S production and methionine concentration for yeast strain EC1118 but there was a correlation for yeast strain UCD522 (regression analysis, $p < 0.05$).

was no difference in the percentage of the total amount of H_2S produced in a given quartile of the fermentation duration among different YAN sources fermented with EC1118 (Fig. 2A) or in fermentations with added methionine (Fig. 2C). All YAN sources added at a concentration of 140 mg nitrogen L^{-1} increased H_2S production in the second quartile of the fermentation duration as compared with the control.

Additions of nitrogen sources arginine, asparagine and ammonium resulted in greater H_2S production than the control when fermented with UCD522 (Table 1). However, there was no difference in H_2S production among nitrogen sources in UCD522 fermentations. Contrary to the findings of fermentations with EC1118, there was no difference in H_2S production between the control and additions of methionine at any concentration in UCD522 fermentations (Table 1). Although methionine did not decrease H_2S production, there was a linear correlation of decreasing H_2S production with increasing methionine in UCD522 fermentations (Fig. 1). There was no difference in H_2S production during the time course of fermentation in any of the UCD522 fermentations (Fig. 2B and 2D).

Fermentation kinetics

In EC1118 fermentations, the arginine treatment had a lower maximum fermentation rate as compared with ammonium treatment ($p < 0.05$), but the maximum fermentation rate with added asparagine was intermediate and was not different from either ammonium or arginine (Table 1). There was no difference in maximum fermentation rate between additions of nitrogen sources in UCD522 fermentations. Additions of 140 mg L^{-1} YAN increased the maximum fermentation rate as compared with nitrogen-

Table 1. Parameters of cider fermentation by two yeast strains treated with different sources of amino nitrogen[a, b]

Yeast strain	Experimental treatment	Total H_2S production (μg)	Maximum fermentation rate (g CO_2 h^{-1})	Fermentation duration (h)	Total CO_2 production (g)	Residual sugar[c] (mg L^{-1})
EC1118	Control[d]	3.9 ± 0.8 a	0.059 ± 0.000 a	282 ± 0 a	9.45 ± 0.11 a	95 ± 19 a
	Asparagine[e]	8.9 ± 3.3 a	0.121 ± 0.005 bc	183 ± 3 b	10.30 ± 0.17 bc	6 ± 9 b
	Arginine[e]	13.2 ± 8.9 a	0.114 ± 0.002 b	179 ± 10 b	10.21 ± 0.55 b	0 b
	Ammonium[e,f]	11.3 ± 4.5 a	0.124 ± 0.003 c	172 ± 6 b	10.34 ± 0.05 c	1 ± 1 b
UCD522	Control[d]	19.2 ± 9.8 a	0.057 ± 0.002 a	304 ± 5 a	10.03 ± 0.11 a	416 ± 262 a
	Asparagine[e]	80.0 ± 4.8 b	0.122 ± 0.016 b	192 ± 5 b	10.24 ± 0.55 a	3 ± 3 b
	Arginine[e]	68.8 ± 18.1 b	0.113 ± 0.009 b	195 ± 0 b	10.26 ± 0.44 a	9 ± 15 b
	Ammonium[e,f]	75.3 ± 4.3 b	0.114 ± 0.001 b	184 ± 12 b	9.89 ± 0.32 a	2 ± 2 b

[a]Values expressed as means ± standard deviations.
[b]Means which do not share a common letter in the specified yeast strain are significantly different (ANOVA, $p < 0.05$).
[c]Quantified using the method described in the 'Materials and methods' section.
[d]Control juice contained 52 mg L^{-1} yeast assimilable nitrogen (YAN) with no added nitrogen.
[e]Added at concentrations of 140 mg L^{-1} nitrogen.
[f]Added in the form of diammonium phosphate.

Table 2. Parameters of cider fermentation by two yeast strains treated with methionine[a,b]

Yeast strain	Methionine[c] (mg L^{-1})	Total H_2S production (μg)	Maximum fermentation rate (g CO_2 hr^{-1})	Fermentation duration (h)	Total CO_2 production (g)	Residual sugar[d] (mg L^{-1})
EC1118	0[e]	3.9 ± 0.8 a	0.059 ± 0.000 a	282 ± 0 a	9.45 ± 0.11 ab	95 ± 19 a
	5	1.0 ± 0.1 b	0.054 ± 0.007 ab	293 ± 10 ab	10.20 ± 0.04 bc	135 ± 25 a
	20	0.1 ± 0.2 b	0.059 ± 0.002 a	288 ± 10 a	10.22 ± 0.07 c	311 ± 295 a
	50	1.0 ± 0.6 b	0.049 ± 0.002 b	307 ± 0 b	9.54 ± 0.12 b	165 ± 87 a
UCD522	0[e]	19.2 ± 9.8 a	0.057 ± 0.002 a	304 ± 5 a	10.03 ± 0.11 a	416 ± 262 a
	5	20.2 ± 0.2 a	0.057 ± 0.001 a	318 ± 9 ab	9.97 ± 0.08 a	343 ± 103 a
	20	18.4 ± 8.8 a	0.055 ± 0.002 a	323 ± 0 b	9.84 ± 0.10 a	606 ± 89 a
	50	15.5 ± 2.4 a	0.058 ± 0.001 a	307 ± 0 a	9.94 ± 0.04 a	498 ± 134 a

[a]Values expressed as means ± standard deviations.
[b]Means which do not share a common letter in the specified yeast strain are significantly different (ANOVA, $p < 0.05$).
[c]Denotes total juice methionine concentration.
[d]Quantified using the method described in the 'Materials and methods' section.
[e]Control juice contained 52 mg L^{-1} YAN with no added nitrogen.

deficient juice in fermentations with both yeast strains ($p < 0.001$; Table 1, Fig. 3). There was no difference in fermentation duration among different sources of added nitrogen in fermentations with either yeast strain (Table 1). Additions of nitrogen sources all resulted in shorter total fermentation duration in fermentations with both yeast strains ($p < 0.0001$; Table 1). In treatments with different sources of added nitrogen, ammonium additions led to a greater mass of CO_2 lost, as compared with addition of arginine in EC1118 fermentation, but neither differed from CO_2 production with added asparagine (Table 1). There was no difference in CO_2 production in UCD522 fermentations across nitrogen addition treatments. There was no difference in RS between any nitrogen sources in either yeast strain (Table 1). However, additions of all nitrogen sources at 140 mg L^{-1} decreased the amount of RS in both yeast strains (Table 1).

Methionine additions at 50 mg L^{-1} resulted in a lower maximum fermentation rate compared with unsupplemented juice for EC1118 fermentations ($p < 0.05$; Table 2). Maximum fermentation rates did not differ at any concentration of added methionine

in UCD522 fermentations. Additions of methionine resulted in longer fermentation duration when added at concentrations of 50 mg L^{-1} in EC1118 fermentations as compared with unsupplemented juice ($p < 0.01$; Table 2). For EC1118 fermentations, methionine additions at 20 mg L^{-1} increased CO_2 production (Table 2). There was no difference observed in CO_2 production when methionine was added at any concentration in UCD522 fermentations. CO_2 production increased when juice was supplemented with all nitrogen sources at 140 mg L^{-1} in EC1118 fermentations, but was not different in UCD522 fermentations (Table 1). There was no difference in RS between methionine additions and the control in fermentations with either yeast strain (Table 2).

Post-fermentation, there was a greater amount of residual nitrogen in treatments with DAP supplementation compared with both arginine and asparagine supplementation in fermentations with EC1118 fermentations (Fig. 4). There was no difference in residual arginine or asparagine in fermentations conducted by either yeast strain (Fig. 4). There was no difference in residual YAN across nitrogen sources in UCD522 fermentations.
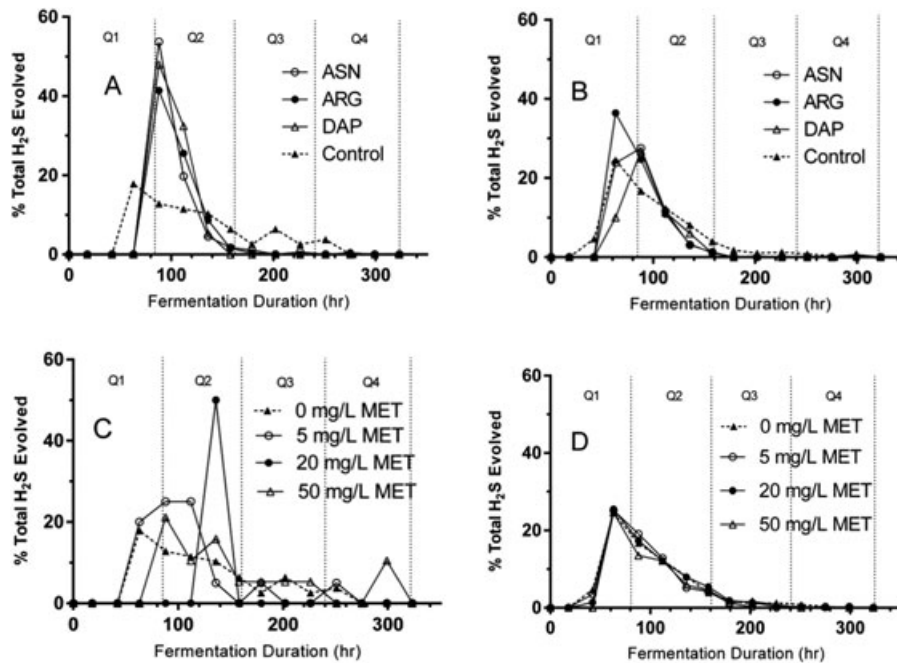
Figure 2. Time course of H_2S production during experimental fermentations. (A, C) Fermentations conducted with yeast strain EC1118; (B, D) fermentations conducted with yeast strain UCD522. Descriptors 'Q1–Q4' represent each quartile of fermentation used to compare the rate of H_2S evolution over the time course of fermentation. There was no significant difference in percentage of total H_2S production over the time course of fermentation between sources of nitrogen or between methionine treatments and control.
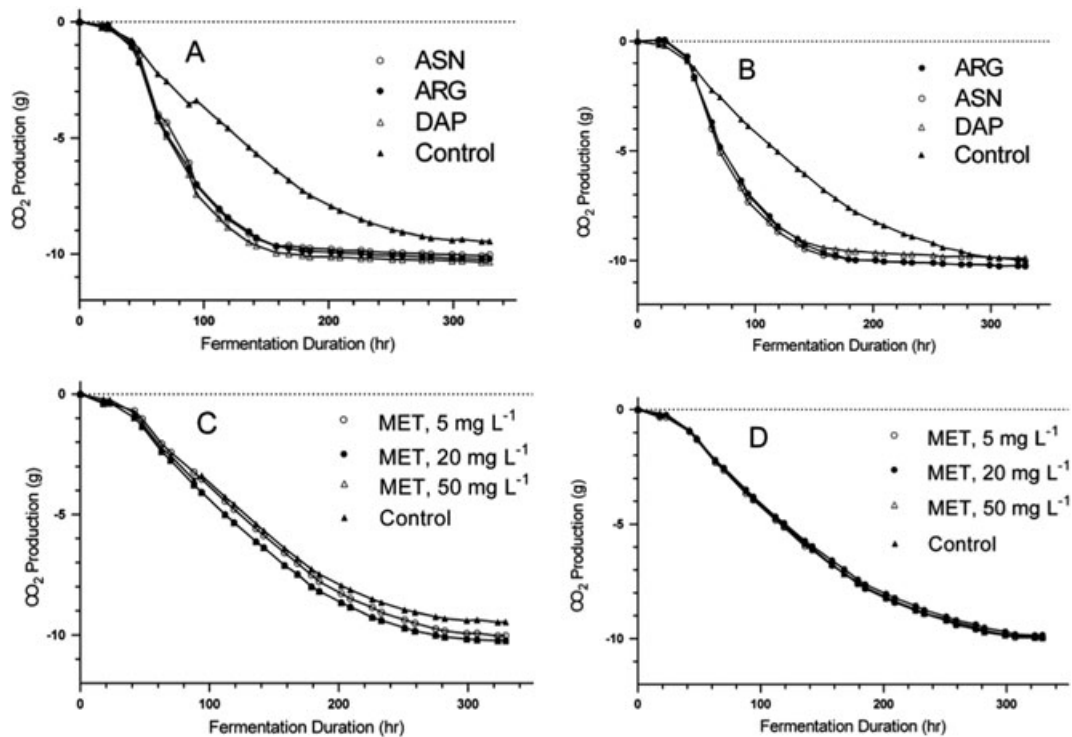

Figure 3. Fermentation curves for treatments containing added methionine and different sources of nitrogen plotted as the mass difference of the fermenter owing to the loss of CO_2, which correlates to fermentation rate. The slope of the curve during the logarithmic phase of growth was used to calculate and compare maximum fermentation rates. (A, C) Fermentations using strain EC1118; (B, D) fermentations using strain UCD522. Treatments containing higher concentrations of added nitrogen fermented significantly faster as compared with low-nitrogen treatments. There was no significant difference in fermentation rate between treatments with methionine.

Interactive effect of methionine and total YAN

As previously stated, additions of methionine resulted in decreased H_2S production in EC1118 fermentations at all concentrations of added methionine to nitrogen-deficient juice. When fermentations were supplemented to 153 mg L^{-1} total YAN, only additions of methionine at 50 mg L^{-1} resulted in decreased H_2S production ($p < 0.05$; Fig. 5). Additions of methionine
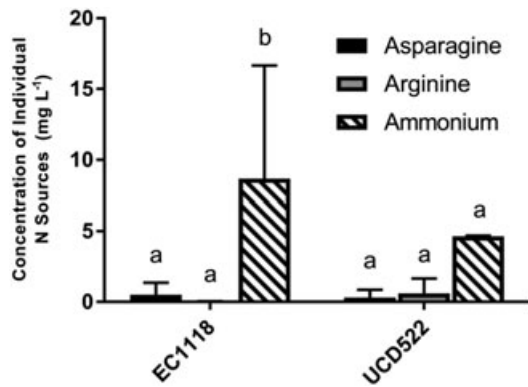
Figure 4. Residual yeast assimilable nitrogen (YAN) concentration for fermentations treated with different sources of YAN. Concentrations represent the residual concentration of the respective YAN sources post-fermentation after supplementation at 140 mg nitrogen L^{-1} each within each treatment. Means not labelled with a common letter are significantly different from one another within the specified yeast strain [one-way ANOVA with Tukey's honest significant difference (HSD), $p < 0.05$].
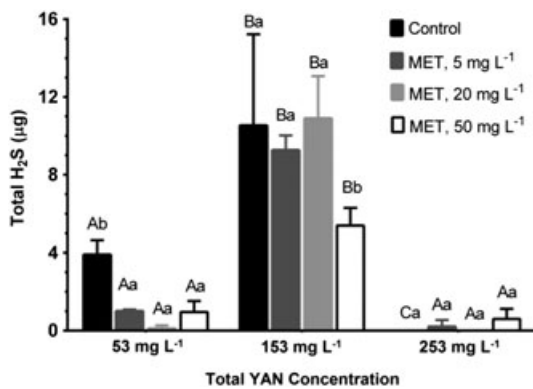

Figure 5. Total H_2S produced during fermentations comparing the interactive effects of total nitrogen and methionine. Columns are grouped corresponding to total juice YAN listed on the x-axis. Values expressed as mean with error bars expressing standard deviation. Control juice contained no added methionine. Means not labelled with a common lower-case letter are significantly different within the specified YAN concentration. Means not labelled with a common upper-case letter are significantly different across YAN concentrations (two-way ANOVA with Tukey's HSD, $p < 0.05$).

did not decrease H_2S production at any concentration when fermentations were supplemented to 253 mg L^{-1} YAN. When juice was supplemented to 153 mg L^{-1} YAN there was an increase in H_2S production across all treatments and in the control as compared with juice at 53 mg L^{-1} YAN ($p < 0.05$; Fig. 5). Conversely, there was no difference in H_2S production between juice containing 53 mg L^{-1} YAN and juice containing 253 mg L^{-1} YAN in any methionine treatment. In fact, H_2S production was lower when supplemented to 253 mg L^{-1} YAN as compared with unsupplemented and 153 mg L^{-1} YAN in the control juice. Across all treatments, the highest volume of H_2S produced occurred when total juice nitrogen was 153 mg L^{-1}.

Additions of methionine did not affect fermentation rate at YAN concentrations of 253 mg L^{-1} (Table 3). In fermentations with YAN concentrations of 153 mg L^{-1}, methionine added at concentrations of 5 mg L^{-1} led to lower fermentation rates, but had no effect when methionine was added to unsupplemented juice (Tables 2 and 3). Higher YAN concentrations decreased fermentation duration in both 153 and 253 mg L^{-1} as compared with juice not supplemented with YAN (Table 3, Table 2). There was no significant difference in fermentation duration between treatments with methionine when juice was supplemented with YAN, but in unsupplemented juice additions of methionine led to decreased fermentation durations (Table 2, Table 3). CO_2 production was not affected by treatments of methionine nor additions of YAN. RS decreased when juice YAN increased from 53 to 253 mg L^{-1}.

Sensory evaluation

Sensory analyses were conducted to determine whether panelists could detect differences in cider aroma between control fermentations and fermentations containing added methionine at 20 mg L^{-1} and nitrogen added as DAP at 140 mg L^{-1} in ciders fermented with either EC1118 or UCD522. Aroma of ciders produced using DAP additions in both strains were different from the control ($p < 0.01$) and methionine additions in EC1118 fermentations were different from the control ($p < 0.05$) (Table 4). No difference from the control was detected in the UCD522 fermentations with methionine added (Table 4).

Table 3. Parameters of cider fermentation by yeast strain EC1118 investigating interactive effects of methionine and total YAN[a–c]

YAN (mg L^{-1})	Methionine (mg L^{-1})	Maximum fermentation rate (g CO_2 h^{-1})	Fermentation duration (h)	Total CO_2 production (g)	Residual sugar[d] (mg L^{-1})
153	0	0.141 ± 0.000 a	160 ± 4 a	9.69 ± 0.12 a	70 ± 32 a
	5	0.132 ± 0.003 b	165 ± 0 a	9.64 ± 0.14 a	105 ± 82 a
	20	0.135 ± 0.003 ab	160 ± 4 a	9.68 ± 0.12 a	82 ± 36 a
	50	0.137 ± 0.002 ab	165 ± 0 a	9.72 ± 0.12 a	83 ± 16 a
253	0	0.162 ± 0.001 a	141 ± 0 a	9.90 ± 0.12 a	13 ± 3 a
	5	0.162 ± 0.003 a	138 ± 5 a	9.71 ± 0.05 a	18 ± 9 a
	20	0.164 ± 0.002 a	135 ± 5 a	9.78 ± 0.13 a	22 ± 8 a
	50	0.164 ± 0.007 a	138 ± 5 a	9.69 ± 0.03 a	24 ± 12 a

[a]Values expressed as means ± standard deviations.
[b]Means which do not share a common letter in the specified yeast strain are significantly different (ANOVA, $p < 0.05$).
[c]Data for EC1118 fermentation with 53 mg nitrogen L^{-1} juice with methionine supplementation are presented in Table 2.
[d]Quantified using the method described in the 'Materials and methods' section.

Table 4. Perceived aroma differences in cider fermented in two yeast strains as determined by triangle sensory testing[a]

Sensory comparison	Total observations	Correct observations	Significance
EC1118			
Control vs ammonium[b] (140 mg L^{-1})	40	25	*p* = 0.0002
Control vs methionine (20 mg L^{-1})	40	20	*p* = 0.0214
UCD522			
Control vs ammonium[b] (140 mg L^{-1})	40	23	*p* = 0.0014
Control vs methionine (20 mg L^{-1})	40	10	*p* = 0.9034

[a]Triangle test $\alpha = 0.05$, $\beta = 0.20$ and $P_d = 30\%$.
[b]Ammonium added in the form of diammonium phosphate.

Discussion

The typical amino acid composition of apple juice has not been well established. Apple juice used for cidermaking varies drastically based on the specific production facility and method. Many apple varieties may be used, and apple juice may or may not undergo pasteurization prior to fermentation. While both pasteurized and unpasteurized apple juice are used commercially to produce hard cider, currently there is no research that indicates how pasteurization affects the amino acid composition of apple juice, and amino acids have been observed in low concentrations in unpasteurized juice (16,17). This suggests the need to investigate the impact of pasteurization on YAN concentration and composition, and the subsequent impact on fermentation kinetics and aroma formation in cider fermentations. There was no difference in fermentation rate or H$_2$S production between the nitrogen sources examined in this study. Although none of the studied nitrogen sources appeared to be assimilated preferentially by yeast, in fermentations treated with sources of YAN, there was higher residual ammonium remaining in cider fermented by strain EC1118, but not in cider fermented by strain UCD522 (Fig. 4). For both yeast strains, the nitrogen source that resulted in the highest residual concentration post-fermentation was ammonium. The higher residual ammonium may indicate that amino nitrogen is preferentially assimilated by yeast, or that the excess amino acids were taken up and stored by the yeast in intracellular pools, then subsequently removed with the yeast upon racking at the end of the fermentation. Despite this, fermentations treated with ammonium fermented to completion in the shortest amount of time with both strains. While no specific nitrogen source contributed to an increase in fermentation rate, higher concentrations of total YAN led to increased fermentation rate (Fig. 3) which is in agreement with previous work (27). Fermentations with added DAP, asparagine and arginine produced similar quantities of H$_2$S in both yeast strains. Our findings may be better explained by other factors which are known to influence fermentations kinetics, including other yeast nutrients such as biotin and pantothenic acid in the fermenting medium which were not investigated in our study, but could potentially have interactive effects with PAN sources (7). Further research is required to determine the influence of YAN sources on the fermentation kinetics and H$_2$S formation during cider fermentation.

Fermentations conducted in the low nitrogen juice produced significantly less H$_2$S when containing additions of methionine for EC1118 fermentations. Additions of methionine at concentration of 5 mg L^{-1} decreased total H$_2$S produced. This is notably lower than the 20 mg L^{-1} concentration previously prescribed as necessary to inhibit H$_2$S production in grape fermentations (21). It is possible that concentrations of methionine <5 mg L^{-1} may

also lead to decreased H$_2$S production, although lower concentrations were not investigated in our study. Additions of methionine did not decrease H$_2$S production in UCD522 fermentations, but as stated earlier, H$_2$S production is highly dependent on yeast strain. The strain UCD522 is particularly prone to greater H$_2$S production owing to its inefficiency in the basal expression of the SRS or the inability to effectively incorporate reduced sulphur in the SRS (28). Previous research indicated that methionine additions can be effective in decreasing H$_2$S production when fermented using the strain UCD522 in artificial media (28), but this effect may be confounded when fermented in actual juice by other yeast nutrients and micronutrients present in the complex apple juice matrix. Within the range of concentrations examined in this study, there was a significant correlation between increasing methionine concentration and decreasing H$_2$S production in UCD522 fermentations even if total H$_2$S production was not significantly decreased (Fig. 1). This finding is particularly important for cidermakers, since apple juice often contains <5 mg L^{-1} methionine, whereas grape juice typically contains >5 mg L^{-1} methionine (18). The results of our study suggest that methionine is essential for decreasing H$_2$S production during fermentation, and that its use as a yeast nutrient may play an essential role in decreasing the incidence of H$_2$S production during cider fermentations.

Since pre-fermentation concentrations of YAN >140 mg L^{-1} have been reported to decrease H$_2$S production, and our control low-YAN juice contained <140 mg L^{-1} YAN, the interactive effect of total YAN and additions of methionine was investigated. Others previously found that additions of methionine and ammonium could both decrease H$_2$S production in an interactive manner when fermented in artificial media (28), but this effect has not been investigated in cider fermentations. Only methionine at concentrations of 50 mg L^{-1} decreased H$_2$S production when juice was supplemented to 153 mg L^{-1} total YAN, as compared with juice at 53 mg L^{-1} YAN where any additions of methionine at 5, 20 and 50 mg L^{-1} resulted in decreased H$_2$S production. This may be due to the highly complex interactions of yeast strain, yeast nutrient deficiencies, amino acid concentration and composition, and temperature, which all are known to affect H$_2$S production (7). Fermentation rate increased when supplemented with YAN, which is known to be correlated with overall yeast biomass (29). Therefore, it is possible that increased yeast biomass may require a higher overall concentration of methionine to effectively inhibit the SRS and prevent H$_2$S production. This is consistent with results obtained when juice was supplemented to 253 mg L^{-1} YAN, where methionine additions at any concentration did not affect total H$_2$S production. However, H$_2$S production was too low to accurately quantify in fermentations containing 253 mg L^{-1} YAN, hence some effects may not be observable owing to the limit of

quantification of our method exceeding the actual minimum H_2S production levels present in this study. H_2S production was almost completely inhibited in juice supplemented to 253 mg L^{-1} YAN. Therefore, under the conditions evaluated in this study, additions of high concentrations of YAN are more effective at decreasing H_2S production than additions of methionine. Clearly, the relationship between YAN concentration and composition and H_2S production in cider fermentations is complex.

Contrary to previous findings, YAN supplementation to 153 mg L^{-1} resulted in a significant increase in H_2S production as compared with nitrogen-deficient juice in all experimental treatments. This effect was observed regardless of YAN source supplemented, indicating that differences in naturally occurring apple juice vs apple juice supplemented with amino acids to more resemble grape juice YAN composition are not a factor in the observed increase in H_2S production. There is conflicting research on the effect of YAN on H_2S production. Some studies indicate that increasing YAN concentration leads to increased H_2S production (2,3), whereas others have observed the opposite effect (6). The minimum YAN requirement typically recommended to avoid a stuck or sluggish fermentation in wine is 140 mg L^{-1} (30) but recently a higher minimum value of 267 mg L^{-1} was recommended (31), with that minimum increasing even further with increasing juice soluble solids concentration (32). However, H_2S may still be produced even when fermentations contain sufficient YAN to avoid stuck or sluggish fermentations. Additions of YAN have been shown to simultaneously increase fermentation rate and H_2S production in wine fermentations (6), which is in agreement with the findings of this study. This is most likely due to the regulation of the SRS sequence in response to overall nitrogen limitation. Low nitrogen concentrations (66 mg L^{-1} YAN) down-regulated the SRS sequence and led to decreased H_2S formation, where high nitrogen concentrations (267 mg L^{-1} YAN) upregulated the SRS and contributed to maximum H_2S production (33). In the current study, YAN concentrations of 253 mg L^{-1} resulted in decreased H_2S production as compared with lower concentrations of 153 and 53 mg L^{-1}. However, fermentation rate increased and fermentation duration decreased with increasing YAN. At concentrations of 53 mg L^{-1} YAN, yeast growth is likely to be inhibited, most likely owing to nitrogen starvation. When supplemented to 153 mg L^{-1} YAN, it is possible that higher yeast biomass leads to depletion of YAN during the early stage of fermentation, leading to nitrogen deficiency during the logarithmic growth phase. This nitrogen deficiency can lead to increased H_2S production as compared with low-nitrogen fermentation owing to the higher yeast biomass without sufficient nutrients (6). If YAN is supplemented to 253 mg L^{-1}, starvation can be avoided altogether, thereby inhibiting H_2S production. For these reasons, other researchers have suggested a two-step addition of nitrogen supplements, with half the total supplementation added prior to fermentation and the other half added at one-third sugar depletion (34). The findings of our study therefore suggest that, if YAN supplementation is made in a one-step approach, prior to fermentation, a minimum YAN concentration of 250 mg L^{-1} is required in apple cider fermentations to prevent stuck fermentation as well as prevent H_2S formation.

Sensory evaluation revealed that additions of DAP resulted in differences in aroma between supplemented ciders when compared with unsupplemented ciders. Although sensory difference testing alone does not identify specific aroma compounds, prior research has shown that several aroma compounds other than H_2S are also influenced by YAN in wine fermentations (19). Analytical results showed higher H_2S production in DAP-supplemented ferments, suggesting that the perceived sensory difference may be related to the observed increase in H_2S production. High H_2S concentrations would lead to a prominent increase in 'reduced' and 'sulphur-like' aromas in the finished cider. This is contradictory to the perception by many cidermakers that additions of YAN in the form of DAP necessarily protect against the occurrence of 'reduced' aromas in finished ciders and wines. This also refutes the assumption by some cidermakers that H_2S is sufficiently purged by CO_2 during the fermentation such that the aroma of H_2S is not present in the finished ciders. Similarly, ciders containing added methionine had differences in cider aroma compared with unsupplemented ciders, but only in ciders fermented using yeast strain EC1118. As indicated by our analytical results, methionine additions significantly decreased H_2S production by EC1118 fermentations but did not affect H_2S production by UCD522 fermentations. It is possible that the perceived difference in aroma could be attributable to a decrease in the 'reduced' and 'sulphur-like' aromas in finished ciders containing added methionine as compared with control fermentations, although further sensory evaluation such as descriptive analysis would be required to test this hypothesis, and to thus identify the nature of the differences we observed through triangle tests. Limitations of this study that could potentially impact the outcome of sensory evaluation include the use of rubber stoppers, which could contribute off-aromas. Use of silicone stoppers in future work would eliminate this potential complication, and would be especially important if descriptive analysis were to be conducted.

Conclusions

Differences in sources of YAN which simulate a more apple juice-like matrix (asparagine rich) have no significant impact on the formation of H_2S in the fermenting juice when compared with arginine-rich juices more like the grape juice matrix. Supplementation of apple juice with methionine to concentrations normally observed in grape juice decreased H_2S production in EC1118 fermentations. This corresponds to a difference in cider aroma, which may be due to a decreased presence of sulphur off-aromas. This finding suggests that relatively small additions of methionine pre-fermentation could contribute to decreased H_2S formation in ciders fermented with some yeast strains, leading to more desirable aromas and flavours in the finished cider. Further research is necessary to examine the overall impact of methionine supplementation on cider flavour and aroma. This research also indicates that additions of YAN do not necessarily decrease H_2S formation, but could in fact increase H_2S production when only supplemented to the commonly recommended YAN concentration of 140 mg L^{-1}. This finding suggests that cidermakers may encounter detectable H_2S production even at YAN concentrations generally considered sufficient for the prevention of sulphur off-aromas. The complexity of this phenomenon points to the need for continued research on optimal pre-fermentation juice chemistry to decrease the incidence of H_2S off-aromas in cider.

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Supporting information

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